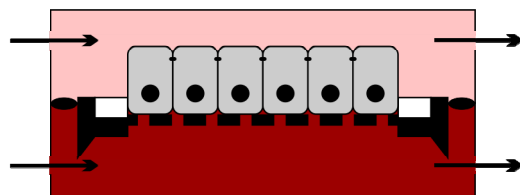
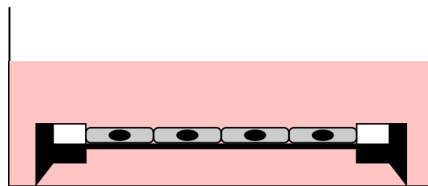
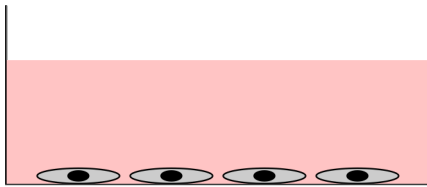


# Advanced Culture Experiments with Adherent Cells

From single cells to specialized tissues in perfusion culture



**Will W. Minuth and Lucia Denk**



**Important note:**

Since research and clinical work are constantly expanding our knowledge, we would like to emphasize that when the book was written all information about dosage and application specifications reflected the state of the art. However, users are strongly asked to check the instructions that come with the presently used preparations and medical products. Each researcher has to use its own judgement on dosage, toxicity and biologic safety risk according to specific recommendations in the own country.

**Search of actual literature:**

In cell and tissue culture technique a rapid growth of information is found, which is constantly transforming our knowledge in the area of biomedicine and cell biology. Consequently it made sense to place beside cited literature a number of search criteria. Putting them in medical or biological databases such as PubMed, Scopus, Web of Knowledge or Biological Abstracts these search criteria will lead the reader to the most up to date published literature on the subject.

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# Preface

Since decades a multitude of cells was cultured in various kinds of plastic culture dishes resembling more or less the classical Petri dish. Also our group was working over many years with these culture vessels and flasks. Although the cells could be multiplied and kept over prolonged periods of time, important functional features were not sufficiently developed. The obvious cellular dedifferentiation was recognized by an altered morphology, a changed physiology and an atypical expression of proteins. Since within tissues and organs perfect functionality is contained, missing characteristics in culture must be caused by a suboptimal environment.

For that reason we decided to improve the environment for cultured cells and tissues. The strategy was to offer an atmosphere resembling as most as possible the physiological environment found in the individual tissues and organs. So we were looking for sophisticated culture equipment on the market. However, we could not find the suitable tools to create a tissue-specific environment for cells in culture. In consequence, we decided to design and to construct a system consisting of individually selected adhesion sites, tissue carriers, perfusion culture containers and adapted culture media.

In this textbook a series of innovative concepts is described in detail to improve the environment for cultured cells and for the generation of specialized tissues. Students, technicians and young scientists shall obtain an insight how to perform sophisticated cell and tissue culture experiments urgently needed in the field of biomedicine, biomaterial research and tissue engineering. On the one hand the necessary theoretical background information is given and on the other hand practical hints facilitate the entrance for the generation of specialized tissues. An essential part of the book is therefore consequently dealing with challenging aspects of perfusion culture technology developed in our laboratory.

Despite increased actual knowledge one should consider that the culture of anchorage-dependent cells and the generation of specialized tissues is not yet perfectly established but has still to be improved within the next decades. In so far young scientists do not stand at the end but at begin of an exciting and future orientated scientific development.

During the course of our earlier experiments we recognized that despite technical improvements multiple aspects have to be overworked and adapted. Thus, the presented manuscript reflects the actual state of the art and not the things that have to be done in future. The permanent learning by doing process told us that the therapeutic steering of regeneration with cultured stem/progenitor cells will be one of the most challenging processes for the future. For this experimental work innovative local drug delivery systems, improved growth factors, smart extracellular matrix, increasing rheological stress, hydrostatic pressure and a suitable provision with tissue-specific culture media will play essential roles. The declared aim will be to develop tools for innovative cell and tissue culture so that the required cellular functionality can be obtained and arise of risks is minimized.

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# Table of contents

Page

<b>1. Cellular basics</b>	<b>10</b>
1.1. Occurrence of cells	11
1.2. Nucleus	13
1.3. Plasma membrane and cytoplasm	14
1.4. Endoplasmic reticulum	18
1.5. Golgi apparatus	18
1.6. Mitochondria	19
1.7. Endosomes, lysosomes and peroxisomes	20
1.8. Cytoskeleton	20
1.9. Cell division	22
1.10. Individual life cycle of a cell	24
<b>2. Cells contact extracellular matrix (ECM)</b>	<b>26</b>
2.1. Structural characteristics of ECM	28
2.2. Multifunctional tasks of ECM	30
2.3. Synthesis of ECM	33
2.4. Linking molecules in ECM	38
<b>3. Crosstalk between cells and ECM</b>	<b>42</b>
3.1. Cells in contact with cells	42
3.2. Cells contact ECM	44
3.3. ECM contacts cells	46
3.4. ECM controls proliferation	49
3.5. ECM influences development	50
3.6. ECM modulates cell seeding	55
3.7. ECM and matricellular functions	56
<b>4. Conventional culture with adherent cells</b>	<b>60</b>
4.1. Selection of the suitable dish	62
4.2. Choice of cells and culture environment	64
4.3. Typical protocols for the culture of adherent cells	68
4.4. Visualization of cells on a transparent substrate	71
<b>5. Initial reactions of cells in culture</b>	<b>72</b>
5.1. From adherent cells to cell suspension	73
5.2. From suspension to first contacts	75
5.3. From cell adhesion to adherence	78
5.4. Inconstant adherence of cells	81
<b>6. Adhesion, adherence and affinity</b>	<b>83</b>
6.1. Affinity tells about adhesion and adherence	83
6.2. Adhesion meets differentiation and dedifferentiation	87
<b>7. Cells meet materials</b>	<b>89</b>
7.1. Visualization of cells on non transparent surfaces	89
7.2. Polish of polystyrene surface	91
7.3. Modulation of adhesion	92

<b>8. Improvements for cell adhesion</b>	<b>95</b>
8.1. Coating substrates with molecules mimicking ECM	96
8.2. Adaption of coating for an individual cell type	100
8.3. Adhesion on biological materials	102
<b>9. Cells contact biomedical materials</b>	<b>104</b>
9.1. Kinds of materials	105
9.2. Variety of polymers	108
9.3. Smooth polymers	109
9.4. Biodegradable polymers	111
9.5. Metals and alloys	114
9.6. External and internal volume	115
<b>10. Pattern of adherent cells in culture</b>	<b>117</b>
10.1. Cell growth on flat surfaces	118
10.2. Growth on external and internal surfaces	121
10.3. Spreading of cells	124
<b>11. Improving adhesion parameters</b>	<b>128</b>
11.1. Anchorage on spherical supports	129
11.2. Flat supports with an inner and an outer surface	132
11.3. Filter as cell support	136
11.4. Experiments with filter inserts	139
11.5. Co-culture experiments with filter inserts	142
11.6. Culture at the interface between fluid and air	144
11.7. Imaging cells on spatial materials	146
<b>12. Proliferation versus differentiation</b>	<b>148</b>
12.1. Phases of the cell cycle	149
12.2. Interphase replaces mitosis	153
12.3. Stepwise to more differentiation	155
<b>13. Limitations in static culture environment</b>	<b>158</b>
13.1. Unstirred layers of fluid	158
13.2. Alterations in nutrition	160
<b>14. Options for dynamic culture</b>	<b>162</b>
14.1. Mass transfer in a spinner bottle	163
14.2. Microgravity in a rotating reactor	164
14.3. Exchange of medium via hollow fibers	166
14.4. Perfusion cultures	168
14.5. Perfusion housing with exact placement of cells	169
<b>15. Adaption of environment to specific needs</b>	<b>173</b>
15.1. Selection of optimal adhesion sites	174
15.2. Punching out an adhesion substrate	175
15.3. Protection of adhesion substrate in a tissue carrier	177

<b>16. Handling a tissue carrier</b>	<b>181</b>
16.1. Mounting an adhesion substrate	181
16.2. Dismantling a tissue carrier	182
16.3. Sterilization of a tissue carrier	183
16.4. Transfer of a tissue carrier to a dish	184
16.5. Seeding of cells on a tissue carrier	186
16.6. Preparing contacting co-cultures with a tissue carrier	188
<b>17. Bridging static with dynamic culture</b>	<b>190</b>
17.1. Versatile containers for perfusion culture	193
17.2. Transport of nutrition and respiratory gas	198
17.3. Dynamic fluid environment for adherent cells	199
<b>18. Performance of perfusion culture</b>	<b>203</b>
18.1. Selecting the suitable tube for medium transport	203
18.2. Assembling a perfusion culture set up	206
18.3. Starting perfusion culture	209
<b>19. Supplementary tools for perfusion culture</b>	<b>211</b>
19.1. Innovative closure for media bottles	211
19.2. Transport of culture medium	213
19.3. Maintenance of the right temperature	216
19.4. Transfer of respiratory gas	217
19.5. Modulation of respiratory gas content	220
19.6. Elimination of harmful gas bubbles	223
19.7. Maintenance of a constant pH in the medium	225
19.8. On-line monitoring of perfusion cultures	229
<b>20. Selection of a suitable culture medium</b>	<b>232</b>
20.1. View to the variety of culture media	233
20.2. Selection of a medium for perfusion culture	237
20.3. Basic compounds of a culture medium	238
20.4. Driving forces within a culture medium	240
<b>21. Applying the best culture medium</b>	<b>244</b>
<b>22. Use of serum as a culture additive</b>	<b>246</b>
22.1. Nutritional influences of serum	247
22.2. Origin and plurality of serum	248
<b>23. Risk of serum application</b>	<b>251</b>
<b>24. Replacement of serum in medium</b>	<b>253</b>
24.1. From serum to serum free conditions	253
24.2. Additives in serum free media	255
24.3. Preventing risks of infection	256
<b>25. Exposure of cells to always fresh medium</b>	<b>258</b>
25.1. Offering the same cells different culture media	259

<b>26. Offering an improved fluid environment</b>	<b>264</b>
26.1. Providing external and internal surfaces with nutrition	264
26.2. Distribution of fluid within a perfusion culture container	267
26.3. Parameters of medium inside a perfusion container	270
26.4. Examples to generate specialized connective tissues	272
<b>27. Simulation of an environment for epithelia</b>	<b>274</b>
27.1. Necessity of epithelial models in biomedicine	275
27.2. Selecting a suitable strategy for the culture of epithelial cells	277
<b>28. Technical prerequisites for gradient culture</b>	<b>280</b>
28.1. Adherence of cells on an individually selected substrate	281
28.2. Protection of cells within a tissue carrier	283
28.3. Epithelia within a gradient perfusion container	285
<b>29. Fluid environment in gradient culture</b>	<b>289</b>
29.1. Providing always fresh medium	289
29.2. Presence of gas bubbles during medium transport	291
29.3. Elimination of gas bubbles during medium transport	294
29.4. Preventing gas bubbles inside the gradient container	296
<b>30. Medium flow in gradient perfusion culture</b>	<b>298</b>
30.1. Sealing of the epithelium pilots flow	299
30.2. Possible leaks within the gradient container	301
30.3. Destruction of epithelia due to embolic obstruction	303
30.4. Monitoring the epithelial barrier over long periods	305
<b>31. Harvest of differentiated epithelia</b>	<b>308</b>
31.1. From embryonic to adult epithelial characteristics	309
31.2. Applications of epithelia kept in gradient perfusion culture	311
<b>32. In vitro regeneration of parenchyma</b>	<b>315</b>
32.1. Using a pad for implantation of stem/progenitor cells	316
32.2. From stem/progenitor cells to polarized cells	318
<b>33. Simulation of the interstitial space</b>	<b>324</b>
<b>34. Creation of an artificial interstitium</b>	<b>328</b>
34.1. Covering stem/progenitor cells with polyester fleeces	329
34.2. Keeping stem/progenitor cells within an artificial interstitium	330
<b>35. Tubule growth in an artificial interstitium</b>	<b>333</b>
35.1. Whole mount label of generated tubules	333
35.2. Cell biological differentiation on growing tubules	335
35.3. Electron microscopic view	338
<b>36. Tubulogenic effect of aldosterone</b>	<b>342</b>
36.1. Inducing tubulogenic development	342
36.2. Interfering the tubulogenic action of aldosterone	344

<b>37. Steroid hormones and stem/progenitor cells</b>	<b>350</b>
37.1. Culture conditions as near as possible to organ-specific sites	350
37.2. Comparing the effect of aldosterone with its precursors	353
37.3. Action of aldosterone versus other steroid hormones	355
<b>38. Interface between tubules and fleece fibers</b>	<b>360</b>
38.1. Site to site orientation of generated tubules	363
38.2. View to the outer surface of tubules	366
38.3. Bridging elements within the artificial interstitium	369
<b>39. Searching a strategy for implantation</b>	<b>372</b>
39.1. Finding a strategy for stem/progenitor cell application	372
39.2. Piling and paving of stem/progenitor cells	375
39.3. Finding a path for microvascularization	378
<b>40. Selecting polyester fleeces for implantation</b>	<b>381</b>
40.1. Finding the correct type of fleece	382
40.2. Testing tubule formation on promising polyester fleeces	384
40.3. Pathologists need to look more closely	390
<b>41. Challenging aspects for the next future</b>	<b>393</b>
41.1. Offering an interstitium promoting typical development	394
41.2. Shaping constructs with suitable tissue carriers	397
41.3. Providing developing tissues within a controlled environment	399
41.4. Urgent need of powerful culture media	401
<b>42. Biologic security</b>	<b>404</b>
<b>43. Publications of authors related to cultures</b>	<b>406</b>
<b>44. Authors</b>	<b>416</b>



# 1. Cellular basics

By the first view it is not recognizable but the second view demonstrates that working with adherent cells in culture is embedded in a broad and multidisciplinary field of knowledge in the area of basic science and medical research. For that reason it is advantageous to know about cell biology, physiology, biochemistry, biomaterial research, tissue engineering, microscopic anatomy including aspects of tissue and organ development. Further some knowledge in pathology is needed and a broad spectrum of laboratory techniques is required so that related cell functions can be critically analyzed and interpreted.

Performing experiments under the sterile bench with adherent cells one has to consider in all of the cases that the generated cells and tissue cultures are model systems. In consequence, in none of the cases it is therefore allowed to perform with them medical applications! Further to avoid any thinkable biological risks, one has carefully to elaborate in how far typical features or dedifferentiated cells with atypical structures are developing. Of special importance is a permanent, critical, constant and personal control, when any cells derived from the organism or stem/progenitor cells are transformed cells for the use in culture experiments.

Sophisticated cell and tissue culture experiments cannot be explained without exact information about a general building plan of cells including their structural environment. For that reason a short introduction is given about typical elements of the cell. However, to obtain more detailed insights a series of excellent textbooks is available on the market.

**[Search: human cell morphology electron microscopy]**

**Self-study: Inform you about the types of human cells**

## 1.1. Occurrence of cells

The smallest functional unit of the animal and human organism is the cell. Typical characteristics are the cellular metabolism and adequate reactions initiated by external stimuli. Except blood cells a typical feature of cells is their capability to perform mitosis so that daughter cells within various periods of time can arise.

In a human organism about  $1 \times 10^{13}$  cells are living in close association within the specialized tissues. Moreover about  $3 \times 10^{13}$  cells within the blood are circulating through the body. The size of cells varies dependent on location within specialized tissues. The diameter of sperm cells is between 3 to 5  $\mu\text{m}$ , glial cells within neural tissue show about 5  $\mu\text{m}$ , while hepatocytes exhibit 30 to 50  $\mu\text{m}$  (Fig. 1). The human non fertilised egg cell has a diameter of about 120  $\mu\text{m}$ .

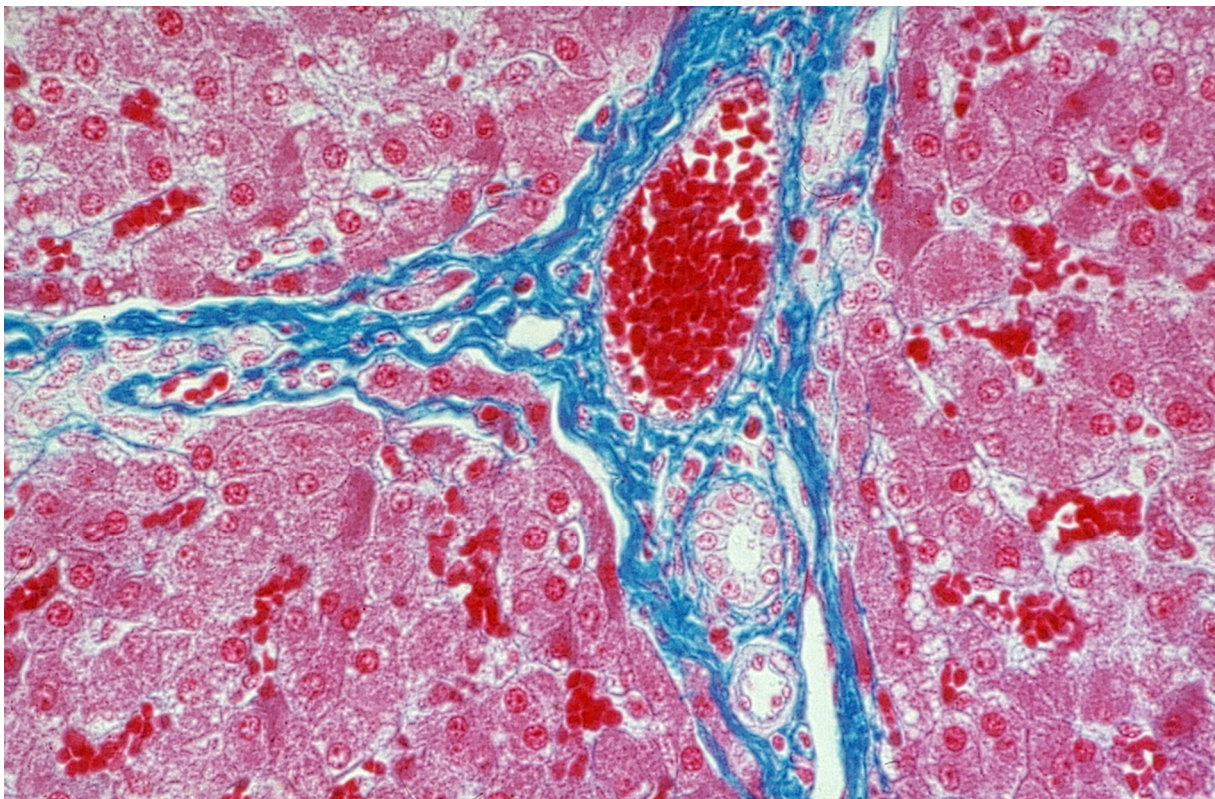


Figure 1: Microscopic illustration of hepatocytes within liver parenchyma. In the centre a triad can be recognized consisting of a bile duct epithelium, a small artery and a vein filled with erythrocytes.

Not only the size but also the morphology of cells shows extreme differences. Between ball like structures, cube shape and spindle like forms all thinkable transitions can be found. The surface of a cell can be smooth or can show numerous protrusions so that a rough surface is given. Plasma membrane specializations are known occurring in a wide range between basal infoldings, microvilli, microplicae or brush border.

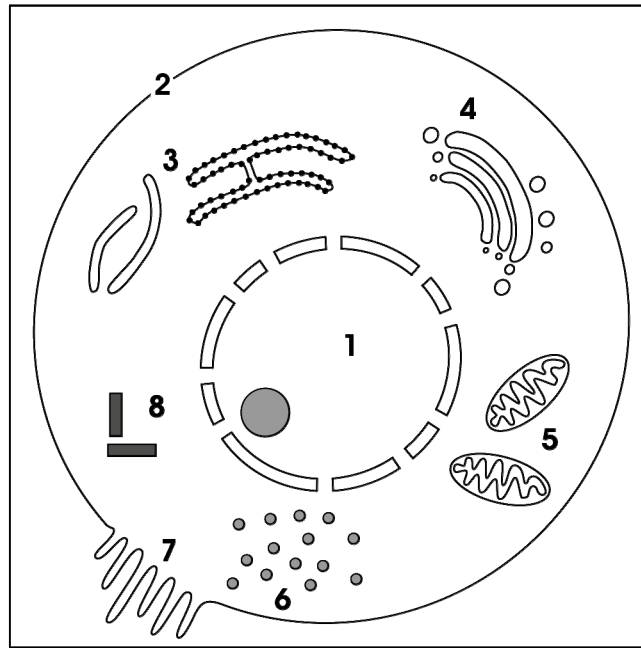


Figure 2: Schematic illustration of a cell with organelles. Demonstrated is nucleus (1), plasma membrane (2), endoplasmic reticulum (3), Golgi apparatus (4), mitochondria (5), granulae (6), mikrovilli (7) and centrioles (8).

An animal or a human cell consists of a nucleus (Fig. 2.1) and is surrounded by a selectively permeable plasma membrane (Fig. 2.2). In the interior many organelles such as endoplasmic reticulum (Fig. 2.3), Golgi apparatus (Fig. 2.4), mitochondria (Fig. 2.5), granula (Fig. 2.6), protrusions of the plasma membrane (Fig. 2.7) and centrioles (Fig. 2.8) are found. Under the light microscope the basophile nucleus and the acidophil cytoplasm can be easily recognized.

For adherent cells in culture all of these mentioned organelles play essential roles regulating the life cycle, the adhesion or adherence and the general cellular metabolism. It is obvious



that all of cell functions closely interact so that a special meaning cannot ascribed to a single organelle.

**[Search: animal human blood cell morphology]**

**Self-study: Inform yourself about the sizes and morphology of cells**

## 1.2. Nucleus

Except erythrocytes all of human cells have a nucleus (Fig. 2.1). The main compounds within the nucleus are the chromosomes including all the necessary genetic information.

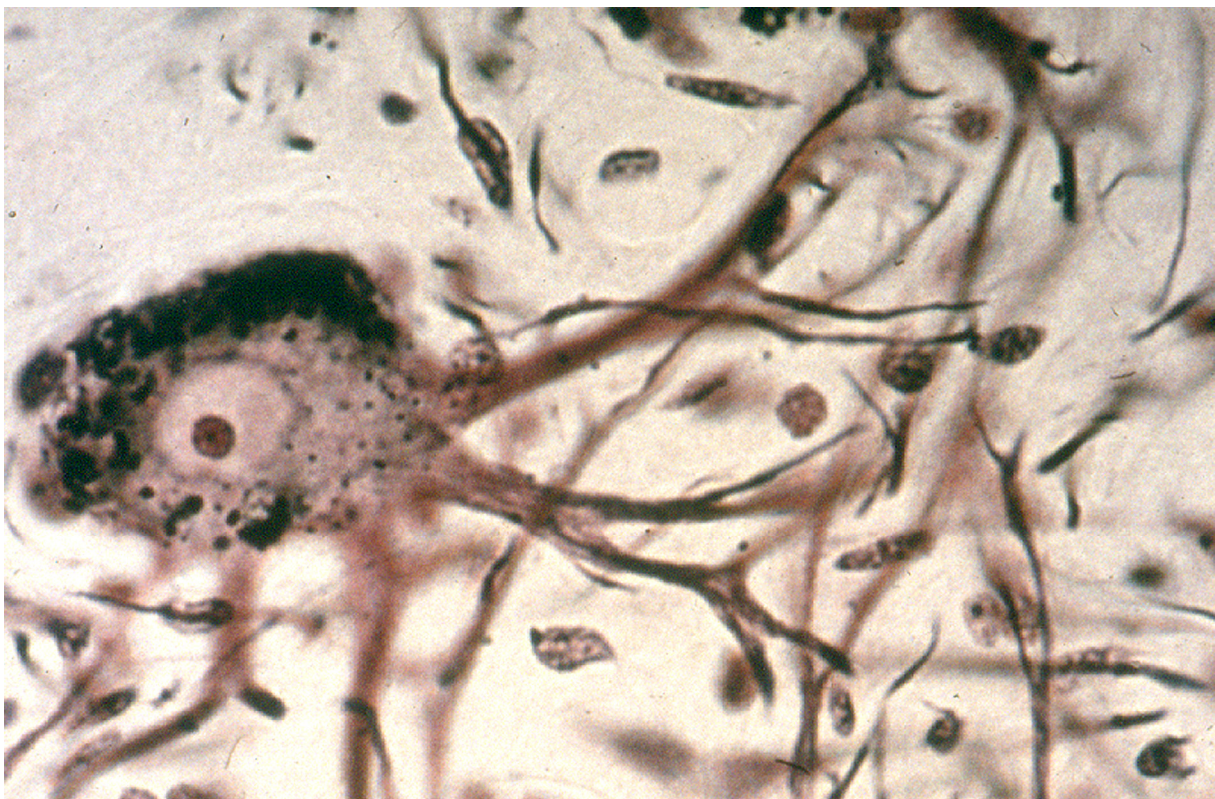


Figure 3: Microscopic view to a human neuron containing a nucleus and a nucleolus. At the upper side of the cell body intense endoplasmic reticulum in form as a Nissl body can be seen.

Further the nucleus is a centre triggering not only the mitotic cycle but many other cell biological functions. Using the light microscope the stained chromosomes can be clearly

recognized during the interphase between two cycles of mitosis.

The contained nucleolus within the nucleus is only seen in the interphase. This is an important sign for adherent cells in culture. When the nucleolus becomes visible the cells stay in the functional interphase to fulfil their physiological tasks.

In the most cases only a single nucleus is found within a cell. However, in some types of cells such as hepatocytes, osteoclasts, syncytiotrophoblasts and skeletal muscle fibers two or even more nuclei occur.

Working with adherent cells in culture one has to consider that they contact their neighbouring cells, the extracellular matrix or a multitude of very different biomaterials. Thus, adherent cells are social elements intensively communicating with each other, the contacts site and the individual fluid surrounding. It is obvious that all of these related functions have to be controlled by the nucleus.

**[Search: cell nucleus morphology]**

**Self-study: Inform yourself about the nucleus and nucleolus**

### **1.3. Plasma membrane and cytoplasm**

Roughly spoken, the plasma membrane is a sophisticated bag, which delimits the interior of the cell from the surrounding (Fig. 2.2). It consists of a phospholipid double layer, where respiratory gas molecules such as O<sub>2</sub> and CO<sub>2</sub> can freely diffuse. In contrast, for cytoplasmic proteins, amino acids, electrolytes, carbohydrates and water it is a functional barrier. When the plasma membrane is seen under the electron microscope, a tri-laminar structure can be detected with a dark-light-dark contrast. In the phospholipid layer numerous proteins are

inserted, which are involved in physiological tasks such as transport of molecules or transmission of signals.

An essential task for adherent cells in culture is to establish either the contact to the naturally occurring extracellular matrix, to the bottom of a culture dish or to an offered biomaterial. In case an optimal site for cell adhesion is recognized, for example special integral proteins such as integrins are inserted in the plasma membrane so that the contact to the adhesion substrate is intensified.

Molecular pumps or hormone receptors are mediators steering processes between the outer and inner side of the cell. However, a plasma membrane is not a rigid outer bag, but represents a very fragile envelope. As well single phospholipids as integral proteins are able to move within the plasma membrane so that they distribute all over the cell surface. Beside the phospholipids still other molecules such as cholesterol are contained, which mechanically stabilizes to a certain degree the plasma membrane.

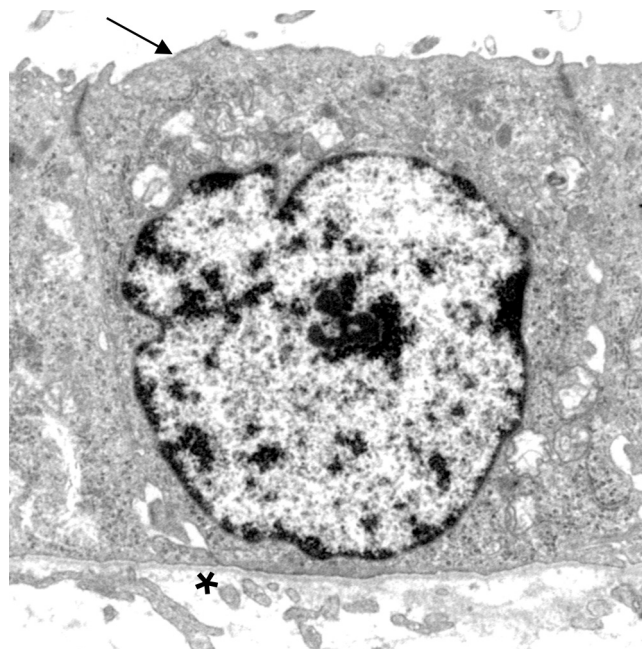


Figure 4: Transmission electron microscopic view to an epithelial cell. The luminal plasma membrane (arrow) borders a lumen, while the basal plasma membrane rests on a basal lamina (asterisk). In the centre of the cell a nucleus can be seen.

In the outer lipid layer of the plasma membrane different glycolipids and glycoproteins are contained. The carbohydrate residues protrude to the outer side of the cell to form the glycocalyx. The contained proteins include hydrophobic and hydrophilic residues. They are integrated within the plasma membrane or associated at the outer side. The hydrophobic parts of proteins are in contact with the lipid layer, while the hydrophilic parts protrude either outwards to the extracellular space or inwards to the cytoplasm. Numerous of these proteins are glycoproteins involved in the transport of electrolytes and amino acids, reception of hormone influences or anchorage of cells.

A main task of the plasma membrane is the function as a physiological barrier as recognized in epithelia (Fig. 4). It controls many active or passive transport features so that molecules can pass for example from the outside to the inside of the cells. A further important task is the capability to form tight junctions or gap junctions so that barrier functions in epithelia are established and communication between neighbouring cells becomes possible. All these characteristics serve the controlled exchange of molecules, the processing of signals and the recognition between cells and the surrounding extracellular matrix. All of these functions are essential, when isolated cells in culture form specialized tissues.

Many functions of a cell are triggered over the action of hormones. Especially the plasma membrane plays an important role (Fig. 2.2). Hydrophobic steroid hormones diffuse through the lipid layer to interact with their receptors localized at the inner side of the plasma membrane or within the nucleus. The resulting hormone-receptor-complex activates regulatory sequences at the DNA within the nucleus, which in turn leads to an up- or down-regulation in protein synthesis at the endoplasmic reticulum (Fig. 2.3).

A primary non-genomic response of steroid hormone action is observed after minutes, while the secondary genomic response needs many hours so that the response to the hormone can last for days.

However, non-steroid hormones can also act on cells within seconds. This quick non-genomic response is steered via four different groups of hormone receptors integrated in the plasma membrane (Fig. 2.2). Hormones such as calcitonin, adrenaline, vasopressin or glucagon act over receptors with 7 trans-membrane domains. The receptor is coupled to G regulatory proteins modulating in turn the amount of cyclic adenosine monophosphate (cAMP) or activating phospholipase C leading then to an increase of  $\text{Ca}^{++}$  within the cytoplasm.

Tyrosine kinase receptors are activated for example by insulin or different growth factors frequently used in cell culture. The receptor is a complex tetrameric molecule exhibiting a glycosylated domain at the outside of the plasma membrane and on the inner side tyrosine kinase domain. When the hormone is binding on the receptor a phosphorylation of tyrosine residues takes place inducing in turn an intracellular cascade of signals.

Binding of erythropoietin or many cytokines on the receptor is leading to a phosphorylation of the receptor and target proteins belonging to cytoplasmic tyrosine kinase. Hormones such as atrial natriuretic peptide (ANP) bind to a guanylcyclase receptor, which produces by the cytoplasmic guanylcyclase cyclic guanosine-3', 5'-monophosphate (cGMP) acting as second messenger.

It is shown that different hormones are acting on different kinds of receptors modulating the level of second messengers such as cAMP, cGMP or  $\text{Ca}^{++}$  within the cell. An increase in the concentration of the second messenger in turn stimulates specific cell biological reactions over intracellular signal cascades. The binding of the hormone on the receptor, the resulting signal cascade and the resulting reaction of the cell is known as transduction.

**[Search: cell cytoplasm plasma membrane morphology]**

**Self-study: Read about the action of hormones on cells**



## **1.4. Endoplasmic reticulum**

Ribosomes represent non-jacketed macromolecules consisting of proteins and ribonucleic acid (Fig. 2.3). On free ribosomes (polysomes) proteins are synthesized in the cytoplasm of the cell for its personal use. In contrast, within the endoplasmic reticulum proteins of the plasma membrane and secretory proteins are synthesized. The endoplasmic reticulum consists of a tubular network protruding to nearly all parts of the cell. At distinct sites numerous ribosomes are attached to the network. Those places are called rough Endoplasmic Reticulum (rER). In a neuron this site is called Nissl body (Fig. 3). The synthesis of glyco- and lipoproteins starts on ribosomes connected with a strand of messenger-RNA (mRNA) at the reticulum. The proteins are then transferred to the lumen of the endoplasmic reticulum.

Thus, on the one hand proteins for essential cell functions are synthesized, while on the other hand proteins for export are constructed. To this group belong proteins of the extracellular matrix urgently needed for cells to find the correct molecular site for adhesion.

**[Search: cell endoplasmic reticulum morphology]**

**Self-study: Inform yourself about the synthesis of proteins**

## **1.5. Golgi apparatus**

In close vicinity of the endoplasmic reticulum the Golgi apparatus is found (Fig. 2.4). Dependent on the cell type it consists of numerous dictyosomes and Golgi vesicles. The dictyosomes or Golgi areas appear in the electron microscopic view as piles of membranes surrounded by numerous vesicles. Within the Golgi apparatus synthesized proteins are processed, which were delivered from the endoplasmic reticulum. At this site specific

carbohydrates are linked to proteins so that glycoproteins or proteoglycans can arise. Proteins lacking the correct link with carbohydrates do not exhibit biological activity.

Since proteins of the extracellular matrix are glycosylated to a high degree, the Golgi apparatus plays an essential role for adherent cells not only in the organism but also for adherent cells in culture.

**[Search: cell Golgi apparatus morphology]**

**Self-study: Read about the function of the Golgi apparatus**

## **1.6. Mitochondria**

Living cells have to generate energy in form of adenosine triphosphate (ATP). This process happens within mitochondria including enzymes for the citrate cycle and the  $\beta$ -oxidation of fatty acids (Fig. 2.5). Cellular sites with an increased demand of energy attract mitochondria. For example, an increased folding of the plasma membrane indicates an increased deposition of pumps for the transport of electrolytes. In consequence, this process needs an increased amount of energy. As a result numerous mitochondria can be seen in close vicinity of the infolding of the plasma membrane by electron microscopy.

Since an intact cellular metabolism depends on a permanent synthesis of ATP, adherent cells in culture have to be permanently provided by a suitable culture medium and oxygen.

**[Search: cell mitochondria morphology]**

**Self-study: Read about the function of mitochondria**

## **1.7. Endosomes, lysosomes and peroxisomes**

The granula, endosomes and lysosomes belong to a heterogeneous group of cell organelles involved in a series of different synthetic and metabolic processes (Fig. 2.6). In granula for example synthesized proteins, hormones or glycogen can be stored. Endosomes and lysosomes are membrane vesicles containing specific enzymes for uptake, separation and digestion of molecules. On the one hand the internalized molecules are screened and transferred to the cytoplasm. On the other hand the lysosomes are a store for residual molecules recognized by light microscopy as pigment or lipofuszin granula. When lysosomal proteins are not enclosed in the endosomes but have contact with the cytoplasm, it leads to autolysis and destruction of the cell.

Beside many other molecules in a culture medium serum proteins can be contained. These proteins are internalized from the cells by endocytosis digested in turn in the endosomal compartment.

Peroxisomes are not found in all cell types. In contrast, some cells detected in liver and kidney contain numerous peroxisomes. The most important function is that they contain  $H_2O_2$  synthesizing oxidases and catalases playing essential roles during gluconeogenesis, fatty acid metabolism and detoxification.

**[Search: cell endosomes morphology]**

**Self-study: Find out about lysosomes and endosomes**

## **1.8. Cytoskeleton**

One important task is that the specific cytoskeleton of a cell are made to hold the numerous cell organelles such as nucleus, mitochondria, endoplasmic reticulum and the Golgi

apparatus in position (Fig. 5). In the organism the complex network is leading to the typical form of a cell known from neurons or epithelial cells. Further the spatial net of endoskeleton fibers helps to maintain communication from one cell pole to the other.

The cytoskeleton consists of microfilaments (Fig. 5.1), intermediate sized filaments (Fig. 5.2) and microtubuli (Fig. 5.3) forming a spider net to transport molecules through the cell and to maintain the inner and outer shape of the cell. The composition for each cell type is very specific.

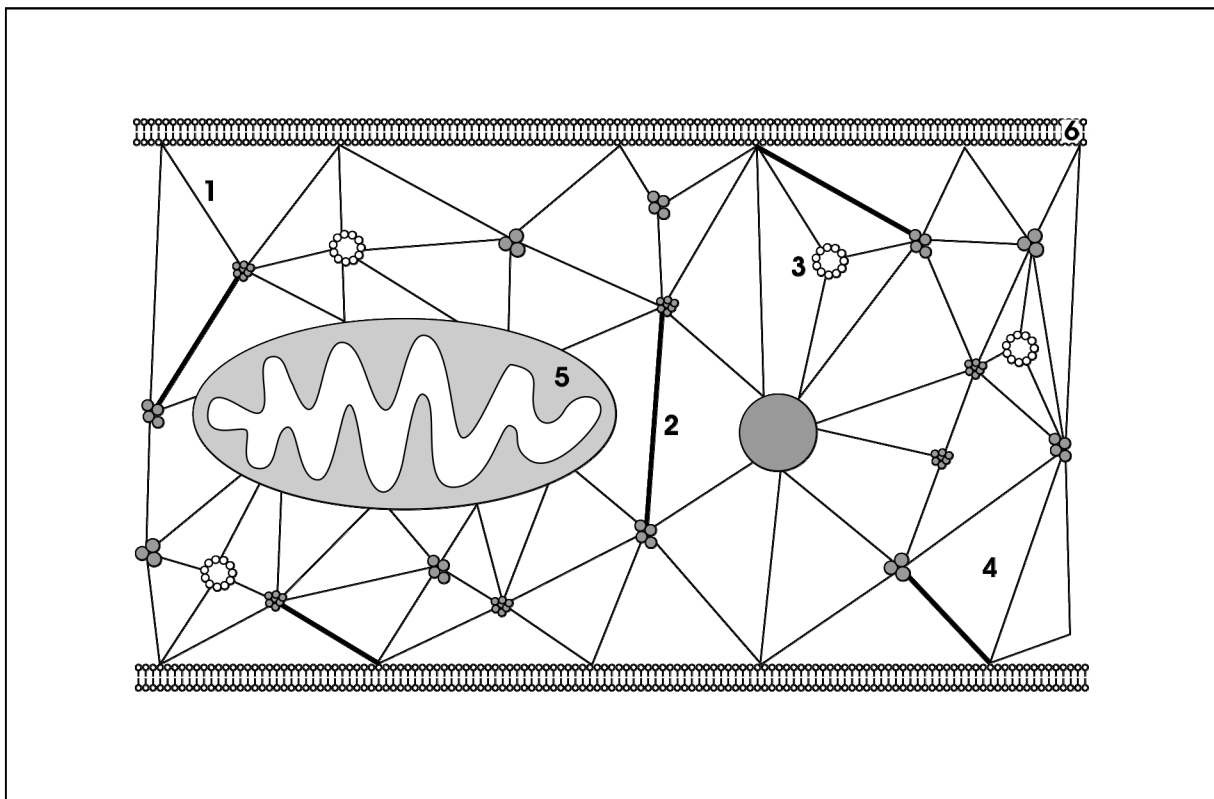


Figure 5: The cytoskeleton of a cell consists of microfilaments (1), intermediate sized filaments (2) and microtubuli (3) lining through the cytoplasm (4). The tree-dimensional web of these structures is keeping organelles such as nucleus or mitochondria (5) in position. All is covered by the plasma membrane (6)

When the culture conditions are optimal, for example adherent epithelial cells are able to express the same profile of cytokeratins as found within the organism. However, when the conditions are not optimal, the same cells will down-regulate individual cytokeratins or can even up-regulate atypical cytokeratins by de-differentiation.

Other compounds of the skeleton web are tubulin, actin, myosin, different kinds of cytokeratins, nexin, vimentin, desmin and finally the neurofilaments. Microtubuli for example are able to transport molecules within the cell. In neurons a transport takes place over dendrites or axons with a length of more than 1 meter. Also the synapses at the end of dendrites or axons have to be provided by the transport of molecules via the microtubulus system. The system transports molecules up to 400 mm per day and guarantees that also the most distant cellular sites are provided with all necessary molecules.

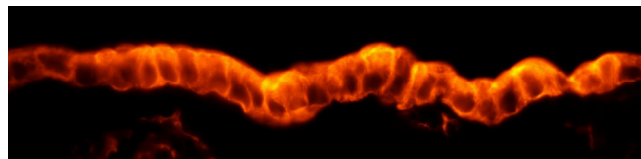


Figure 6: Immunohistochemical label of cytokeratin Endo-A (TROMA-I) is detected on a renal collecting duct epithelium kept for 13 days in perfusion culture. The same cytokeratin is expressed of the cells within the kidney.

Microfilaments such as actin and myosin are distributed within a cell in various amounts. Cells forming long protrusion exhibit more microfilaments than others. Intermediate sized filaments such as cytokeratins are forming endoskeletal structures in epithelial cells guaranteeing stability and equal morphological appearance (Fig. 6).

**[Search: cell cytoskeleton morphology]**

**Self-study: Read about cytokeratins, actin and myosin**

## 1.9. Cell division

When a tissue within an embryo is developing or when in the adult organism a tissue is regenerating multiple cells are observed showing mitotic division. An analogous observation is made, when a rich culture medium is offered. This treatment promotes multiple cell types

to divide so that an increasing number of cells is registered. Consequently numerous cell divisions are the prerequisite to rise the mass of cells in culture.

A division takes place at a certain point in the life cycle of a cell (Fig. 7). First a cell is doubling its content in DNA, then the cell is dividing in the course of mitosis. The nucleus itself is dividing during karyokinesis, while division of the cell body including organelles is made during cytokinesis.

The state of a differentiated cell within the interphase ( $G_0$ -phase) can be recognized in light microscopy by the formation of a distinct nucleolus (Fig. 4). When a cell decides to divide the  $G_1$ -phase is opened (Fig. 7). Yet important compounds such as RNA, proteins and lipids are synthesized in the course of 24 hours. This synthesis results in an increase of the cell volume. In the following S-phase the nucleus and the contained DNA is replicated. When this phase comes to the end, the cell starts with the  $G_2$ -phase to control mistakes in transcription. Now the replication of DNA is terminated and the cell is preparing for the ultimate task of division.

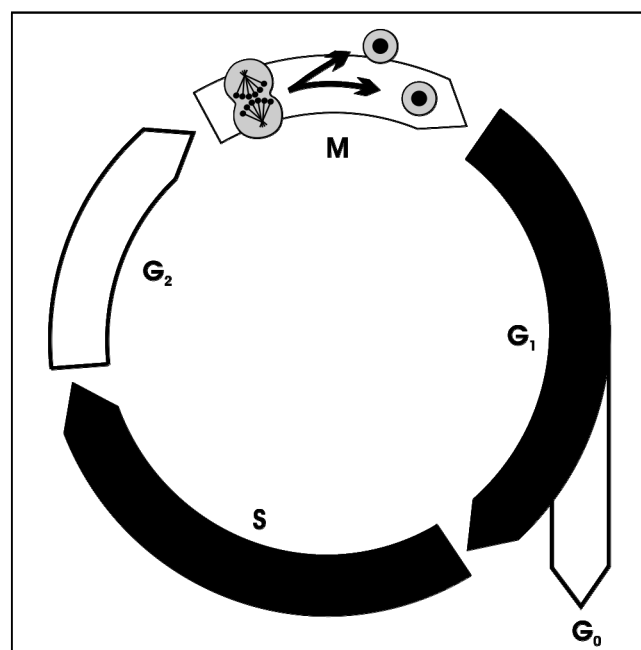


Figure 7: Schematic illustration of the cell cycle consisting of  $G_0$ -,  $G_1$ -, S- und  $G_2$ -phases. In the M-phase the ultimate division of the cell takes place, while in the  $G_0$ -phase (Interphase) functional features are developing.

The period of mitosis takes about four hours. In the starting prophase the DNA including histones is condensed so that for example 46 human chromosomes arise. At the centrioles the spindle for mitosis is forming (Fig. 2.8). The envelope of the nucleus and the nucleolus disappear (Fig. 2.1). In the metaphase the chromosomes are orientating at the equator of the cell indicating the geometrical site of cell division. At that time each of the chromosomes consists of two chromatides. In this state the short and long regions of the single chromosomes can be analyzed. In the further course of division the chromosomes are separating into the chromatides so that they can be transported in the anaphase to the both centrioles. In the following telophase a new envelope for the nucleus is synthesized. The cell division is terminated by a ring consisting of actin and myosin, which cuts the cell body in the middle. In this phase of cytokinesis each of the daughter cell receives a newly synthesized nucleus, the half of the cytoplasm and the necessary organelles. In so far the cell has copied her genetic material. After a clearly determined period within the interphase the cell is ready for a new cycle of cell division.

**[Search: cell mitotic cycle]**

**Self-study: Get updated about cell division**

## **1.10. Individual life cycle of a cell**

For the specialized tissues within an adult organism very specific differences in the frequency of mitosis are seen. Epithelial cells within the intestine can be renewed within days, while hepatocytes or tubule cells within the kidney normally divide after years. Skeletal muscle, cardiomyocytes and neurons do not divide within the life span. Thus, the renewal of cells takes place in a very individual time frame within days, month and up to many years. In contrast, cells are observed, which do not divide any more. These non proliferating cells stay in the G<sub>0</sub>- phase of the life cycle (Fig. 7).

Thus, depending on specialized tissues the differentiated cells remain for various periods of time in the interphase ( $G_0$ - phase) or  $G_1$ -phase. Up to date it is not known, which mechanism keeps cells in different tissues to remain over years respectively for hours in the interphase ( $G_0$ - phase) or  $G_1$ -Phase (Fig. 7). On cultured cells it could be shown for example that addition of foetal calf serum, application of growth factors or alteration of the electrolyte environment stimulates cells to promote from the interphase into the S-phase so that mitosis and proliferation proceeds.

An important question is the molecular mechanism promoting cells to go from the  $G_1$ - into the S-Phase of the cell cycle. Normally this step is inhibited by the protein Sic1. Sic1 is blocking a protein complex including kinases such as Cdk1. As long kinases are inhibited, the cells cannot reach the S-Phase. However, when Sic1 is phosphorylated multiple times, the step into the S-phase becomes possible. Yet the protein complex SCF aggregates together with ubiquitin and Sic1. Now Sic1 is degraded in the proteasome, while the S-phase can start.

**[Search: cell mitotic cycle arrest]**

**Self-study: Read about the cell cycle**



## 2. Cells contact extracellular matrix (ECM)

In an animal or human organism both anchorage dependent cells and non-anchorage dependent cells are present. Non-anchorage dependent cells are contained in the peripheral blood, represent erythrocytes and lymphocytes, which occur in the lumen of vessels.

Depending on the situation also stem/progenitor cells circulate within the peripheral blood. However, many of them are resident within the bone marrow or adhere within an organ or tissue specific stem/progenitor cell niche. Further on all sites of the organism and within individual tissues migrating cells such as lymphocytes, plasma cells and macrophages are recognized, which react upon degradation of cell products, infection by bacteria and exposure to antigens supporting thereby the immunological reaction.

Beside multiple blood cells an animal or human organism contains a multitude of anchorage dependent or even called adherent cells. They occur within the four basic tissues including nervous tissue (Fig. 8.1), epithelium (Fig. 8.2), connective tissue (Fig. 8.3) and muscular tissue (Fig. 8.4). A tissue is defined as a group of cells that work together to perform a special role in the structuring and functioning of the body.

It has also to be considered that cells within the individual tissues communicate with each other as well as the surrounding extracellular matrix and the interstitial fluid. The specialized tissues are widespread and contained in parenchyma organs, the cardiovascular system, the respiratory system, the urinary system and the brain. Within these structures about 200 different cell types are represented cooperating in a more or less close contact with a tissue or an organ specific extracellular matrix (ECM).

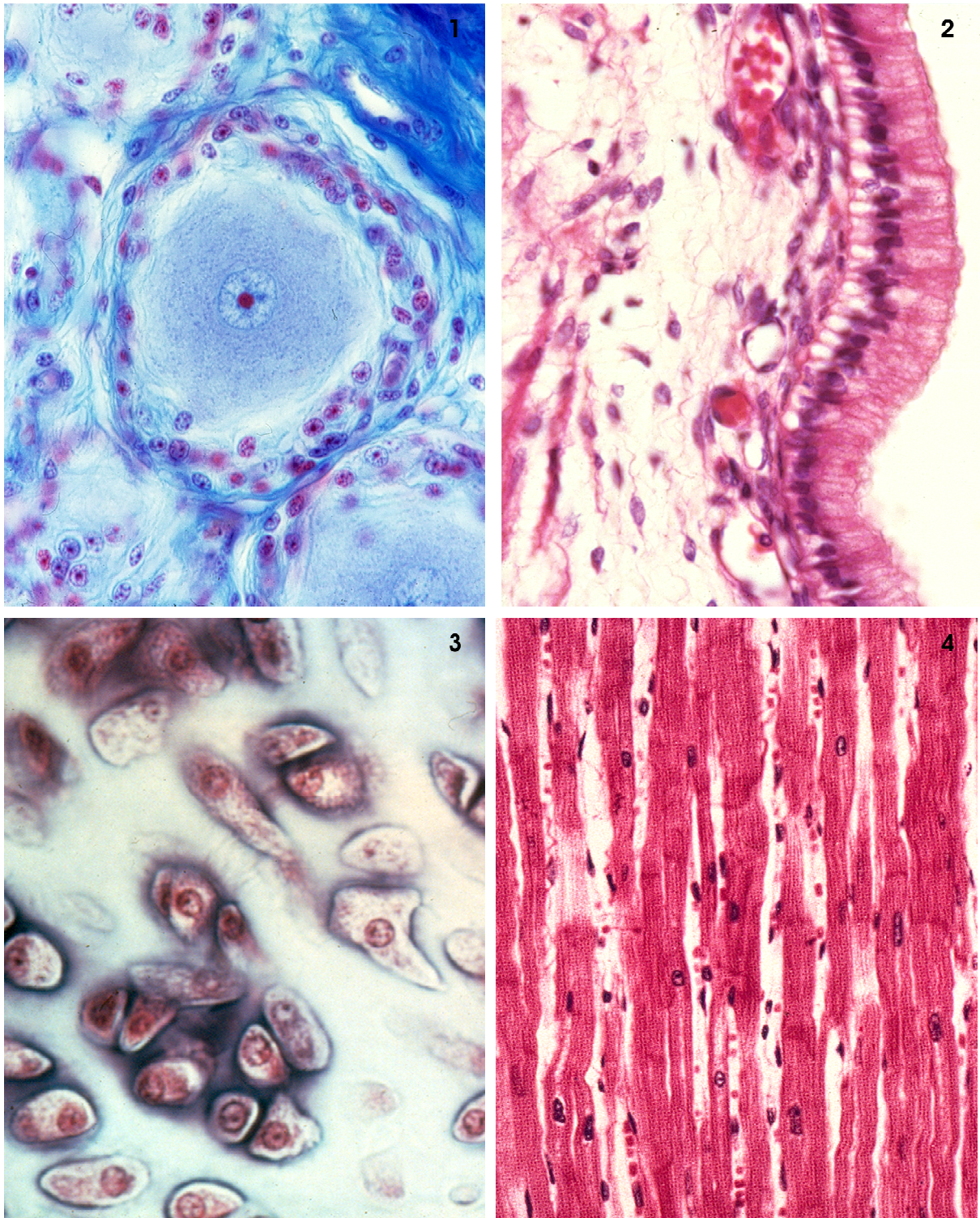


Figure 8: Microscopic illustration of the four basic tissues consisting of neural tissue (1), epithelium (2), connective tissue (3) and muscular tissue (4).

Anchorage dependent or even called adherent cells develop in the organism functional features, when they are in contact with a specific ECM. Under these conditions the cells start to form new ECM. However, frequently it happens that under culture conditions the synthesis

of intact extracellular matrix is lacking, decreased or disturbed. For example, epithelial cell lines such as LLC PK1 or MDCK synthesize proteins of the ECM, but they do not link them into a functional basal lamina as found at the basal aspect of epithelia within the organism. To balance the lack of an intact basal lamina synthesis, cells can be cultured in dishes coated with proteins of extracellular matrix. This technique is often chosen to offer an adequate anchor for the cells stimulating in turn the own synthesis of ECM proteins.

Much cell biological information is available about morphological features and physiological functions of individual cell types within tissues of the animal and human organism. However, relatively few information is given about the individual contacts between the plasma membrane of cells and the surrounding extracellular matrix. Anyway, beside multiple morphogenic information the extracellular matrix is of essential importance for the primary adhesion, anchorage, maintenance and ongoing spatial development of cells. For that reason detailed information about the construction and the related cell biological interactions with anchorage dependent cells shall be given in this context.

**[search: cell extracellular matrix]**

**Self-study: Read about extracellular matrix**

## **2.1. Structural characteristics of ECM**

All of the cells within the specialized tissues are surrounded by a surprisingly individual ECM. Synonymous terms for the extracellular matrix are pericellular matrix or intercellular substance. It consists mainly of fibrillar proteins found within collagenous, reticular und elastistic fibers. A minor portion contains globular proteins and specific matricellular molecules featuring a structural and functional connection between the plasma membrane of cells and ECM. The space between the fibers of extracellular matrix is filled with the interstitial fluid, the plasma membrane of cells and the outer side of vessels.



In light microscopy ECM is recognized as a more or less homogenously appearing material, which is synthesized in various amount and individual composition by nearly all of the anchorage dependent cells. Especially large portions of ECM are synthesized by connective tissue cells found within tendon, cartilage (Fig. 9.1) and bone (Fig. 9.2). Depending on daily variations of the water and electrolyte content in the body the biophysical state of ECM is either in a sol or in a gel like aggregate state resulting in turn in changes of molecular conformation status.

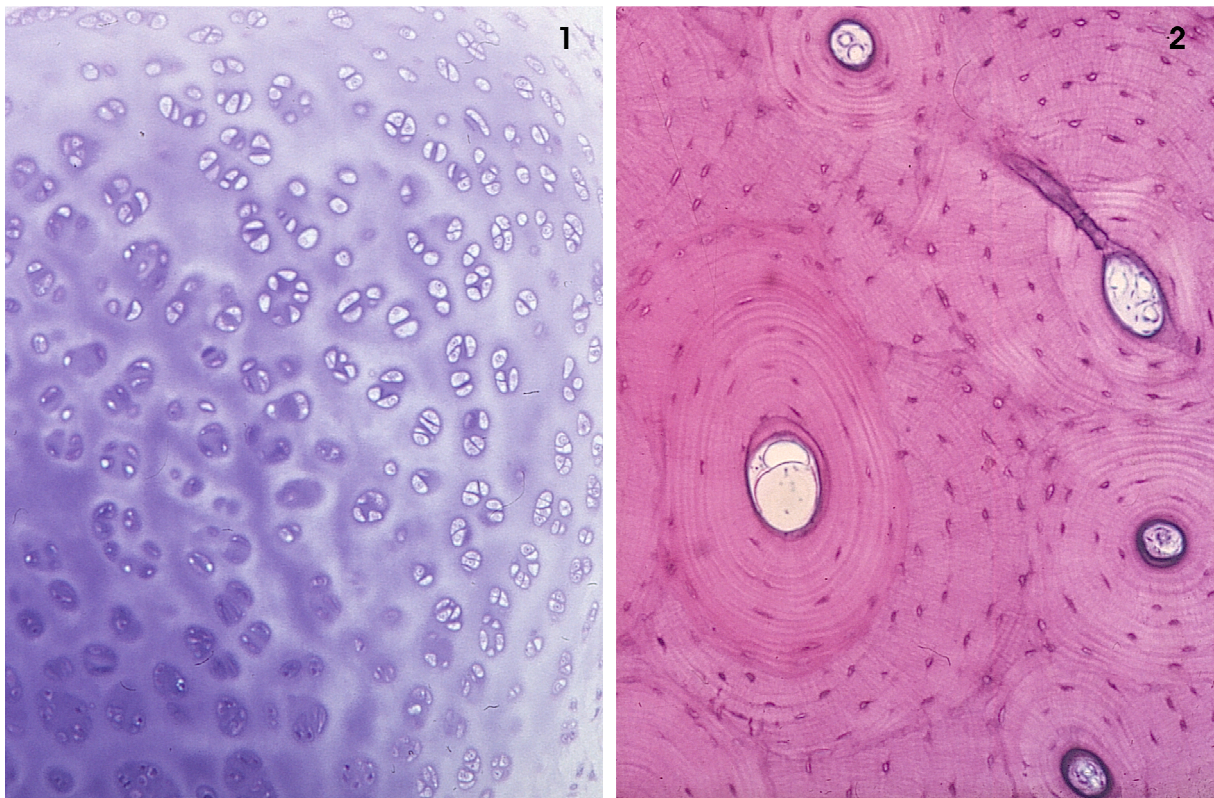


Figure 9: Microscopic illustration of hyaline cartilage (1) and bone (2). Between the cells a large amount of extracellular matrix can be recognized.

The comparison of ECM in various specialized tissues shows different composition and function. The web like structure consists of macromolecules found within collagenous, elastic and reticular fibers. The contained fibrillar proteins can be orientated in parallel or woven orientation. Linking molecules are hyaluronic acid and different kind of proteoglycans.

In the space within the three-dimensional meshwork of fibers interstitial fluid including electrolytes, water, nutrition and respiratory gas occurs. Depending on the individual tissue the content of water varies within the different species of ECM during the course of the day and the actual physiological situation. This feature makes it possible to compensate hydraulic pressure and mechanical strength.

The construction and degradation of ECM is a surprisingly dynamic process. Primary construction of it happens in a more or less temporary form. After appearance parts of ECM is degraded by matrix metalloproteinases. Into the arising molecular openings newly synthesized elements are secreted. These permanently repeating steps ranging between molecular construction and degradation are leading finally to the typical functional ECM recognized in the specialized tissues. However, depending on proceeding age the composition of the extracellular matrix is changing and degrading. A clear indication for this alteration process is for example arise of wrinkles in the skin.

**[search: cell extracellular matrix morphology]**

**Self-study: Get updated about metabolism of extracellular matrix**

## **2.2. Multifunctional tasks of ECM**

The ECM is not only a sophisticated skeleton element but has also a great meaning for the selective exchange of molecules between blood vessels and the different tissue layers. In the space between the fibers nutrition and respiratory gas is provided and elimination of metabolic products takes place via the permanent exchange of interstitial fluid. Last not least, the ECM contains multiple information motives so that development, differentiation and maintenance of distance in neighbouring cells and within tissues can take place.

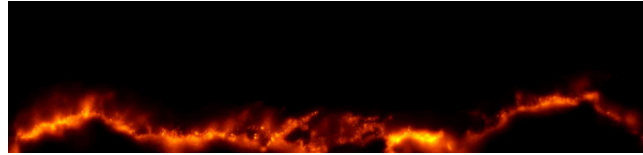


Figure 10: Immunohistochemical illustration of laminin  $\gamma 1$  in the basal lamina of a renal collecting duct epithelium kept for 14 days in perfusion culture.

The construction of ECM varies. At the interface between epithelia and the connective tissue the basal lamina as a special flat form of extracellular matrix is found (Fig. 10). At least six different genes are steering development for the basal lamina so that collagen type IV is woven into a fine meshwork localized at the basal aspect of epithelia. Collagen type IV is connected with a series of non-collagenous proteins such as different isoforms of laminin, nidogen and perlecan. On positions with strong mechanical stress collagens type XVII and VII are detected. Collagen type XVII is a transmembrane molecule, which is able to bind epithelial cells with fibrils onto the basal lamina. Focused sites of mounting can be found for example in hemidesmosomes connecting the plasma membrane of cells with the basal lamina. The collagens type XV and XVIII belong to the group of multiplexins (multiple triple-helix domains and interruption). Such proteins are detected in blood vessels at the basal lamina of the endothelium. When collagen type XVIII is digested with proteases, the process liberates heparin-binding fragments, which are able to prevent the spreading of new vessels. One of these fragments is endostatin bound to the carboxy-terminal end of collagen type XVIII. When this peptid is tested in culture, it prevents the multiplication and migration of endothelial cells. This effect can be used to prevent the growth of endothelial cells within a tumour.

In contrast to epithelia in muscular, connective and neural tissues a more close or a wider neighbourhood of cells is defined. This spatial distribution of cells determines in these specialized tissues the intercellular spaces, the synthesis of ECM and consequently the amount of contained interstitial fluid. Epithelia show between the lateral sides of cells a very narrow fluid space, which does not show any deposition of ECM. In connective tissue the

cells secrete large amounts of extracellular matrix in form of cartilage or bone matrix into the surrounding resulting in an extended interstitial space. However, such an increased deposition of ECM finally hinders the transport of nutrition and respiratory gas. For that reason permanent compression of cartilage must take place or multiple capillaries in bone must be synthesized so that unstirred layers of fluid are avoided and continuous provision with nutrition and respiratory gas is possible.

Regarding the numerous specialized tissues within an organism it can be seen that ECM is not homogeneously composed but exhibits in each tissue very individual properties. Cells of connective tissue occur within a multitude of three-dimensional matrices with mechanically resistant characteristics. In contrast, epithelial cells are attached to an extremely flat basal lamina, while myocytes are surrounded by a matrix with astonishingly elastic features.

For a long time it was believed that ECM reflects only a simple skeleton element harbouring cells in various densities. However, during the last years it became more and more evident that a close functional interaction exists between cells and the surrounding ECM. Exchange of molecular information occurs via peptide signal sequences integrated in ECM. The interaction starts, when special proteins such as integrins in the plasma membrane of adhering cells come in contact with these signal sequences. As a result the information is transmitted via the plasma membrane to the cytoplasm and then into the nucleus of the cell.

This signalling is able to activate or to inhibit signal cascades within the cell influencing thereby gene regulation and protein expression. By this mechanism not only various physiological cell functions but also the synthesis of ECM can be influenced. This effect in turn leads first to an increased degradation of extracellular matrix followed by an increased synthesis of ECM. The cellular activity is controlled by a process, which is called dynamic reciprocity. The same mechanism triggers in parallel mitotic activity, migration, attachment, adhesion, differentiation, dedifferentiation and apoptosis of anchorage dependent cells.

ECM consists not only of structural elements such as fibrillar collagens, reticular und elastic fibers but also of attached multiple glycoproteins, hyaluronic acid and glycosaminoglycans. Further growth factors, cytokines, matrix-degrading enzymes and related inhibitors are attached or integrated. Many of these molecules are acting in an interactive manner so that they influence not only internal cellular functions but also synthesis and degradation of ECM. However, the interactions are more complex than described here. Transforming growth factor  $\beta$  (TGF $\beta$ ) stimulates for example cells to synthesize extracellular matrix proteins, while at the same time matrix-degrading enzymes such metalloproteinases are inhibited.

Cells can show adhesion to ECM for a short period of time or they can show adherence for their whole life time. For such a long term binding on ECM specific binding sites on the surface of cells are necessary. In consequence, the molecules of extracellular matrix contain specific signal motives in form of amino acid sequences, which allow in turn binding of integrins occurring at the outer side and in the plasma membrane of cells. The most investigated motive is the tripeptide RGD (R for arginine, G for glycine und D for asparagine). This specific sequence of amino acids is for example contained in fibronectin so that cells can adhere to this molecule. The same motif sequence is present also in other proteins of ECM such as laminin, entactin, thrombin, tenascin, fibrinogen, vitronectin, collagens type I and VI, bone sialoprotein or osteopontin.

**[search: cell extracellular matrix morphology RGD]**

**Self-study: Read about signal motives in extracellular matrix**

## **2.3. Synthesis of ECM**

Anchorage dependent cells within specialized tissues synthesize proteins of ECM themselves or in combination with neighbouring cells. The cooperation of synthesis leads



then to a specific network so that cells can find the correct distance to each other resulting in a narrow aggregation or a wide separation. A perfect parallel deposition of collagen fibers can be organized by fibroblasts (Fig. 11)

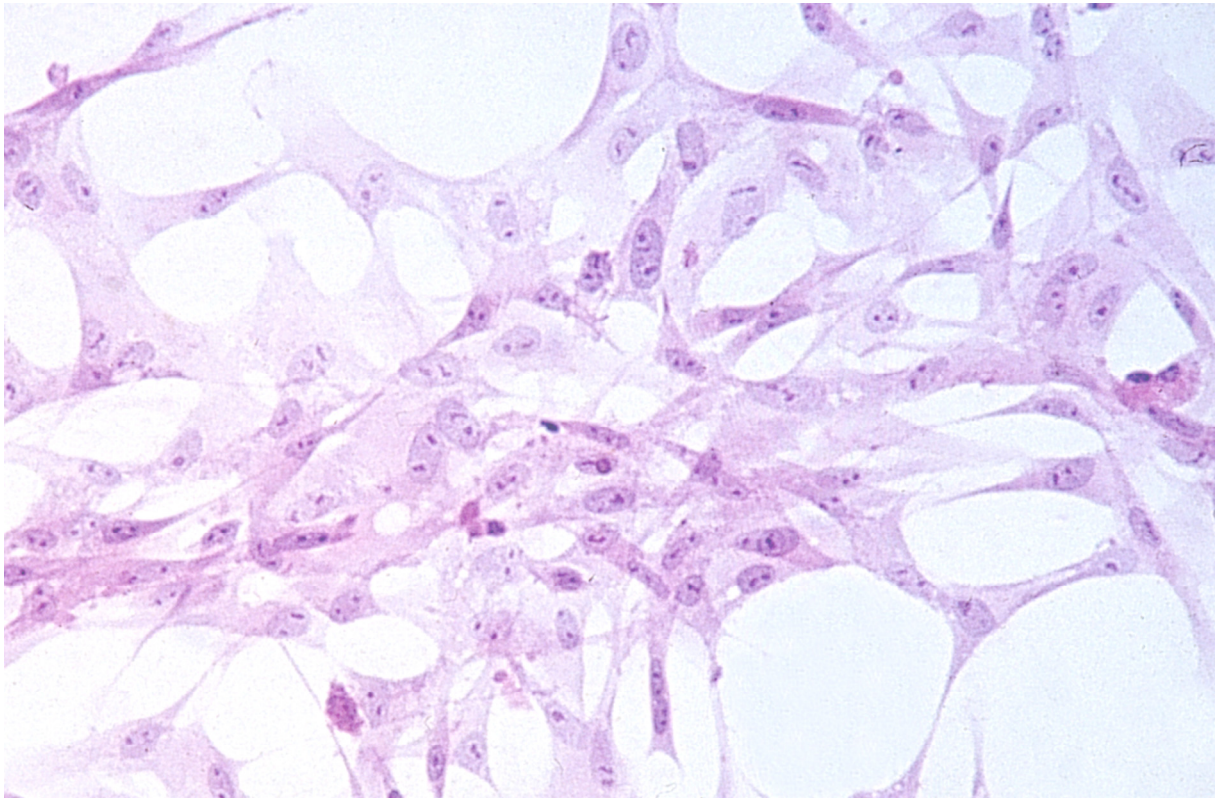


Figure 11: Microscopic view to numerous fibroblasts cultured at the bottom of a culture dish.

Independent from cellular orientation the basic molecules of ECM such as collagens, fibronectin and proteoglycans are constantly contained, while their individual amount and composition varies. The situation is complicated by the fact that more than 20 different collagens and collagen-like molecules exist. When these molecules are linked with each other or with fibronectin, laminin and proteoglycans a variety of three-dimensional possibilities of a network can appear. This variety of formulations explains the occurrence of an individual ECM in each specialized tissue. Moreover, beside the structural elements the contained amino acid sequences are an enormous source of information motifs for attachment, adhesion, adherence, migration and cell division.

It is evident that the synthesis of ECM must be a complex process. An insight in the complex synthesis and construction of ECM can be given by the synthesis of collagen. Since collagen molecules are barely soluble, the synthesis comprises three phases (Fig. 12). Within the cell collagen is synthesized in a soluble form (Fig. 12.1-3). Then collagen is secreted into the interstitial space (Fig. 12.4) and finally linked to other molecules in ECM far away from the cell (Fig. 12.5-7).

Collagenous molecules can be synthesized by many kinds of cells especially by fibroblasts on polyribosomes within the rough endoplasmatic reticulum (rER). First pro- $\alpha$ -polypeptides are synthesized containing special cargo signal sequences rich on amino acids such as proline and lysine (Fig. 12). The polypeptides are then incorporated into cisterna of the rER, where degradation of the signal sequence occurs. Yet a hydroxygroup is added to contained proline and lysine molecules by enzymes such as peptidyl-proline-hydroxylase and peptidyl-lysine-hydroxylase. The hydroxylation is followed by a glycosylation so that carbohydrates are linked with the hydroxylated group (Fig. 12.2). Specifically in the basal lamina of epithelia a high degree of such a glycosylation sites is found.

All of the fibrillar collagens consist of three subunits, which are called  $\alpha$ -chains. An  $\alpha$ -chain contains about 1000 amino acids. Thus, in the next step of collagen synthesis  $\alpha$ -chains are drilled with each other so that a tripple helical structure arises (Fig. 12.3). Collagen type II and III contain for example the three  $\alpha$ -chains (homotrimer), while collagens type I, V and XI contain different kinds of  $\alpha$ -chains (heterotrimer).

The synthesized collagens are due to their aliphatic fibrillar structure barely soluble molecules. After synthesis within the cytoplasm and excretion they must reach their target sites within the ECM and far away from the cell surface.

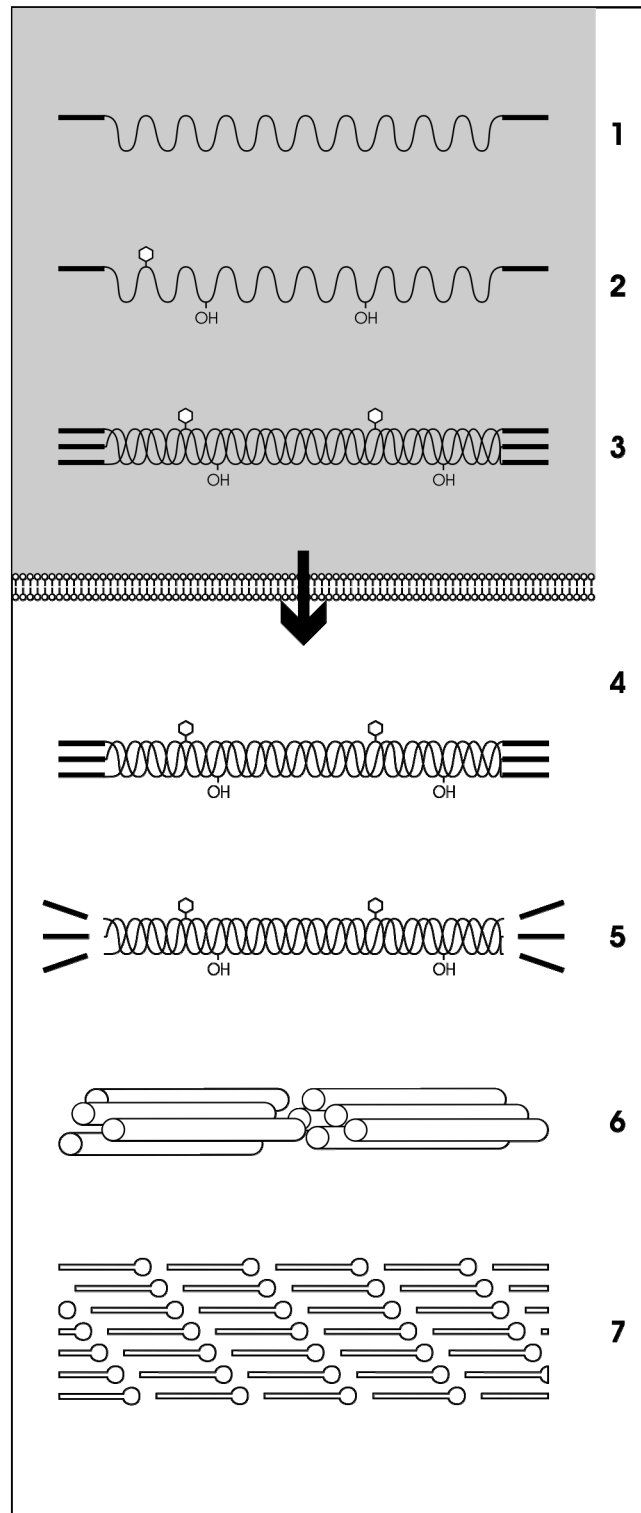


Figure 12: Illustration shows synthesis of collagen within the cell (1-3), secretion by exocytosis (arrow) and extracellular polymerization of fibers (4-7). Collagen synthesis starts for example with an  $\alpha$ -chain containing at the ends register peptides (1). Then hydroxylation and glycosylation takes place (2). In parallel a triple helix is formed (3). In the next step exocytosis of the procollagen occurs (4). In the interstitial space the register peptides are separated. The arising tropocollagen (5) is aggregating to primary filaments (6) and then to collagen fibers (7).

To facilitate the diffusion of collagen over a wide distance additional amino acids in form of register peptides are added within the endoplasmic reticulum on both ends of the  $\alpha$ -chains. This modification of the molecule increases its solubility and prevents at the same time a wrong polymerization with other molecules occurring in the vicinity. In the next step the molecules are processed from the Golgi compartment by exocytosis into the surrounding interstitial fluid (Fig. 12.4). The register peptides control thereby the process so that the three  $\alpha$ -chains can hold the right position to each other. During this process the helical structure must not precipitate.

After exocytosis of the molecule the register peptides at the carboxyterminal end are degraded by peptidases, while the register peptides at the aminoterminal end are dismantled later far away from the cell at the site of the aggregating collagen fibrils (Fig. 12.5). The molecule is yet called tropocollagen. After the complete degradation of the register peptides the molecule becomes insoluble so that it can be linked to primary filaments and to the arising collagen fibrils (Fig. 12.6).

By a parallel aggregation and covalent linking filaments, microfibrils and finally recognizable collagen fibrils arise (Fig. 12.7). The microfibrils can spontaneously appear by the aggregation of collagen type I und type III filaments, while other types of collagens do not show spontaneous aggregation. Of great importance during this aggregation process are glutaraldehyde groups, which arise by enzymatic desamination of lysine and hydroxylysine. This kind of cross linking is later essential for the mechanical strength of a collagen fibril within a tendon (Fig. 16). The limited healing of an unphysiological stretching can be mainly explained by a fragile cross linking of fibrils during regeneration.

The collagen fibers recognized in a microscope are heterotypically composed consisting of different kinds of collagen molecules. Fibers of collagen type I contains also collagen type V,

while collagen type II coexists with collagen type XI microfibrils. By a certain relation of mixture it is determined if more thin or more thick fibers of collagen develop.

When tissues or organs are exposed to an increased pressure or tension, mainly collagen type III (reticulin) or the heterotypically constructed elastin are synthesized in high amounts. This kind of extracellular matrix proteins is able to compensate an elastic deformation. Collagen type III is found as an endoskeleton element beside lymphatic tissues in many parenchyma organs such as liver, kidney or intestine. In contrast, elastine is found in arteries near the heart, within the skin and lung. Elastic fibers show a five times higher elasticity as compared to rubber. On the surface of elastin fibers fibrillins as a special kind of proteins are found. Fibrillins contain repeats of the amino acid sequence of EGF (epithelial growth factor) und TGF- $\beta$  (transforming growth factor  $\beta$ ).

**[search: cell extracellular matrix morphology synthesis]**

**Self-study: Inform yourself about synthesis of extracellular matrix**

## **2.4. Linking molecules in ECM**

ECM has to stand pressure and tension coming from all directions. To compensate optimally these mechanical and biophysical influences fibrillar collagens have to be linked three-dimensionally with other kinds of molecules (Fig. 13).

An important structural element in this coherence is fibronectin connecting cells and different kinds of proteins within ECM. Fibronectin is a glycoprotein consisting of dimers with a molecular weight of about 250 000. By alternative splicing different kinds of fibronectin are synthesized. Most interesting, the liver produces an isoform of fibronectin, which is circulating in soluble form within the blood serum. Within the different tissues a multitude of fibronectin

isoforms was detected. Each of the subunits is multiple times folded and contains repeats of amino acids, which are called FN1, FN2 und FN3. With the FN1/2 region the fibronectin molecule is able to bind to collagens, while the FN3 region contacts the cells. The contact sites are found within the fibronectin molecule on amino acid sequences Arg-Gly-Asp binding in turn to specific integrins found at the outer side of the plasma membrane of cells. Especially integrin  $\alpha 5 \beta 1$  located in the plasma membrane of individual cells shows a high affinity to fibronectin.

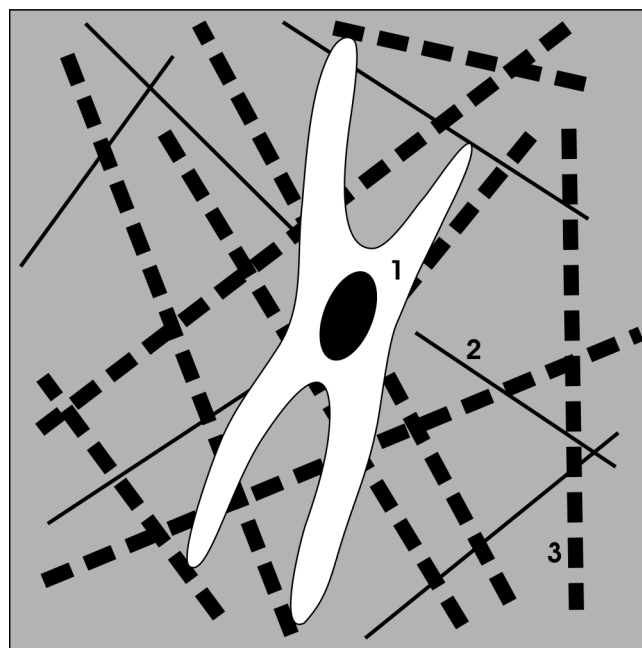


Figure 13: Schematic illustration of a fibroblast within a mesh of extracellular matrix proteins. The fibroblast (1) is able to contact elastin or collagen type III (2) beside collagen (3) fibers.

A further linking molecule within the ECM is laminin. Its molecular weight ranges between 140 000 und 400 000. Laminins are especially found within the basal lamina of epithelia and as a cover of skeleton muscle fibers. The molecule consists of an  $\alpha$ -,  $\beta$ - and  $\gamma$ -chain. By polymerization of the different chains cross-like molecules arise. On the short arm of the molecule repeating amino acid sequences are located as detected also in the epidermal growth factor (EGF) molecule. Between these repeating sequences globular domains are present, which bind in the presence of calcium to other proteins of the ECM.

Comparable to the fibronectin isoforms the laminins are able to bind on the one hand to collagens and on the other hand to proteins on the cell surface. Further it was shown that nidogen has a high linking affinity to laminins. The binding sites are found on the  $\gamma 1$ -chain in the crossing center of the molecule. The globular domain of laminin is binding on collagen type IV to increase further the mesh like polymerisation of the basal lamina. Beside nidogen also heparin, perlecan und fibulin-1 act as linking elements between laminins and collagens.

Beside the classical collagens with typical triple-helical structure also molecules in the ECM are contained exhibiting globular protein structure. To this group belong collagens type IX, XII, XIV and XIX. Collagen type IX is found for example on the surface of collagen fibers type II and XI. The elongated part of the molecule is attached in parallel to the course of fibers, while the short part protrudes into the perifibrillar space. It is believed that collagen type IX contacts on the one hand neighbouring fibers and on the other hand other proteins within the surrounding ECM. Also from collagen type IV it is known that it binds on the one site of the molecule heparin sulphate and on the other site decorin linking in turn collagen fibers.

Further high molecular weight proteoglycans have to be mentioned, since they fulfil important physiological tasks in ECM. Aggrecan und versican are bridging together with hyaluronic acid wide distances within ECM of cartilage. Syndecan is localized within the plasma membrane of cells serving here as an anchor. Perlecan is found within the basal lamina of epithelia and in the pericellular matrix of many other cell types. In the liver perlecan is synthesized by endothelial cells occurring in the sinusoides. Syndecan is a transmembrane protein connecting the cell surface with molecules contained in ECM such as growth factors, inhibitors of proteases and different kinds of enzymes. Small proteoglycans such as decorin, biglycan, lumican und fibromodulin interact with numerous molecules of ECM. Decorin for example binds to collagen fibers playing in this coherence an important role during ECM protein synthesis, polymerization and spatial construction.

Hyaluronic acid is detected in nearly all kinds of extracellular matrix. The molecule acts as a ligand for the cartilage linking protein, aggrecan und versican. Also anchor proteins on the cell surface such as CD44 are able to bind hyaluronic acid influencing thereby cell proliferation and migration. During the development of tissues deposition of hyaluronic acid molecules leads to spaces not filled with cells. By the activation of hyaluronidase these spaces can be opened so that cells are attracted to form new niches for invading cells so that tissue structures can be formed.

Some proteins or fragments of the extracellular matrix are able to control angiogenesis. For example, endostatin is a derivative of collagen type XVIII that inhibits angiogenesis. Anastellin fragment IIIIC is a peptide within the first type III repeat in fibronectin that inhibits angiogenesis. Canstatin is a fragment of type IV collagen, which blocks endothelial cell migration and proliferation. Finally, restin is the C-terminal fragment of the alpha-chain of collagen type XV that specifically inhibits endothelial cell migration. It is obvious that the presence or absence of such molecules strongly influence the degree of vascularisation within tissues.

**[search: cell extracellular matrix morphology synthesis fiber]**

**Self-study: Inform yourself about extracellular matrix in specialized tissues**



### 3. Crosstalk between cells and ECM

It is barely to believe but during culture of anchorage dependent cells and during development of tissues a spider like net is built up for communication via cell to cell and cell to ECM interactions. Mechanical stability, recognition of morphogenic information and constant flow of interstitial fluid is essential for the maintenance of neighbourhood between cells. The distance between cells can be for example close in epithelia (Fig. 8.2) and extremely wide in cartilage (Fig. 8.3, 9.1) or bone (Fig. 9.2).

**[Search: cell extracellular matrix anchorage]**

**Self-study: Get updated about contact between cells and extracellular matrix**

#### 3.1. Cells in contact with cells

A homophil contact between the same kind of cells is controlled by cadherins acting between the lateral plasma membranes. This intense contact needs the presence of  $\text{Ca}^{++}$  ions. When the extracellular  $\text{Ca}^{++}$  surrounding is disturbed for example by chelating molecules such as ethylenediamine tetraacetic acid (EDTA), the contacts to neighbouring cells are interfered. In contrast, the binding between cadherins and the surrounding ECM is negligible.

Selectins belong to a group of plasma membrane proteins modulating in the presence of  $\text{Ca}^{++}$  ions the heterophil contact between different kinds of cells. The selectin molecules exhibit lectin-like features recognizing short chains of carbohydrates such as Sialyl Lewis X/A on the cell surface of their binding partners.

Cell Adhesion Molecules (CAM) have homophil and heterophil binding characteristics so that they are able to contact the same and different kinds of cells. Their contact to neighbouring cells is not dependent on the presence of  $\text{Ca}^{++}$  ions.

Of special importance is the tight junction complex found at the border between the luminal and lateral plasma membrane in an epithelium (Fig. 14). The tight junction represents the physiological sealing so that molecules cannot pass uncontrolled the intercellular space. In consequence, all of the related molecules have to cross the cell via the plasma membrane.

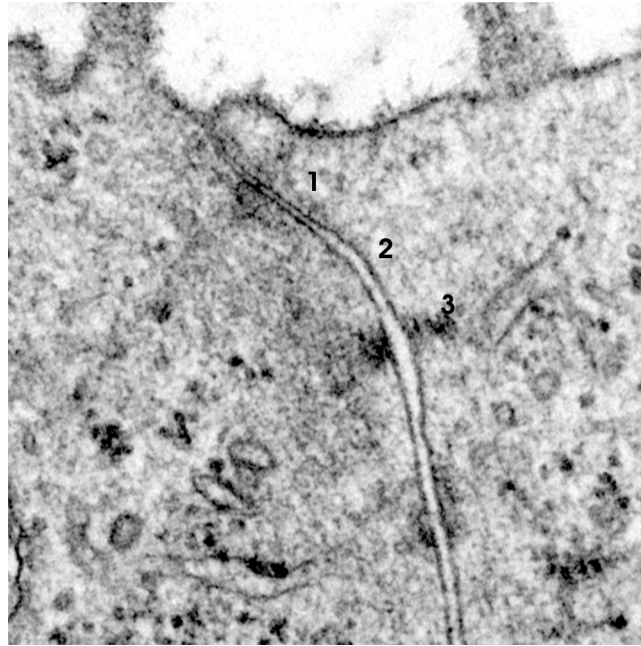


Figure 14: Electron microscopy of the tight junction complex at the border between the luminal and lateral plasma membrane of epithelial cells. The tight junction belt consists of zona occludens (1), zona adhaerens (2) and a desmosome (3).

Often desmosomes are found between neighbouring cells, while hemi-desmosomes are seen between the plasma membrane and the basal lamina. Both can be recognized as a plaque at the inner side of the plasma membrane. In so far desmosomes are mechanical contacts.

Gap junctions serve for the exchange of small molecules and functional, electric and morphogenic information. Numerous gap junctions are found between epithelial cells, cardiomyocytes, smooth muscle cells and osteocytes.

Further cells within the cornea and cells surrounding the oocyte do communicate over gap junctions.

**[search: cell cell contact homophil extracellular matrix]**

**Self-study: Read about cell to cell contacts**

### **3.2. Cells contact ECM**

It is depending on the individual cell type and the kind of specialized tissue if the contact of cells to ECM is loose or narrow. On focal but also on continuous plasma membrane contacts with ECM special anchor proteins are concentrated. At these sites integral plasma membrane proteins of the cell containing specific amino acid sequences exhibit a high affinity to ECM. Such integrins are heterodimer molecules consisting of two different  $\alpha$ - and  $\beta$ -subunits (Fig. 15). From each subunit again 8 isoforms exist, which can interact in various combinations with ECM. These multiple kinds of combination explain that individual cells can bind to individual proteins within the ECM. The constellation of  $\alpha$ - und  $\beta$ -subunits is not constant but varies depending on cell type and developmental stage of tissue.

Collagens are widely distributed proteins within ECM. An anchorage of a cell to collagens is made over integrins  $\alpha1\beta1$ ,  $\alpha2\beta1$  and  $\alpha3\beta1$  incorporated in the plasma membrane. The collagens in turn are linked with fibronectin, which exists in multiple splice variants. However, nearly all of the cells can interact with fibronectin over integrin  $\alpha5\beta1$ . A further binding is possible over integrin  $\alpha v\beta3$ , which interacts more with fibronectin than with collagen. Vitronectin ist a multifunctional protein within ECM, which is an anchor for integrins  $\alpha v\beta3$ ,  $\alpha v\beta1$  und  $\alpha11\beta3$ . Laminin is restricted to the basal lamina of epithelia and skeletal muscle fibers. It is able to bind a multitude of integrins so that related cells are mechanically fixed to ECM.

Integrin  $\beta 2$  is mainly involved in processes of the cell to cell recognition, while integrins  $\beta 1$  and  $\beta 3$  act between cells and the ECM. Predominantly integrins  $\beta 1$  and  $\beta 3$  are able to interact with a series of extracellular matrix proteins such as collagens, fibronectin, vitronectin and laminin. Normally integrin  $\beta 1$  is found in cells of connective tissue searching contacts with fibronectin, laminin and collagen. In contrast, integrin  $\beta 3$  within the blood vessel system shows intensive binding with fibrinogen, von Willebrand-faktor, thrombospondin and vitronectin.

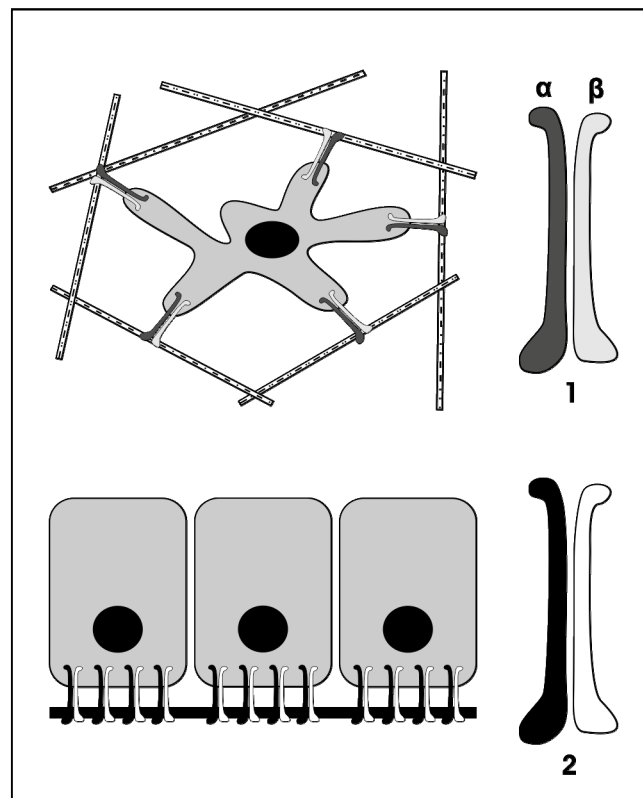


Figure 15: Anchorage between cells and ECM. The illustration depicts the binding over specific integrins of a connective tissue cell within the pericellular matrix (1) and between epithelial cells on a basal lamina (2).

The integrin  $\beta 3$  for example binds endothelial cells resulting in a close affinity to the extracellular matrix of a vessel. In consequence, the streaming of the blood is not able to disturb this cell to ECM interaction so that binding of endothelial cells stays during long periods of time on the site of anchorage.

When new polymers for vessel prosthesis are tested in culture, it may happen that the typical integrin is not expressed or that atypical integrins are synthesized as detected by Western blot assays or immunohistochemistry. In this case the endothelial cells do not further maintain adherence to the selected polymer. In consequence, the surface of the polymer has to be optimized, until endothelial cells do accept the molecular surface structure. In turn these cells will express the correct kind of integrins so that an intense connection between polymer material and endothelial cells is built up and maintained. A comparable reaction is known for osteoblasts producing only within the optimal scaffold the correct integrins leading in turn to an increased synthesis of collagen typ I followed by an intense calcification.

**[search: cell contact integrin extracellular matrix]**

**Self-study: Inform yourself about the contact between cells and extracellular matrix**

### **3.3. ECM contacts cells**

The contact between ECM and cells is often seen as a pure mechanical fastening. However, it is in cell biological sight much more, since a functional coupling exists between ECM and adherent cells. Most of these cellular mechanisms are controlled by Extracellular signal Regulated Kinases (ERK) and Mitogen Activated Protein kinases (MAP). Dependent on the signals coming from ECM the resulting reactions influence a variety of functions such as cell adhesion, adherence, mitotic activity and the period of the functional interphase. Thus, the interaction between cells and the related ECM determines the future cellular functions.

It has been shown that the binding between integrins of the cell and the contact to ECM is dependent on oligopeptide sequences. The amino acid sequences for integrin binding on individual proteins of ECM are orientated either in a linear or cyclic fashion. A well known

sequence contains the RGD motif (R for arginine; G for glycine; D for aspartic acid). This motif was detected in fibronectin and is able to bind not a single but a series of integrins. The experiments further showed that the binding is very specific. However, binding on the RGD motif will only occur, when the correct amino acid sequence is recognized. When the position of a single amino acid is altered, the binding of cells via integrins to the RGD motif is lacking.

Beside the highly specific binding between cells via integrins to ECM via the RGD motif also a less specific one by a heparin-binding domain is possible. In this case proteoglycans containing heparin and/or chondroitinsulphate on the cell surface acting as an anchor. A further typical example for a multifunctional binding is the Neural Cell Adhesion Molecule (NCAM). This molecule is known for its ability to mediate cell adhesion via downstream signalling such as binding of Glial derived neurotrophic Factor Receptor (GFR) resulting in cell migration, development of morphology and synapse formation.

For the synthesis and spatial construction of ECM very different molecules are needed. Further one has to consider that the management of synthesis depends always on individual cell types and occurs always in a tissue specific manner. On the one hand ECM does degrade on demand and on the other hand it must stand over long periods of time in a stable form (Fig. 16). In consequence, the attached cells have to control by a permanent interaction the surrounding ECM. Problematic in this coherence is that ECM is not completed after synthesis for the whole period of life, but must be renewed in a permanently repeating and stepwise process. Beside degradation by proteinases a continuous linking of proteins occurs in parallel for example via an activation of transglutaminases. In turn proteases degrade matrix so that new ECM is synthesized at this specific molecular site.

In consequence, the environment of cells is so attractively constructed that their integrins are searching always contact binding sites on suitable collagens, glycosaminoglycans, fibronectin and laminin. It is evident that these molecules must be present in close

neighbourhood. When a contact is made, the resulting signals can tell the cells to stay and to settle for a long period of time. When these horizontal and vertical signals are not present, the cells will migrate to search a more attractive place for seeding. This process happens during spreading of cells within a culture dish or during seeding of cells within a scaffold.

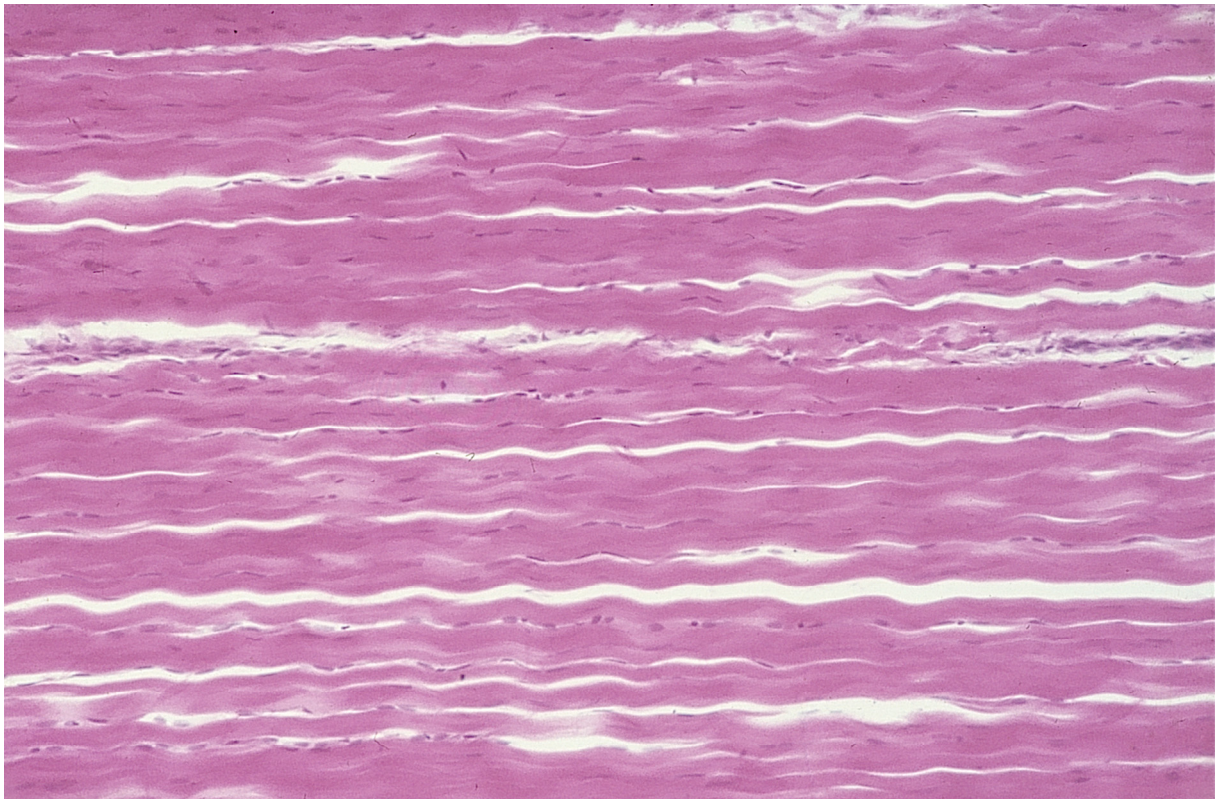


Figure 16: Illustration demonstrates numerous fibroblasts within wave like bundles of collagen of a tendon.

An anchorage dependent cell needs further permanent cell biological information from the contacting ECM. In turn these data are leading to an intense synthesis or even to a degradation of ECM. A special meaning in this coherence has metalloproteinases such as collagenases, gelatinases, serinproteases, kathepsin and plasmin secreted by the surrounding cells. After cleavage of ECM molecules is made by these enzymes new space within the extracellular matrix is created so that newly synthesized proteins such as fibronectin or chondronectin can be inserted in aged collagen structures.

During the period of ECM renewal cells can migrate to intensify the process of restoration. An important task in this continuous process is the functional fixation of different kinds of growth factors. Normally growth factors are present in ECM but they are inactivated for most of the time. In the case of restoration the factors are activated on demand so that they are bioavailable only for a short period of time for the surrounding cells. For example, factors such as basic Fibroblast Growth Factor (bFGF) or Vascular Endothelial Growth Factor (VEGF) bind for most of the time with high affinity to heparin integrated in ECM. As long as the growth factor is attached to heparin, it cannot show any biological activity. However, when the matrix including heparin is degraded by heparinase, the growth factor is activated so that it can exhibit biological activity.

**[search: cell integrin interaction extracellular matrix]**

**Self-study: Read about molecular contacts between extracellular matrix and cells**

### **3.4. ECM controls proliferation**

Between cells and ECM exist on the one hand a mechanical contact and on the other hand numerous reciprocal interactions, which are influencing important physiological functions. The signals are mediated via amino acid motifs integrated in the site of anchorage on ECM. An important meaning has this signal transduction upon proliferation. For example, culture of cells on a laminin coat stimulates an intense proliferation. This effect is due to the  $\alpha$ -chain of laminin, where numerous sequence repeats of Epithelial Growth Factor (EGF) are integrated. When these motifs become accessible, in turn a strong effect on proliferation of numerous cells can be observed.

In contrast, an inhibition on proliferation of cells can be shown by the administration of heparin. When endothelial cells of the aorta are cultured in a medium containing heparin,



proliferation is decreased as compared to cultures with a medium lacking heparin. In contrast, when the heparin coat is treated with heparinase, proliferation of cells is increased. However, applying chondroitinase or other proteases does not induce this effect speaking for a specific action of heparinase.

Another example is the culture of cells derived from the lactoferric duct of the mamma. After isolation these cells permanently divide, when they are cultured in an uncoated dish. In contrast, when the surface is coated by proteins of the ECM, the proliferation rate is strongly reduced. With cultured hepatocytes one could further show that coating of a dish with ECM inhibits activity of immediate early growth response genes, while at the same time C/EBP $\alpha$  is induced leading to the up-regulation of other genes steering the functional cell metabolism.

**[search: cell mitotic cycle extracellular matrix]**

**Self-study: Get updated about cell division and extracellular matrix**

### **3.5. ECM influences development**

Hepatocytes develop many functional features, when they are cultured in dishes coated by ECM derived from Engelbreth Holm Swarm tumour (EHS) cells. This mixture is found for example in Matrigel®. When cells are cultured in such dishes, they are exposed to numerous extracellular matrix proteins including a high amount of laminin. Surprisingly, transcription factors such as eE-TF, eG-TF/HNF-3 and eH-TF are only activated, when cells stay for a longer while in contact with the EHS matrix. Similar results were obtained with epithelial cells of the lactoferric duct of the mamma. In uncoated culture dishes the cells express only a minimum of tissue specific proteins. In contrast, keeping these cells within a dish coated by EHS matrix as found in Matrigel®, alveolar structures arise and expression of typical milk proteins such as casein is up-regulated. Regarding the course of development the cells show

first a change in morphology paralleled by a new program of cytoskeleton proteins. In a secondary step the binding of cells via  $\beta$ 1-integrin to ECM activates a tyrosine kinase signal leading finally to the synthesis of  $\beta$ -casein.

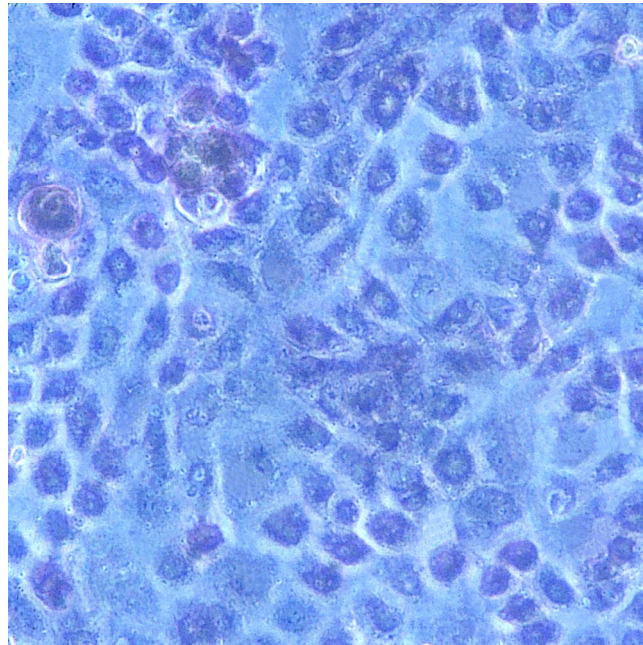


Figure 17: Microscopic view to hepatocytes cultured on the bottom of a dish.

A comparable signaling was observed in hepatocytes cultured in contact with extracellular matrix proteins (Fig. 17). The binding between ECM and  $\beta$ 1-integrin molecules leads to a cytoplasmic signaling resulting finally in the synthesis of albumin. It could further be shown that the coating of a dish with low concentrations of fibronectin or laminin stimulates the synthesis of albumin, while higher concentrations inhibit the synthesis of albumin but stimulate mitotic activity.

During development of tissues beside intense interaction of cells with ECM a cooperation with growth factors is essential. Basic Fibroblast Growth Factor (bFGF), interleukines (IL-1, IL-2, IL-6), Hepatocyte Growth Factor (HGF), Platelet Derived Growth Factor (PDGF-AA) and Transforming Growth Factor (TGF  $\beta$ ) are found in relatively big amounts within the ECM. As

described before their bioavailability is not accidentally but is controlled and depends on demand.

A close interaction between cells, ECM and TGF  $\beta$  can be demonstrated during development of the mammary gland. During pregnancy the epithelial cells have to multiply in a first step so that elongation and arborisation of the ductuli lactiferi can take place. This occurrence is proceeding in close cooperation with the surrounding ECM. After a mitotic phase the extended mammary gland has to develop alveoli with highly differentiated cells. The growth of the gland is now terminated and has to be stabilized by the surrounding ECM. During this important phase the epithelium is synthesizing TGF  $\beta$  inhibiting proliferation. In parallel the enzymatic degradation of ECM by the metalloproteinase stromelysin-1 is inhibited. In contrast, the occurrence of TGF  $\beta$  cannot be shown in newly developing areas of the gland so that proliferation becomes possible at this specific site. Also at this site enzymes for the degradation of ECM are not inhibited. By this mechanism the lactiferic system of the mammary gland can expand by successive activation of cell proliferation and synthesis of ECM.

During angiogenesis the formation of new blood vessels in cooperation with ECM is of great importance (Fig. 18). To find a suitable path for blood vessel formation, endothelial cells migrate along a cue of extracellular matrix proteins. When such a matrix is offered at the flat coat at the bottom of a culture dish, formation of three-dimensional capillaries will not occur.

In contrast, intense formation of capillaries is observed, when endothelial cells are completely embedded in a matrix derived from Engelbreth Holm Swarm (EHS) tumour cells. This kind of ECM contains beside laminin collagen type IV, different proteoglycans and entactin. However, the development of capillaries can be prevented, when the embedding of cells is not performed with EHS matrix but with collagen type I.

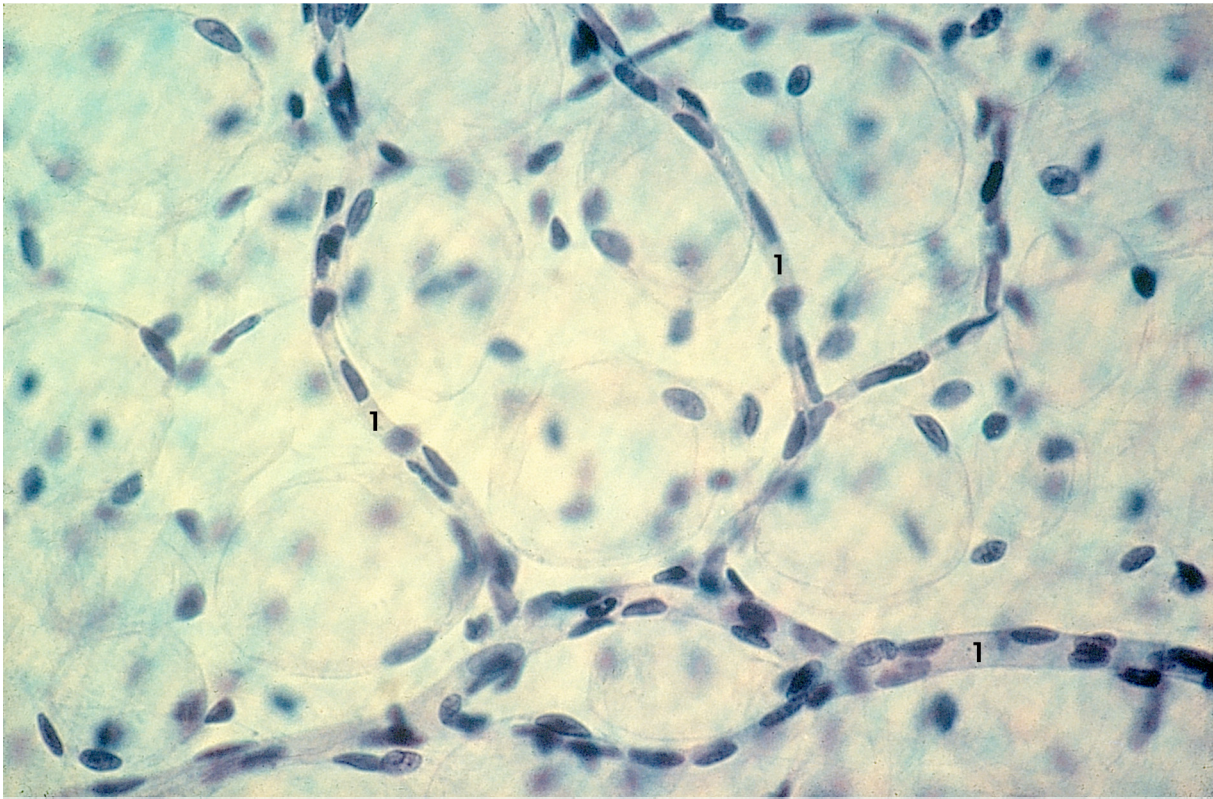


Figure 18: Microscopic view depicts capillaries (1) growing within tissue.

Also laminin has an influence on the development of vessel structures. When endothelial cells are embedded in an EHS matrix containing an antibody against laminin, the formation of capillaries is lacking. It appears that the contact to an intact laminin molecule is essential for the formation of capillaries. Formation of tubules can also be observed, when only a peptide is used containing the amino acid sequence SIKVAV of the  $\alpha$ -chain of laminin. In contrast, in the  $\beta$ -chain of laminin the amino acid sequence CDPGYIGSR-NH<sub>2</sub> is found. Applying this peptide in culture, formation of capillaries is prevented in all cases. Although the exact molecular mechanism of this reaction is not known in detail, it is surprising that within a molecule of laminin on the one hand promoting and on the other hand inhibiting amino acid sequences for development are contained.

Also the development of neural cells needs an intense contact to ECM. With cultured neurons it could be shown that coating of dishes with laminin results in much longer dendrites and axons than found after coating with fibronectin. The experiments exhibited



further that convincing results are only obtained, when the composition of proteins used for coating reflects the situation found under natural conditions.

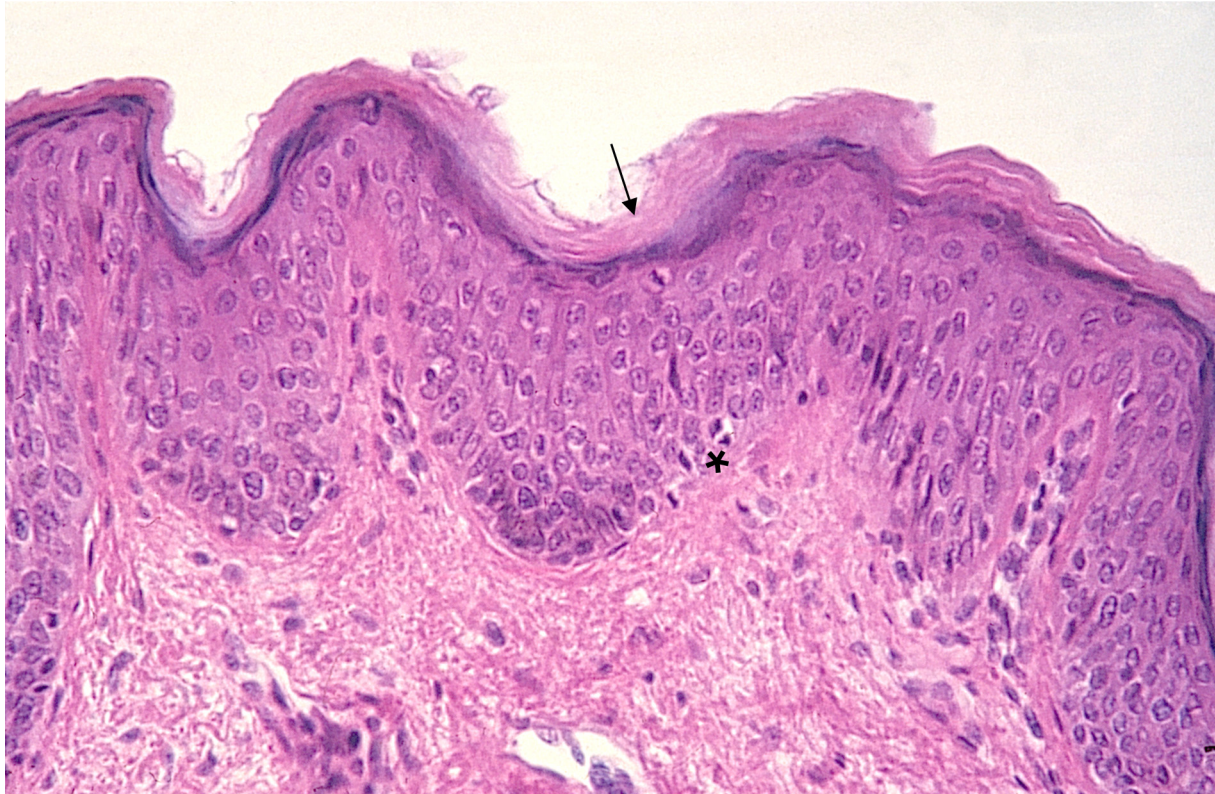


Figure 19: Microscopic view to horny epidermis of the lip. The horny layer (arrow) borders the lumen, while the basal side of the epithelium rests on a basal lamina (asterisk).

ECM has also an enormous influence on the development of skin. The multilayered epithelium of the epidermis is built up by keratinocytes and renewed within 30 days from the stratum basale (Fig. 19). For this constant regeneration the stem cells within the stratum basale have to perform permanent cell divisions. During this phase of development they have a permanent and close contact to the ECM found within the basal lamina. The stem/progenitor cells are integrated in the epithelium, but they do not show a typical expression of proteins normally found in terminally differentiated keratinocytes.

The regeneration of epidermis is started by asymmetric cell divisions of stem cells, which interrupt the contact to the basal lamina so that cells migrate towards the suprabasal cell

layer. During this developmental step the first signs of differentiation become visible such as expression of involucrin. The example shows that detachment from the basal lamina is leading to a new program of differentiation.

A similar situation can be mimicked in culture. When cells of the stratum basale are cultured in suspension without presence of ECM, only a minimal program of differentiation is observed. In consequence, to investigate gene activity during development into a multilayered epithelium, keratinocytes were cultured on a layer of fibroblast like 3T3 cells containing high amounts of ECM. This coat promoted in keratinocytes the synthesis of a basal lamina stimulating in parallel a high degree of differentiation. In contrast, keratinocytes on the bottom of a dish do not show this development.

**[search: cell differentiation extracellular matrix]**

**Self-study: Inform yourself about cell differentiation and extracellular matrix**

### **3.6. ECM modulates cell seeding**

Numerous investigations have shown that the interaction between a cell and ECM is not always close and permanent but can be modulated by various influences. While fibronectin and laminin promote the adhesion of cells to ECM, other neighbouring molecules such as thrombospondin and tenascin are able to intensify or even to decrease the degree of this interaction. The thrombospondins contain for example several Epidermal Growth Factor (EGF) repeats and binding sites for  $\text{Ca}^{++}$  ions leading on the one hand to proliferation and on the other hand to cell adhesion. Thrombospondin 1 is synthesized from fibroblasts, endothelial cells or smooth muscle cells. It can bind to fibrillar collagens, fibronectin, laminin und heparansulfate proteoglycane. The molecule has a stimulating effect on proliferation of fibroblasts destabilizing at the same time the interaction between cells and ECM. An

analogous function is found in cartilage. The Cartilage Oligomeric Matrix Protein (COMP) has molecular similarities to thrombospondins. The protein is synthesized by chondrocytes and secreted into the pericellular matrix. A decreased amount or a missing synthesis of COMP leads to a drastic softening of the cartilage matrix.

Also tenascins are able to modulate the interactions between a cell and the contacts within ECM. These molecules consist of three (tenascin-X) or six (tenascin-C/-R) subunits. In the amino acid sequence repeats for binding to collagen type III, EGF similar domains and binding sites for  $\beta$ - und  $\gamma$ -chains of fibrinogen are contained. The expression of tenascin is depending on the individual cell type and tissue. Tenascin-R is mainly synthesized during development of the nervous system, while tenascin-X occurs in smooth muscle cells, in cardiomyocytes and in skeleton muscle. Tenascin-C is present in embryonic tissues, in healing wounds, in various tumours and in brain. The molecule promotes the adhesion of cells by attracting integrin and proteoglycan molecules. This effect can be interfered by fibronectin. Finally, molecules on the cell surface such as contactin react with tenascin-C/-R influencing thereby the development of neurons.

**[search: cell seeding extracellular matrix]**

**Self-study: Read about cell seeding and extracellular matrix**

### **3.7. ECM and matricellular functions**

The intensity of a contact between cells and the ECM can be controlled by matricellular proteins. These molecules influence numerous activities on the cell surface, intracellular signal cascades and gene expression. The resulting signals influence sociality between cells, migration and differentiation so that specialized tissues can arise and be maintained. Thrombospondin 1, thrombospondin 2, tenascin C, osteopontin and Secreted Protein Acidic

and Rich in Cysteine (SPARC) belong to this interesting group of proteins. The matricellular proteins are found in associated form within ECM without exhibiting a mechanical meaning. All of the matricellular proteins trigger signals of cytokines, steer activity of extracellular proteases and are able to communicate with the ECM as well as cell receptors.

Thrombospondins comprise a group of proteins with a molecular weight of about 450 000. These molecules are found on the outer side of the plasma membrane. At least five extracellular domains are in contact with collagen type I and V or laminin, fibronectin, fibrinogen and SPARC. Thrombospondins are also able to perform contacts with integrin molecules on the cell surface. For example, this interaction can be disturbed, when endothelial cells are co-cultured with smooth muscle cells in combination with an antibody directed against thrombospondin. The interference prevents the formation of new capillaries. Thrombospondin can also influence the adhesion of endothelial cells. Interfering the binding site of thrombospondins is leading to migration of cells. A great meaning has the influence of thrombospondin during healing of skin and migration of tumour cells.

In the adult organism tenascin C is down-regulated. However, during development of tissues it is up-regulated. In the most cases tenascin C is binding to fibronectin. Cellular contacts are made over at least five different integrins and annexin II. The strength of cell adhesion depends on the profile of cellular receptors with tenascin C. During this process EGF and bFGF can interact. Such interactions were shown with cultured smooth muscle cells embedded in collagen. Since the cells secrete metalloproteinases (MPP), the degradation liberates new binding sites for integrins. The binding of integrins in turn leads to synthesis and secretion of tenascin C. Newly synthesized tenascin C embedded yet in ECM stimulates binding of integrins of the cell. This effect in turn leads to a new organization of focal adhesion sites of the cells. At the same time increased expression of EGF receptors is observed at the opposite side of the cell. When soluble EGF is present, the factor is now able to stimulate mitotic activity within the cell.



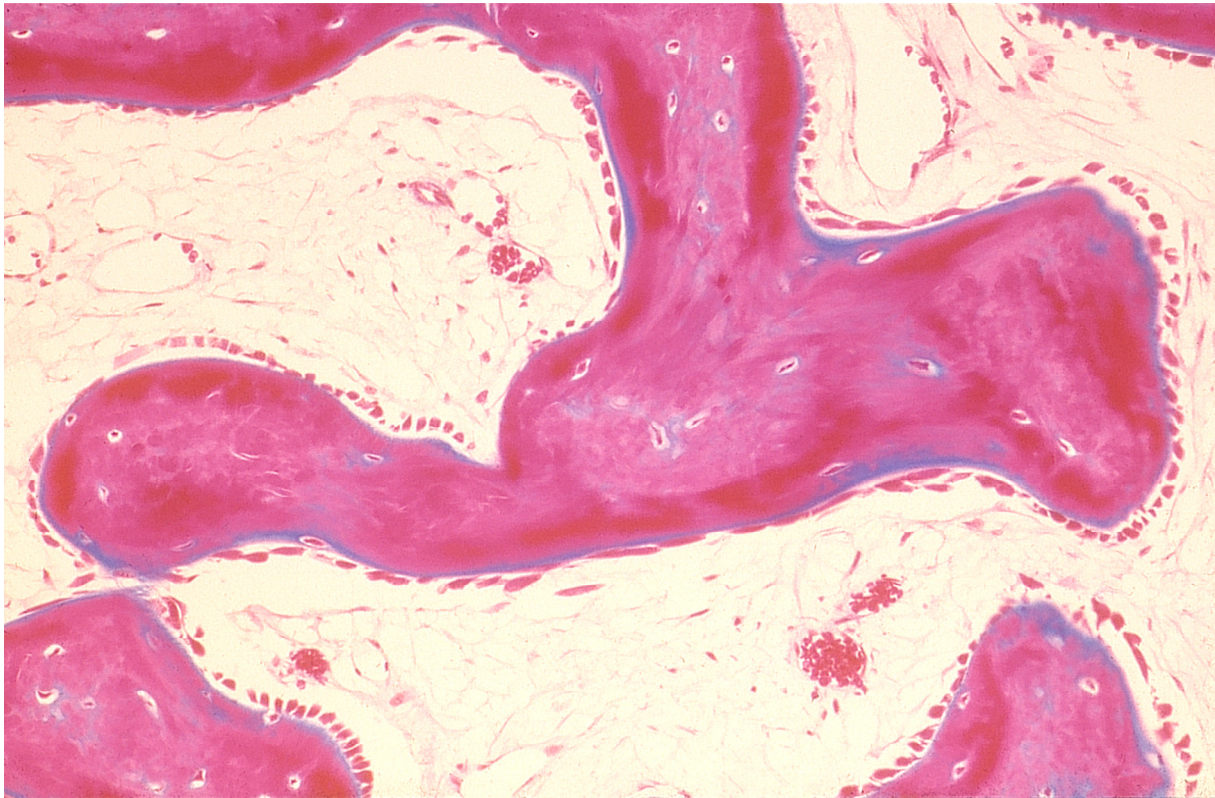


Figure 20: Microscopic view to the development of trabecular bone. To recognize are osteoblasts surrounding a trabeculum. In the interior of the trabeculum some osteocytes are seen. In the space between bone mesenchyme with numerous capillaries is recognized.

Especially in bone but also in other tissues a special level of osteopontin is found (Fig. 20). The molecule binds on the one hand to collagen type I, II, III, IV and V and on the other hand to numerous integrins and CD44 interacting from the cellular side. Blocking the synthesis of osteopontin in smooth muscle cells is leading to a minor adhesion of cells but to an increased spreading in artificial matrices. Binding sites for integrins on the surface of osteopontin can be activated by the protease thrombin. Treatment with thrombin increases the number of integrins binding to osteopontin, which in turn informs the cells to synthesize further integrin molecules.

A special task of osteopontin is the maintenance of cellular differentiation and the prevention of apoptosis. It was shown that endothelial cells undergo apoptosis, when administration of

growth factors was lacking in the culture medium. However, when the cells were cultured on a substrate containing osteopontin, apoptosis is prevented despite the lack of growth factors.

SPARC (BM-40, Osteonectin) is mainly found in bone, but also in many other tissues. Increased amount of SPARC is especially present in regenerating tissues such as the villi of intestine, healing wounds, but also in liver fibrosis, glomerulonephritis and in different tumours. The molecule is binding on the one hand on collagens type I, III, IV and V and on the other hand a close interaction with thrombospondins was found. SPARC is further binding growth factors incorporated in the network of the surrounding ECM. By this interaction SPARC is modulating the biological activity of growth factors. A special effect is its influence on cell division. When cells are cultured in dishes coated with SPARC, mitotic activity is downregulated. It appears that SPARC has a further influence on ECM synthesis, since it controls the amount of synthesized collagen type I.

**[search: cell matricellular proteins extracellular matrix]**

**Self-study: Get updated about matricellular proteins**

## 4. Conventional culture with adherent cells

Regarding the variety of cell culture experiments by the first view two different strategies can be recognized. Numerous groups are using continuous cell lines. Within shortest time cells can be multiplied with high efficiency so that synthesized proteins such as antibodies, vaccines, recombinant proteins or organelles of cells can be harvested (Fig. 21).

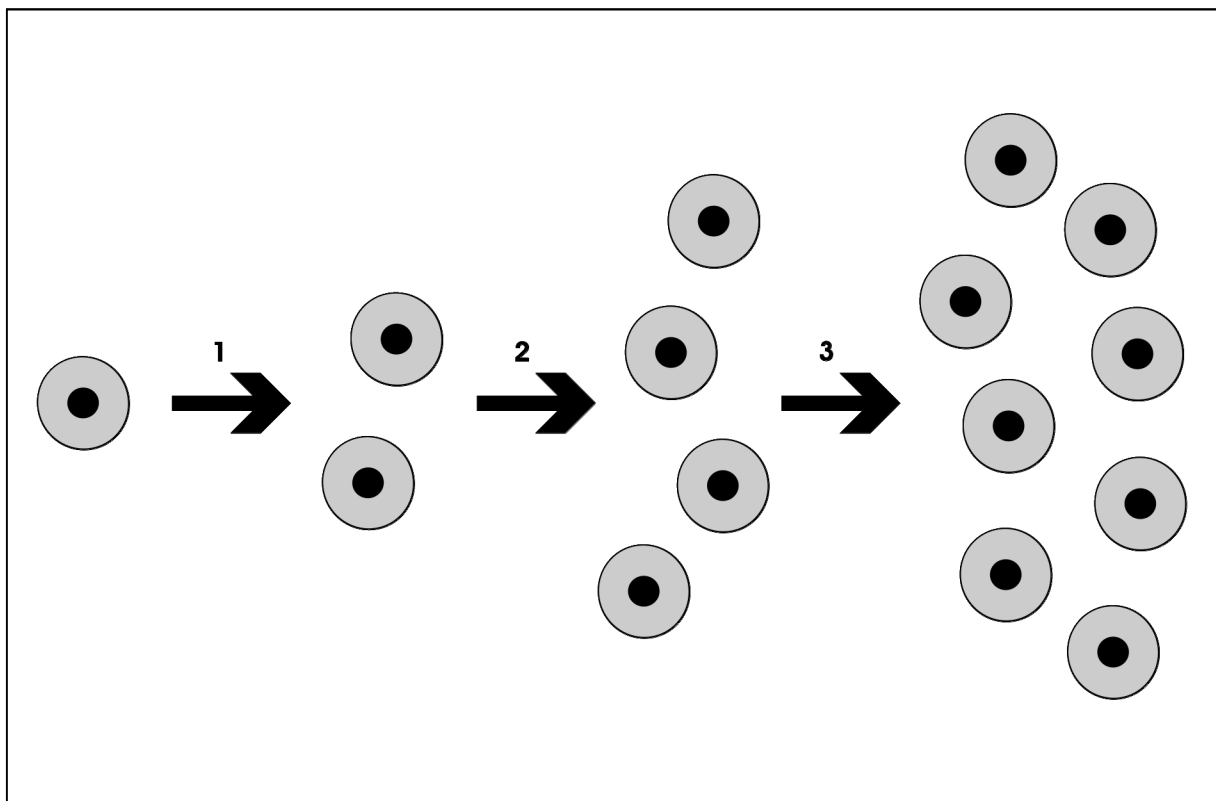


Figure 21: Schematic illustration shows the multiplication of cells after first (1), second (2) and third (3) passage.

Following this strategy only multiplication of cells is in the focus of interest. In this coherence it is not important if special physiological functions or tissue specific features are contained. In so far the aim is directed to increase the number of cells so that the required molecules are synthesized as fast as possible. A multitude of experiences over decades illustrates that the necessary mass of cells can be cultured in conventional cell culture dishes and in flasks before an up scale production is made using roller bottles and different kinds of bioreactors.

In contrast, beside the efficient production and multiplication of cells a second group of scientists needs cells exhibiting quite other features. To investigate very specific cell reactions, to analyze the metabolism of newly developed drugs or to elaborate new strategies for regeneration, highly differentiated cells with clearly defined tissue and organ functions are urgently needed in culture experiments (Fig. 22).

It is obvious that for the production of cells and the generation of tissue specific functions quite different culture protocols have to be applied. While mass production is mostly realized with cells kept in suspension, highly differentiated cells are anchorage dependent and must be kept on a more or less suitable substrate substituting features of the extracellular matrix (ECM).

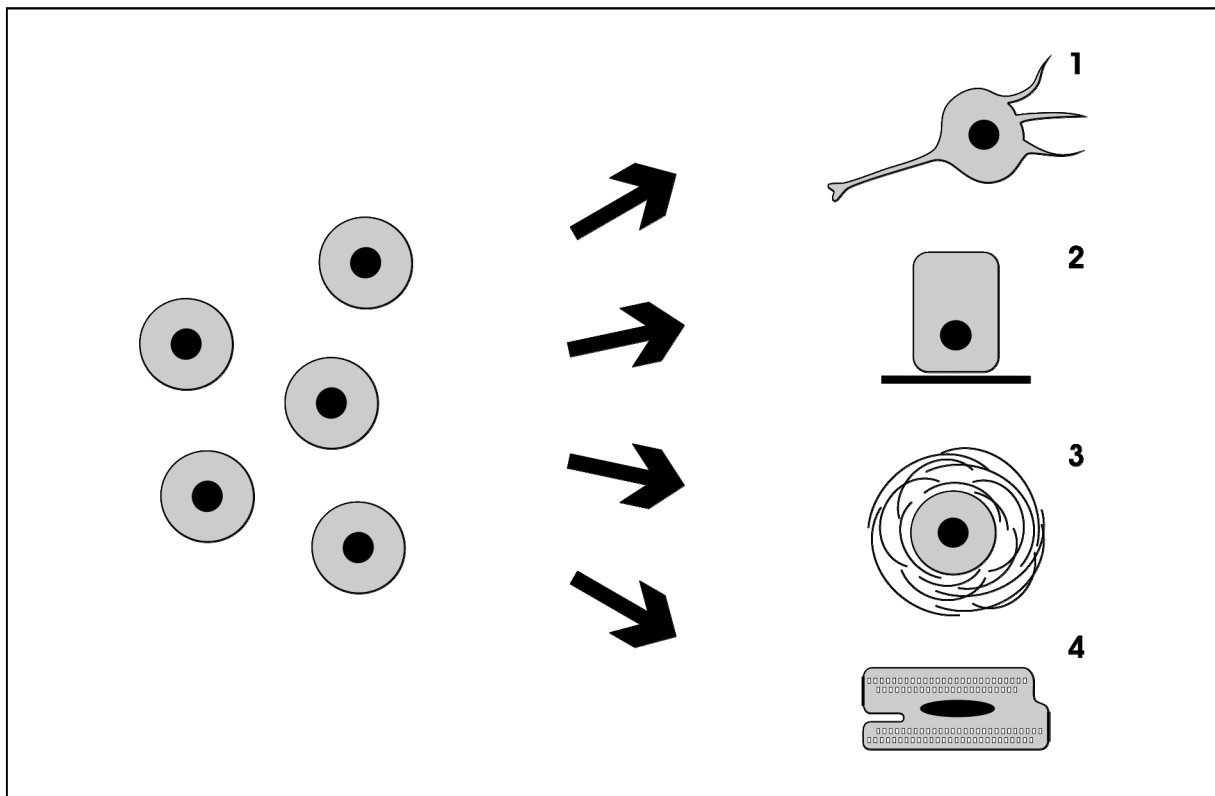


Figure 22: Schematic illustration demonstrates the transition of isolated cells into differentiated cells found in neural tissue (1), epithelium (2), connective tissue (3) and muscular tissue (4).

It is obvious that the number of cells, the size of the culture dishes and the amount of necessary culture medium varies in the individual experiments with adherent cells. Therefore the dimensions of the planned experiments have to be defined.

**[Search: cell culture microscope]**

**Self-study: Read about cell culture dishes**

## **4.1. Selection of the suitable dish**

For the laboratory scale a multitude of culture dishes is commercially available, which can be ordered in different sizes, with a plane, concave or convex bottom. Special dishes have gas permeable or optical bottoms for an optimal microscopic visualization of cells. In principle all of these dishes are derived from the classical Petri dishes made out of glass invented decades ago.

Looking to a catalogue a great choice of disposable culture ware is commercially available (Fig. 23). The offer for the culture of anchorage dependent cells comprises different sizes of flat dishes, multiwell plates, flasks, bottles including roller bottles and trays to pile cells within containers.

According to the various dimensions of cell number respectively medium volume the area of growth can vary from few square millimeters ( $\text{mm}^2$ ) in mini dishes to thousands of square centimeters ( $\text{cm}^2$ ) in multitray. Flasks used for the culture of anchorage dependent cells in the laboratory dimension have in the most cases a growth area between 25 und 175  $\text{cm}^2$ .

Disposable culture dishes are produced according an ISO 9000 certification and are consequently injection molded under permanent quality control. Actual products consist of



polystyrene USP Class V1, are sterilized and do not exhibit pyrogen activity. Using this kind of culture articles it is assured that the surface for growing cells is treated by a vacuum-gas-plasma supporting an optimal attachment, adhesion and adherence of cells. The area for cell growth must be plane, when optimal microscopic analysis has to be performed.

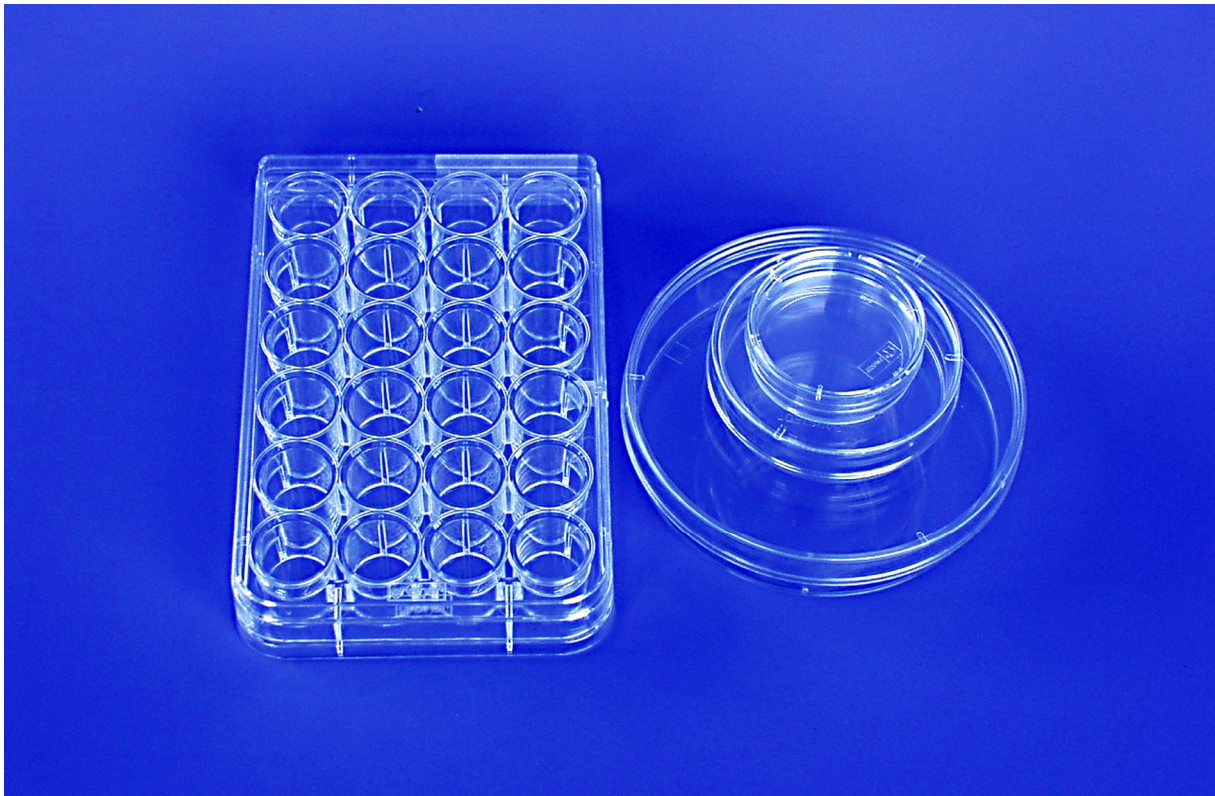


Figure 23: Photography shows a 24well plate and classical culture dishes in three different sizes. In the lid of each dish small spacers are incorporated so that the contained culture medium is continuously exposed to a defined gas atmosphere.

Most interestingly in this coherence are mini-construct chambers (MC-8<sup>®</sup>). The culture module allows researchers to engineer tissue constructs using customer-specified substrates and cell types. The contractility of cells and the stiffness of synthesized extracellular matrix can then be measured for example by a Palpator<sup>®</sup>. Since bottom and top of mini-construct chambers are transparent, also Ca<sup>++</sup> signalling, mitochondrial potentials and other biological parameters can be measured to elaborate the physiological state of growing cells. In consequence, only full transparency will provide optical accessibility for monitoring growth of

cells and quantitative measurement using fluorescence indicators to determine their physiological state.

**[Search: cell culture dish]**

**Self-study: Get updated about cell seeding in culture dishes**

## **4.2. Choice of cells and culture environment**

All of the cells occurring in an organism principally can be isolated. To obtain a suitable cell suspension for culture, tissues or organ parts have to be excised and treated several times by proteases diluted in a medium lacking  $\text{Ca}^{++}$ . After several steps of centrifugation and resuspension the obtained isolated cells are ready to start a culture experiment.

When culture is performed with cells freshly isolated out of a tissue or an organ, it is called primary cell culture. Once the cells are provided with a suitable medium, within several hours adhesion on the bottom of the dish and multiple cell divisions can be recognized under the microscope. After a period of days the complete bottom of the dish will be covered by cells. This state of cells completely covering the bottom of the dish is called confluency. Yet, subculture must be made so that the cells do not die. Either some cells are scratched from the bottom of the dish or a suspension of cells has to be made by the help of peptidases such as trypsin or collagenase. Some scratched cells or an aliquot of suspended cells is then transferred to a new dish for subculture.

When primary cells are subcultured for more than seventy times without restrictions the primary cell line is converting to a continuous cell line. Also by a spontaneous conversion of primary cells or by genetic modifications cell lines can be obtained. In many of the cases the derived cells are cultured in future as anchorage dependent respectively adherent cells.

However, during culture over prolonged period of time the characteristics of the cells do not stay constant but change. Typical features can be lost by dedifferentiation, while other characteristics can be acquired by transdifferentiation.

Generally, when cells are brought in culture, part of them can show adhesion on the interior of a glass or plastic pipette, on the bottom of a dish or on an offered substrate. Surprisingly some of cells exhibit primary attachment, but do not adhere to stay further in suspension (Fig. 24.1). As explained later other cells show in biomaterial research only mediocre adhesion on different substrates (Fig. 24.2). In contrast, again other substrates are so attractive that cells show close adhesion to search constant contact (Fig. 24.3).

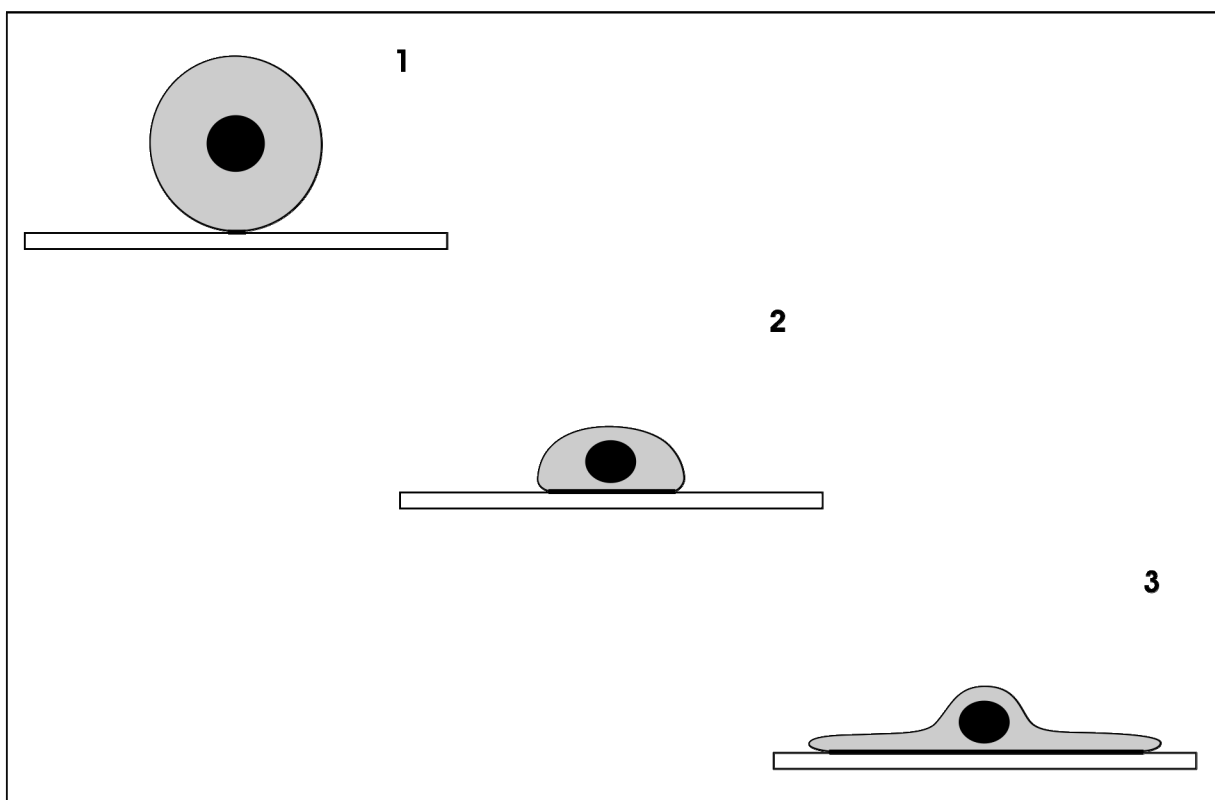


Figure 24: Illustration shows primary attachment (1), mediocre adhesion (2) and close adherence (3) of a cell.

During ongoing culture in an optimal culture medium cells are covering finally as a confluent monolayer the complete bottom of the dish or the offered substrate. Independent from the cell type and the substrate now a subculture has to be made. As described before the cells



are incubated by a protease so that finally cells in suspension arise. An aliquot of these cells is distributed into new dishes. When subculture is not performed, cells will die due to unknown reasons.

In many cases it is too circumstantial to isolate fresh cells from a tissue or an organ to prepare primary cultures. An alternative is the use of a continuous cell line. A well known example are MDCK (Madin Darby Canine Kidney) cells (Fig. 25). This line derived from the kidney of a dog and was taken in culture in 1958 by Drs. Madin and Darbin. The 49th subculture of these cells was given to the American Type Culture Collection (ATCC).

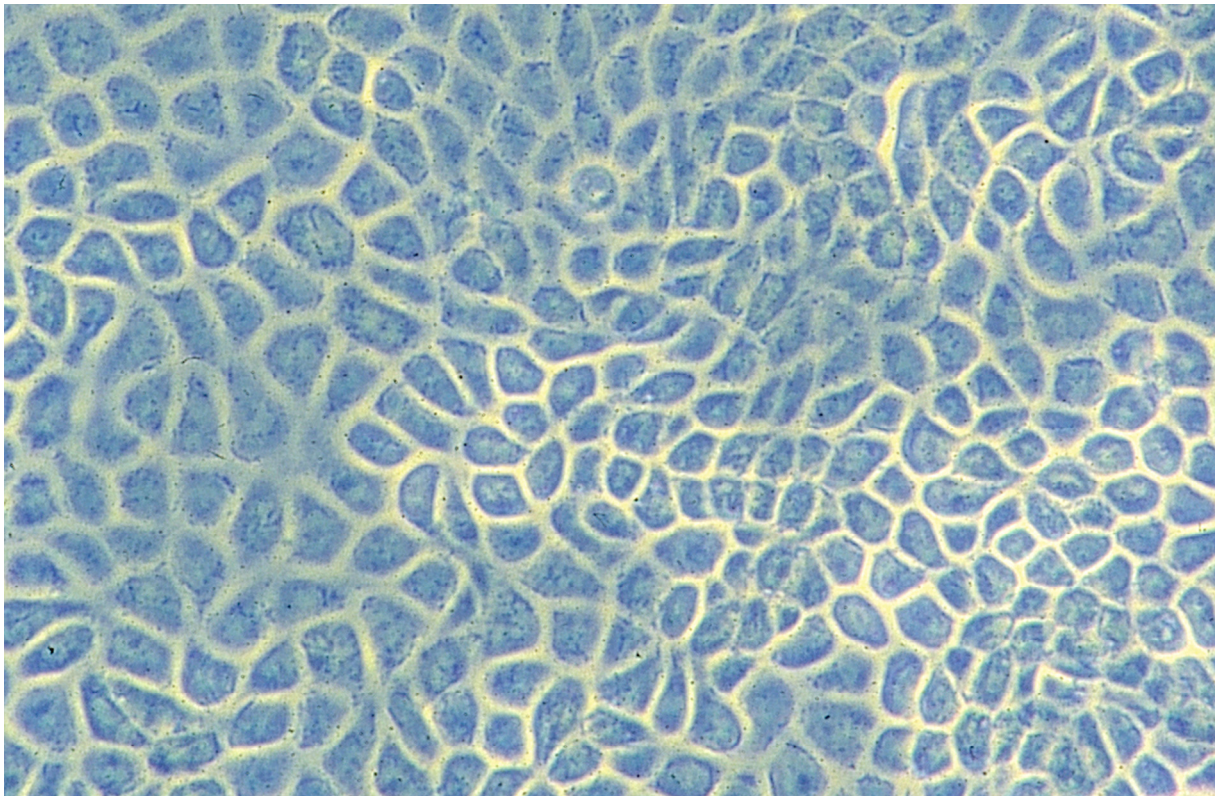


Figure 25: Light microscopic view to MDCK cells developing a confluent monolayer at the bottom of a culture dish.

Up to date different sublines of MDCK cells are commercially available. Of special interest is strain I and II with very different morphological and physiological features. On the bottom of a polystyrene dish MDCK cells are flat and they do not develop so much features of differentiation (Fig. 26.1). In contrast, when they are cultured on the surface of a filter and in

the presence of a serum containing medium, MDCK cells show features of a polarized epithelium with intense physiological transport properties (Fig. 26.2). Regarding their mitotic activity MDCK cells resemble tumour cells. However, in the highly differentiated state the cells do not survive over prolonged periods of time so that a subculture becomes impossible (Fig. 26.2). The cells can be kept on stock under frozen conditions so that they are always available on demand.

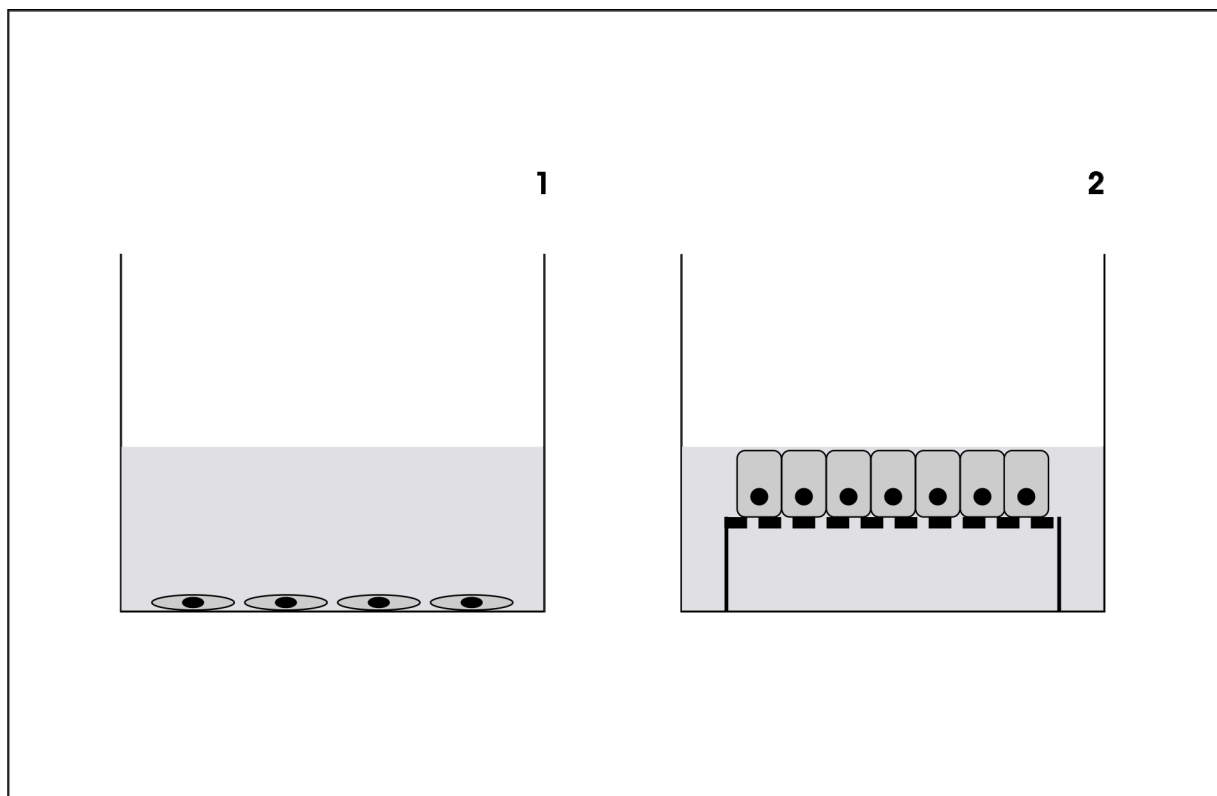


Figure 26: View to MDCK cells growing on the bottom of a dish (1) and on the surface of a filter (2). On the bottom of a dish MDCK cells are growing as a flat monolayer (1), while the surface of a filter and the high content of serum in the medium stimulates polarization (2).

MDCK cells do not represent cells derived from a defined nephron segment but contain mixed features including proximal and distal tubule characteristics. In so far the cells of a continuous cell line do not have all of the characteristics normally found within tissues or organs. In the most cases cells of a continuous cell line exhibit a different number of chromosomes when compared with cells derived from a primary cell line. As many other cell

lines MDCK cells form a confluent monolayer in form of an epithelium in culture (Fig. 25, 26.2).

Surprisingly, MDCK cells and other cell lines do not develop a basal lamina normally found as a typical feature in epithelia. Instead the cultured cells secrete molecules of the basal lamina such as fibronectin or collagen type IV in a soluble form into the culture medium. Despite the presence of these synthesized extracellular matrix proteins the cells have lost their capability to link them at the basal aspect of the epithelium so that not a typically structured basal lamina arises.

**[Search: primary cell culture; MDCK cells]**

**Self-study: Read about cell division in culture dishes**

### **4.3. Typical protocol for the culture of adherent cells**

On the bottom of a culture dish consisting of polystyrene MDCK cells grow as a planar monolayer (Fig. 25). The cells adhere in a reversible manner to the polystyrene of the culture dish, until they can be detached for subculture for example by trypsin, EDTA and a medium lacking  $\text{Ca}^{++}$ . To follow a permanent culture protocol, MDCK cells or other cell lines are kept in conventional dishes or flasks.

When the cells are feed with a suitable culture medium, after a few days the complete bottom of the dish is covered by cells so that the state of confluence is reached. According to the experimental needs one part of the cells can be stored, while a second part of cells can be multiplied by subculture in conventional dishes.

**Storage** of cells in liquid nitrogen can be made in a special freezing medium consisting of:

- 80 % foetal calf serum (FCS) and 20 % dimethylsulfoxide (DMSO).

**Multiplication** of MDCK cells is made in a proliferation medium consisting of:

- 93 % Earle's Minimal Essential Medium (EMEM) with 0.85 g/l bicarbonate
- 5 % foetal calf serum
- 1 % L - glutamine 200 mM in PBS (Phosphate Buffered Saline)
- 1 % penicilline / streptomycine cocktail

Culture is performed in a CO<sub>2</sub> incubator to maintain a correct pH, respiratory gas atmosphere and temperature.

When culture is performed in the described medium, cells quickly divide to promote from one cell division to the next. This intense proliferation leads after a few days to a new confluent monolayer anchored at the bottom of the dish. It is again important to note that cells will die if not a subculture is yet performed. In consequence, detachment of cells from the bottom of the culture dish has to be made so that a cell suspension is obtained to inoculate new dishes with cells for the next culture series.

**Detachment** of cells is made in the following solution:

- 0.05 % trypsin and 0.02 % EDTA (ethylenediaminetetraacetic acid)  
solubilized in phosphate buffered saline (PBS) lacking Ca<sup>++</sup> and Mg<sup>++</sup>

For example, 10 ml of this solution is used in a 75 cm<sup>2</sup> culture flask. Before use the solution is warmed to 37° C and pressed through a sterilization filter.

For detachment of cells the medium is completely drawn out of the culture dish. Then 10 ml PBS is added for rinsing. In the next step 5 ml trypsin / EDTA / PBS solution is added with a pipette. Incubation is performed for 15 minutes at room temperature. The solution is drawn out by a pipette. Then 1 ml of trypsin / EDTA / PBS solution is added for 15 minutes. Incubation is now performed in an incubator at 37°C. At the end normally all of the cells are found to be detached and suspended. Still adherent cells are detached by knocking softly on the culture dish. Then 9 ml of culture medium are added to block the action of trypsin by contained foetal calf serum. Finally the density of cells is determined in a counting chamber.

After detachment, dissociation and counting cells are inoculated for subculture. The suspension is so diluted that a density of  $1 \times 10^4$  cells / cm<sup>2</sup> is reached. For a 75 cm<sup>2</sup> culture flask it means  $1 \times 10^6$  cells / ml. Thus, into the new culture dish 20 ml of culture medium and 1 ml of cell suspension is pipetted. After a 3 to 4 days culture period in a CO<sub>2</sub> incubator a confluent monolayer is observed at the bottom of the dish. After reaching confluence the cells have to be subcultured again.

Beside trypsin different collagenases can be used for the isolation of cells. For example, collagenase A is applied, when an especially high harvest and vitality of cells is required. Collagenase B contains a low concentration of clostripain activity or collagenase D shows low tryptic activity. When hepatocytes are isolated, in the most cases collagenase H is used. In contrast, pancreatic islets are isolated in many cases with collagenase P, since it well preserves important cell surface features.

Finally, dispases can be applied for the isolation of cells. These enzymes are aminopeptidases cleaving peptides in extracellular matrix proteins such as fibronectin, collagen type IV and collagen type I. Also liberases are available for the dissociation of cells. For pancreatic islets for example 4 different liberases can be used.

In all of the cases must be considered that proposed enzymes may be contaminated by endotoxins hampering vitality and physiology of isolated cells. Further all of the enzymes may be contaminated by prions. For that reason enzymes for cell isolation are ordered that were not in contact with animal tissue during fabrication.

**[Search: cell culture dish MDCK]**

**Self-study: Inform yourself about cell culture protocols**

#### **4.4. Visualization of cells on a transparent substrate**

It is obvious that a culture dish has a transparent bottom so that the growing cells can be easily visualized under living conditions and without staining by a conventional inverse microscope containing phase contrast objectives. The only limitation is that the bottom of a dish is thicker than a microscopic cover glass. For that reason long distance objectives have to be applied, when cells are investigated with high optical resolution. In the meantime special culture dishes with an improved optical bottom are available. For example, in Confo Cult® dishes three-dimensional cultures are performed on a slide that can be retrieved from the culture dish to mount it for high resolution on the microscope. Also the CytoWell® plates provide an excellent platform for cell based assays and imaging applications. The film bottoms are extremely flat and only 50 µm thick. This fact facilitates high magnification and results in excellent resolution of micrographs during automated imaging of all cells within the plate. An alternative are Lumox® dishes. They exhibit a 25 µm ultra-thin gas permeable base. The short diffusion path ensures optimal gas exchange via the bottom, high optical transparency and low autofluorescence.

**[Search: cell culture light microscopy]**

**Self-study: read about inverse light microscopy**

## 5. Initial reactions of cells in culture

When tissue or organ specific tasks are required, cells have to grow in a more or less intense adhesion to the extracellular matrix or to a suitable substrate offered under in vitro conditions. When a mimicking of the physiological situation is not given, cells remain more or less in an undifferentiated state so that they will die after a short period of time.

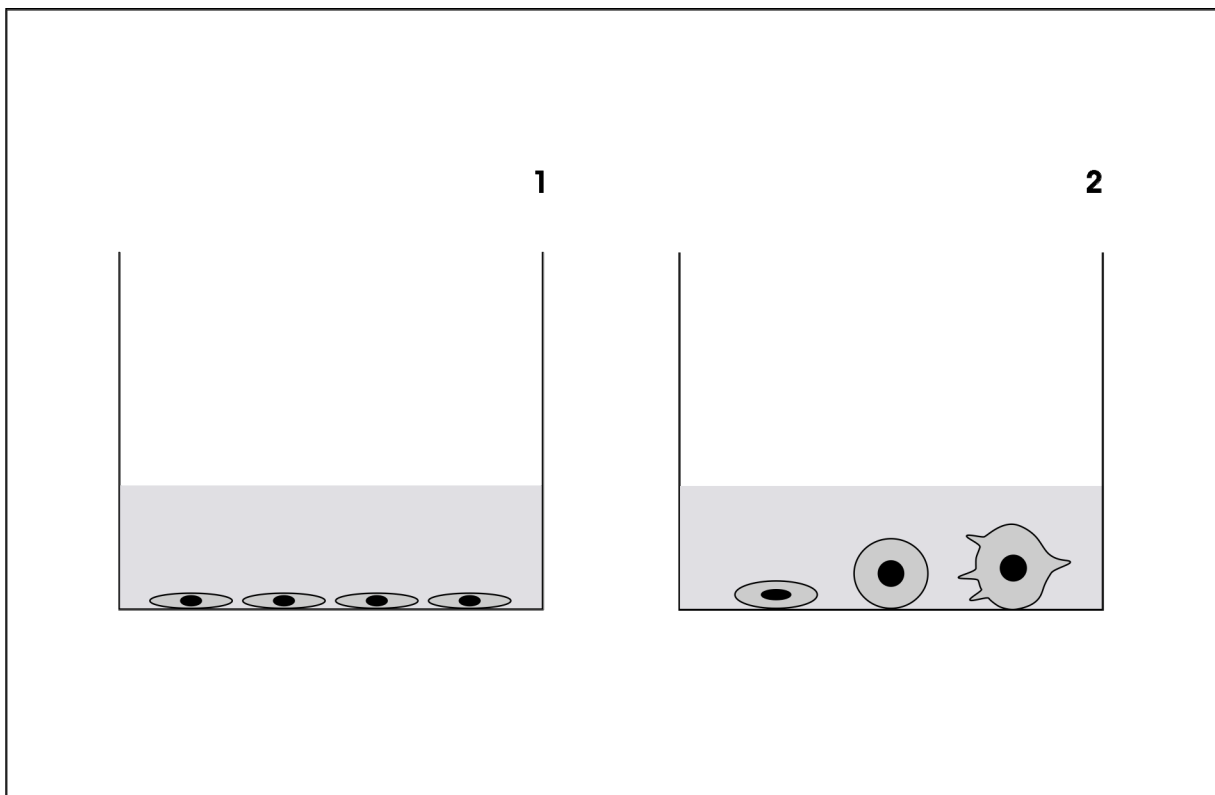


Figure 27: Schematic illustration of adherent cells growing on the bottom of a dish (1). Other cell types may show only primary attachment dependent on the acceptance of the offered substrate (2).

Thus, one of the keys for a successful culture with adherent cells is to offer an optimal site for anchorage mimicking as much as possible the situation within individual tissues or organs.

**[Search: cell culture attachment]**

**Self-study: Get updated about cell adhesion in a culture dish**

## 5.1. From adherent cells to cell suspension

The majority of cells within the organism is searching an intense contact to individual extracellular matrix or basal lamina structures. An analogous situation is given for cells under in vitro conditions.

For example, they can grow in form of a confluent monolayer in contact with the bottom of a polystyrene culture dish or within an offered three-dimensional substrate stripped from extracellular matrix. In the case the offered material appears attractive for the cells in culture primary attachment followed by adhesion will occur. To see some of the theoretical background, the phases of cell adhesion, adherence including affinity shall be explained.

For the start of culture in most cases cells in suspension are applied. For the isolation from an individual tissue or from the culture dish the built up anchorage of cells to the individual substrate has to be disrupted. As described before, this can be performed by incubation of cells and tissues with a medium containing proteases and low concentration of  $\text{Ca}^{++}$ . As a result the cells are separated from their neighbours and from the individual adhesion substrate.

Most important, performing dissociation to obtain a cell suspension, one has to consider that neurons are losing their long axons and dendrites (Fig. 28.1). In epithelia dissociation is disrupting the close neighbourhood of cells and the intense contact to the basal lamina resulting in a complete loss of polarization (Fig. 28.2). Regarding connective tissue collagenase is liberating single chondrocytes out of the isogenic group of cells and the complex surrounding of extracellular matrix (Fig. 28.3). Cardiomyocytes are losing the mechanical as well as functional contacts to their neighbour cells (Fig. 28.4).



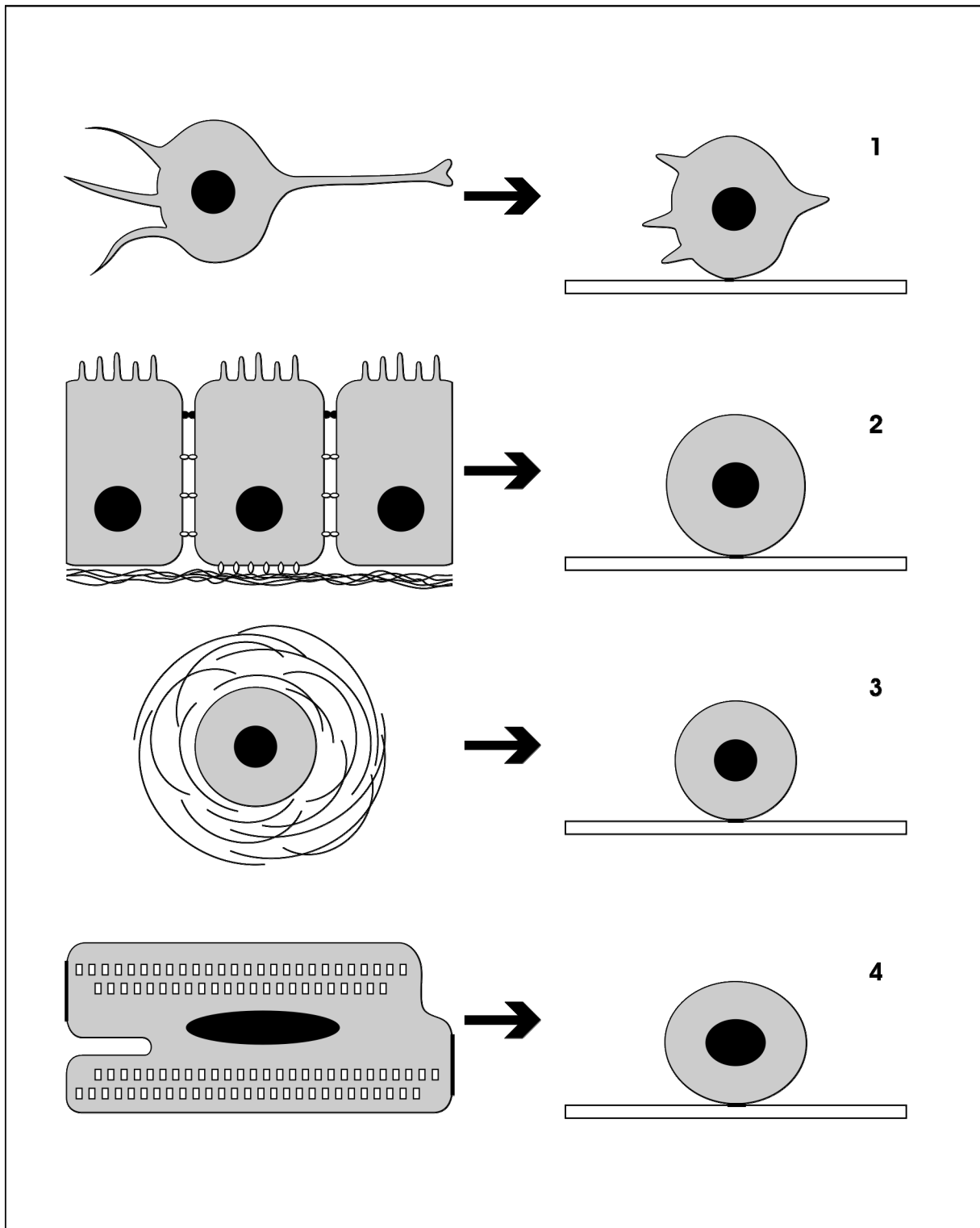


Figure 28: Effect of isolation on single cells used in cell culture experiments. Intact features of a neuron (1), an epithelial cell (2), a chondrocyte (3) and a cardiomyocyte (4) are seen at the left side. Situation of these cells after isolation is seen at the right side. When cells are at the start of culture, typical functional features are lost.

**[Search: cell culture isolation protease]**

**Self-study: Inform yourself about dissociation of cells before culture**

## 5.2. From suspension to first contacts

After disruption of contacts between neighbouring cells and the extracellular matrix cells are suspended within the isolation medium. For stopping the dissociation process and for rinsing cells are centrifuged and resuspended in a defined volume of suitable medium. Yet the number of cells is counted so that correct aliquots can be made to start with the culture protocol. Finally a defined amount of cells is transferred by a pipette into a suitable culture dish.

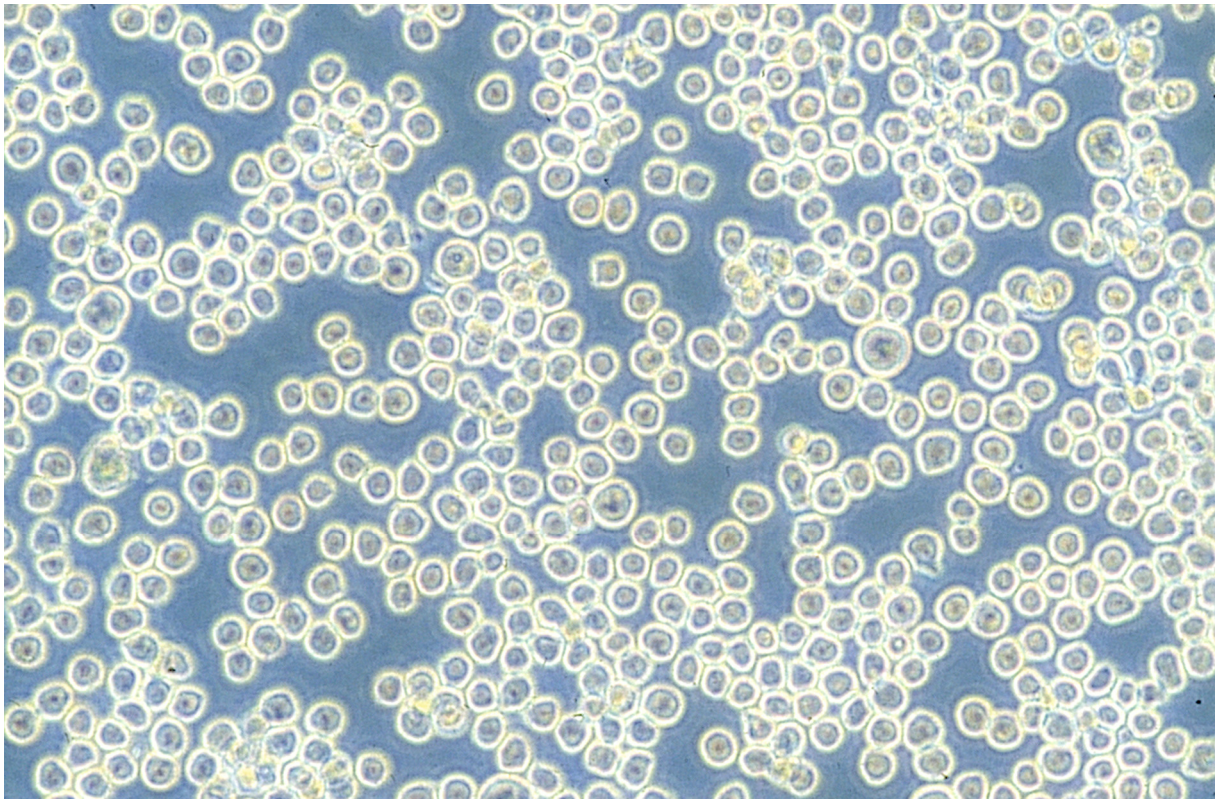


Figure 29: Microscopic view to cells in suspension. Due to the round shape it can not be recognized, which cell type is cultured.

When the start of culture with isolated cells is analyzed under an inverse light microscope, all of the cells exhibit a round shape within the dish (Fig. 29). This effect of rounding-up is made by the cells due to biophysical reasons and to gain an outer surface as small as possible.

Without exceptions it is obvious that after isolation all of the three-dimensional features of the individual cell types are lost. This result is paralleled by a complete change of typical morphological, physiological and biochemical features normally contained in individual cell types.

For a short period of time the cells will remain in suspension. Then they will sink to the bottom of the dish due to gravity ( $1 \times g$ ). Yet the cells show **primary attachment** at the bottom of the culture dish (Fig. 29).

This process can be analyzed by looking through an inverse microscope. First the cells will sink, then attach for a moment, detach and attach again to migrate finally on the bottom.

Depending on the type of cell it will take a few minutes up to many hours, until they remain attached for a longer while. In the following phase of **adhesion** it is observed that the round cells start to flatten, while they intensify the contact to the bottom of the dish (Fig. 30).

Yet specific cell receptors are activated that are involved in adhesion of cells. When the cells feel that the surface is not optimal for further development they detach, round up, attach again, migrate and finally adhere at a different site for a longer period of time.

When the surface is not recognized as suitable for adhesion, the cells can migrate over big distances to search still better adhesion sites.

Observing cells under the microscope during this initial culture phase neural cells cannot be distinguished from epithelial, muscular or connective tissue cells.

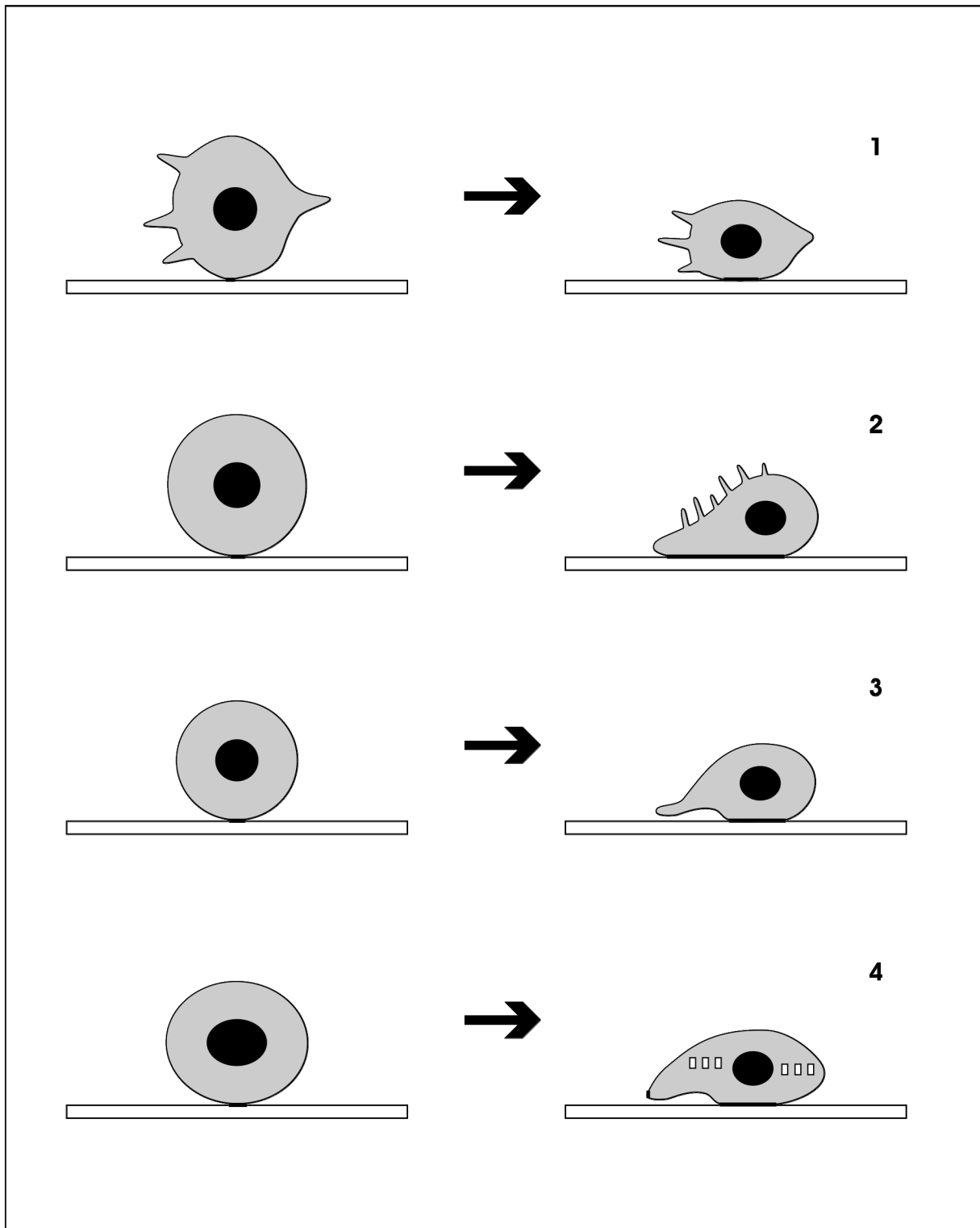


Figure 30: Schematic illustration of cells between first attachment (left side) and primary adhesion (right side) of a neuron (1), epithelial cell (2), connective tissue cell (3) and cardiomyocyte (4). It is obvious that in this stage typical functional features of cells cannot be recognized.

**[Search: cell culture isolation suspension]**

**Self-study: Get updated about cell suspension**

### 5.3. From cell adhesion to adherence

After a primary adhesion of cells is made on the bottom of a culture dish, the synthesis of specific cell receptors is up-regulated as described later. In consequence, the adhesion of cells on the bottom of the dish is boosted. As a result first intensified **adhesion** (Fig. 30) and then **adherence** (Fig. 31) of the cell takes place.

This process occurs over longer periods of time, depends on the individual type of cell and on the quality of the offered substrate for adherence. This reaction can be so sensitive that cells show adherence on the polystyrene dish of company A but not on dishes of company C and D.

In the phase of transition between adhesion and adherence the shape of the growing cells is changing. While all of the isolated cells show in suspension a completely round shape, the process of adhesion and adherence proceeds in parallel with an untypical flattening of cells (Fig. 30, 31, 32.2).

When a culture dish appears to be suitable for an individual cell type, it is observed that cells show within shortest time adhesion and a more or less even distribution on the bottom. During ongoing culture time the adhesion is replaced by adherence. In this phase it can be recognized that the original round shape of a cell more and more disappears so that the border of the cell becomes barely visible. Yet the period of adherence starts.

Beside microscopy the result of transition can be demonstrated, when for example ten different substrates including glass as a reference were tested within a dish. Five of ten tested polymers exhibit after 10 days an excellent surface coverage of cells, two of them allowed less well adherence than the glass reference. Three of them showed only poor cellular growth without perfect adherence.

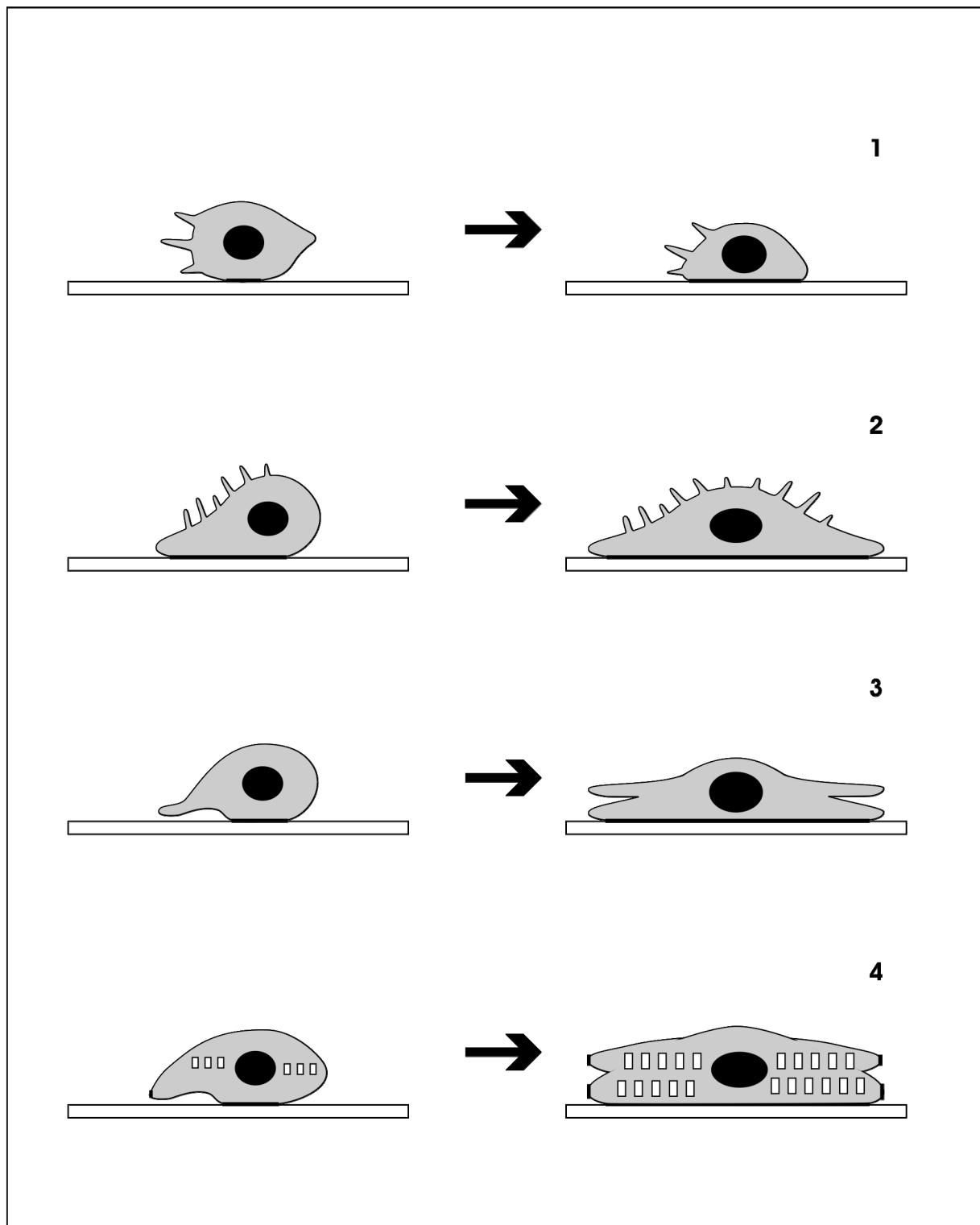


Figure 31: From adhesion (left side) to adherence (right side) of a neuron (1), epithelial cell (2), connective tissue cell (3) and cardiomyocyte (4).

When cells develop adherence on the bottom of a culture dish, they can be compared with fried eggs within a pan (Fig. 32.2). The position of the yolk indicates the highest area of the cell. At this site the nucleus is found. In contrast, the cytoplasmic periphery is remarkably flat.

It is obvious that the visible morphological alterations are correlated with a functional dedifferentiation of the cell. For example, special surface markers are not expressed, typical hormone receptors are down-regulated or special signal cascades are not developed.

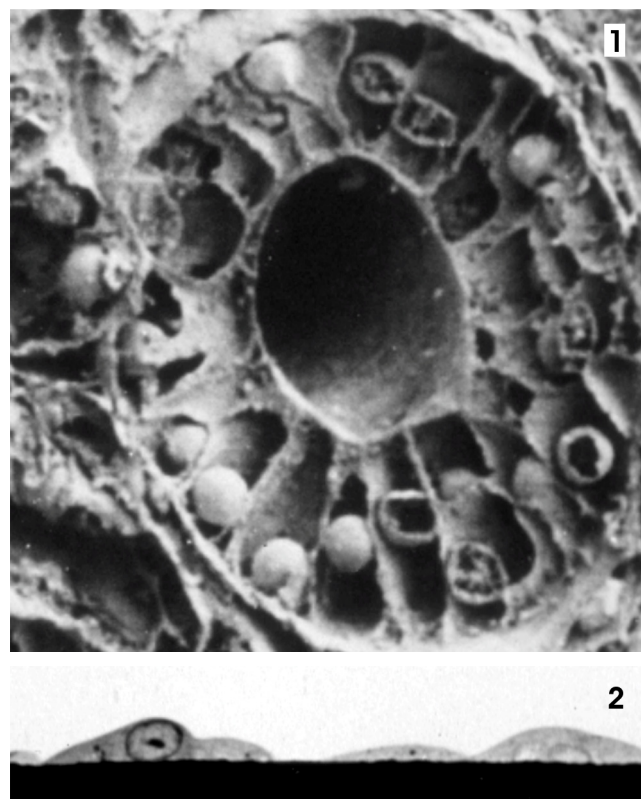


Figure 32: Transition from polarized cells (1) within a renal tubule to an untypically flat cell (2) cultured on the bottom of a dish.

**[Search: cell culture isolation adhesion]**

**Self-study: Get updated about cell adhesion and adherence on a biomaterial**



## 5.4. Inconstant adherence of cells

Most of cell culture dishes are made out of polystyrene. Although this material does not occur in the organism, a multitude of cells shows more or less close adhesion and adherence to polystyrene. However, adherence of cells to a substrate appears to be inconstant, since it is influenced by various factors such as cell density, migration, application of hormones, growth factors, drugs and by autocrine mechanisms.

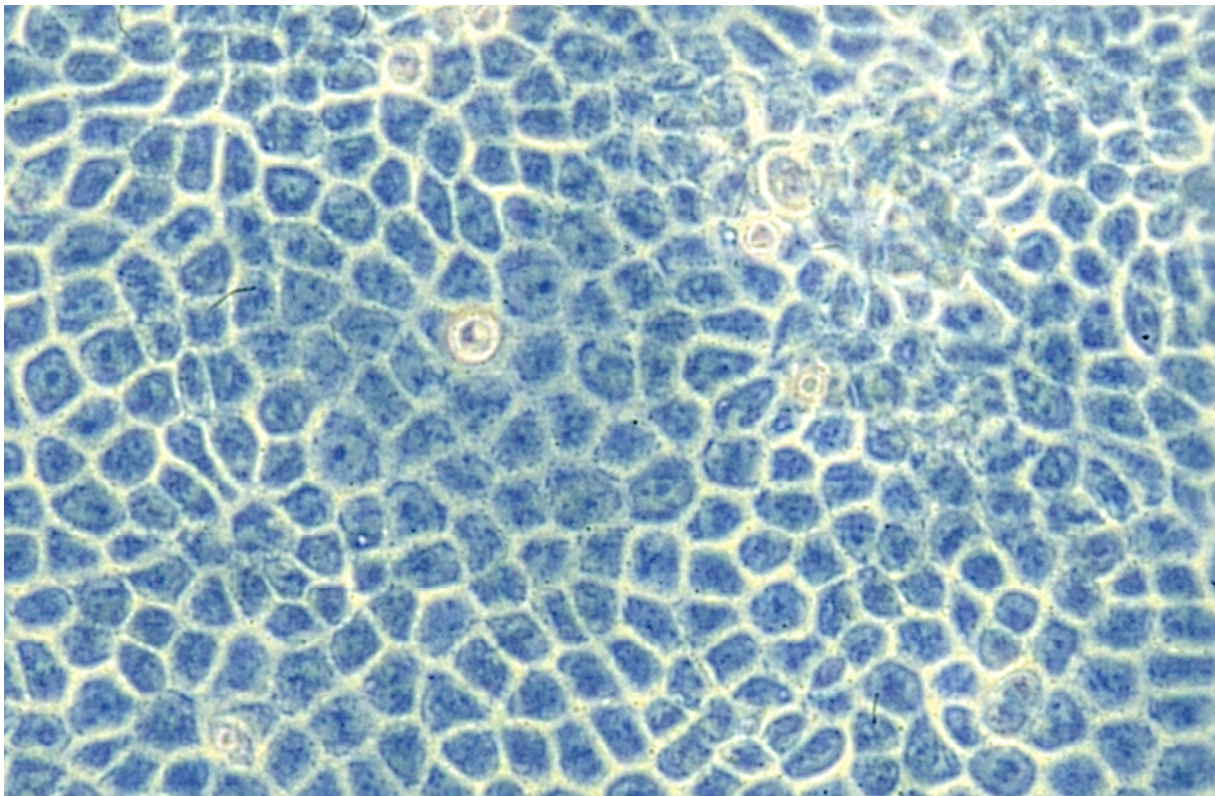


Figure 33: Microscopic view to MDCK cells cultured at the bottom of a dish. The majority of cells exhibits a close contact to the polystyrene surface. In the right upper corner cells form an aggregate. At this site cells do not show anymore adherence to the bottom of the dish.

For example, Madin Darby Canine Kidney (MDCK) cells are growing at the start of culture as a simple monolayer at the bottom of a polystyrene culture dish. After steroid hormone application such as aldosterone or after longer periods of culture time the cells form aggregates and leave the bottom of the dish (Fig. 33). At these sites cells do not exhibit



further adherence to the polystyrene of the dish. It is obvious that the contact to neighbouring cells becomes more important than the adherence to the substrate.

When Baby Hamster Kidney (BHK) cells are cultured for a short period of time, adherence at the bottom of the dish takes place (Fig. 34.1). In contrast, when the culture is performed for longer periods of time, the adherence on the bottom of the dish decreases and cells form aggregates (Fig. 34.2). Here an increased contact to neighbouring cells is detected.

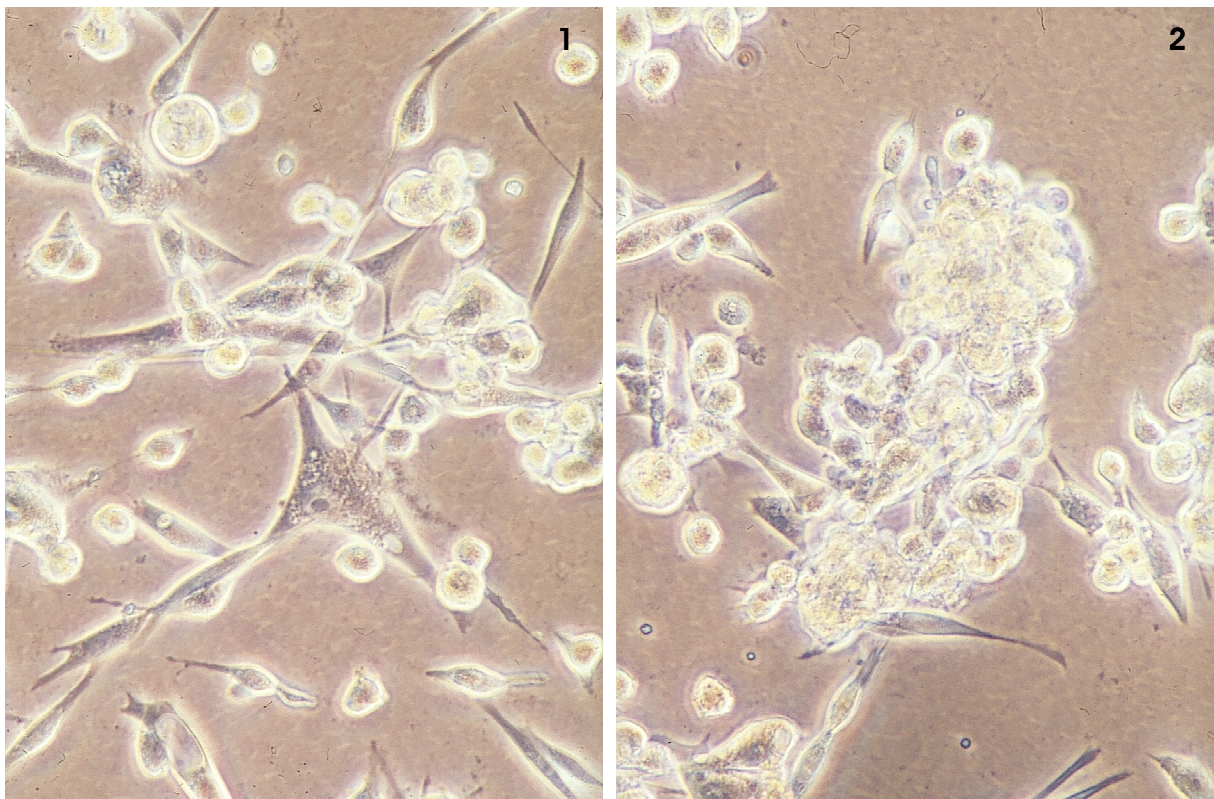


Figure 34: Culture of Baby Hamster Kidney (BHK) cells on the bottom of a dish after a culture period of 3 (1) and 14 days (2). After 3 days most of the cells show adherence on the bottom of the dish (1). After 13 days multiple cells do not show anymore contact to the bottom of the dish but are forming aggregates (2). This is a clear sign that the contact between cells is more favoured than the growth onto polystyrene.

**[Search: cell culture isolation adherence]**

**Self-study: Get updated about cell anchorage**

## 6. Adhesion, adherence and affinity

Culture experiments are not only performed on the bottom of a dish but are also made with a variety of biomedical polymers and different kinds of scaffold materials. Multiple experiments have shown that the primary **adhesion** triggers a series of cell biological reactions for the anchorage of cells. In the case an unsuitable site for adhesion is found, an immediate detachment of the cell is observed. In contrast, when an attractive site for adhesion is recognized, the primary adhesion of the cell is intensified by a solid **adherence**. In consequence, the increasing adhesion of cells on the surface of the material is a mirror for the cell biological quality of the contact site. When this contact appears positive, it stimulates the cell to intensify the binding with ongoing exposure time. The resulting **affinity** reflects the degree of attraction between the material and the cells. It appears that the primary positive contact of a cell is the key supporting a long term contact for cells.

### 6.1. Affinity tells about adhesion and adherence

Exact information about **affinity** and the related cell biological reactions of cultured cells are essential for laboratory and industrial optimization. For example, the inner surface of a blood vessel prosthesis has to exhibit a high affinity for endothelial cells. It is obvious that selection of such a material must guarantee a close and long lasting adherence of endothelial cells. Thus, the degree of **affinity** between the material and endothelial cells is the main presupposition for a faultless function of the prosthesis so that the rheological stress of the streaming blood does not disrupt the endothelial cell layer.

Further experiences show that hydrophilic surface structures result in a better adherence of cells than hydrophobic materials. Thus, when a close anchorage is required, biomaterials must be selected featuring a high **affinity** for cells. It is generally accepted that only biomaterials with a high tissue-specific **affinity** promote a functional development of cells

urgently needed in biomedicine including tissue engineering. However, a biomaterial with a high original **affinity** for the individual cell type does not guarantee the maintenance over the complete culture time or the period of implantation. The quality of **affinity** is not determined alone by the cell or alone by the selected biomaterial, but is triggered in a bi-directional cooperation.

As described before, exact information about adherence of cells and affinity to a new biomaterial and to endothelial cells is essential, when for example a blood vessel prosthesis has to be tested. To evaluate adhesion, adherence and affinity under in vitro conditions, endothelial cells are cultured on selected materials within a dish. Then the specimens are transferred to a tube and centrifuged in cycles with different rotation speeds. Parallel to increasing gravity force cells will stand the centrifugation procedure showing more or less close adherence to the selected material. Specimens made out of isolated blood vessels are serving as control. The quote between non-adherent and adherent cells will illustrate the quality of adherence of endothelial cells and will reflect the degree of affinity between cells and the selected biomaterial.

A further test to investigate the adherence of cells on a selected biomaterial is the exposure to a rheological stress situation. Such an experiment starts with the culture of endothelial cells on different biomaterials placed within a dish. After a few days the specimens with more or less adherent cells are transferred to a perfusion container, where variations in different fluid streaming can be simulated. Here the biomaterials must be kept in parallel to the wall of the perfusion container so that a constant laminar streaming can arise. An increasing transport rate of culture medium will result in an arise of rheological stress on adherent endothelial cells. Long term culture experiments revealed that not only adherence of endothelial cells on a new biomaterial can be elaborated but also positive and negative influences on cell differentiation.

It cannot be predicted if cells are really developing a close adherence to a selected biomaterial. In consequence, for each cell type and for each material culture experiments have to be performed. In a further series of experiments for determination of adherence selected biomaterials were placed in a culture dish. Then cells in suspension were transferred by a pipette onto the materials. After increasing periods of culture time it was possible to analyze with different biophysical methods in how far cells exhibited adherence on a selected material. Since it is not possible to predict if cells will stay on a certain material in close contact or if they detach, the specimens were transferred every day to a new dish.

For example, analysis by light microscopy showed that some cells exhibit close adherence, while others showed only weak adhesion. To investigate better the quality of adherence, specimens were centrifuged. Results showed that cells with stronger adherence remain in conjunction with the material, while cells with weak adhesion were found to be detached. Labelling and counting of both groups of cells with a fluorescent cell marker such as DAPI demonstrated the degree of adherent cells on a selected biomaterial.

When cell lines are used for this kind of experiments, it has to be considered that a specific selection of cells with high affinity to a biomaterial can be made. The procedure is very simple, since in this case only cells are taken for subculture, which exhibit a high adherence on a selected biomaterial. In consequence, each subculture produces by selection pressure more cells exhibiting strong adherence to the biomaterial. In contrast, following this procedure all of the cells are lost showing less good adhesion features.

However, the application of this method is not effective for biomaterial testing, since it does not reflect the real situation. For that reason convincing experiments are always performed with cells showing the original identity.

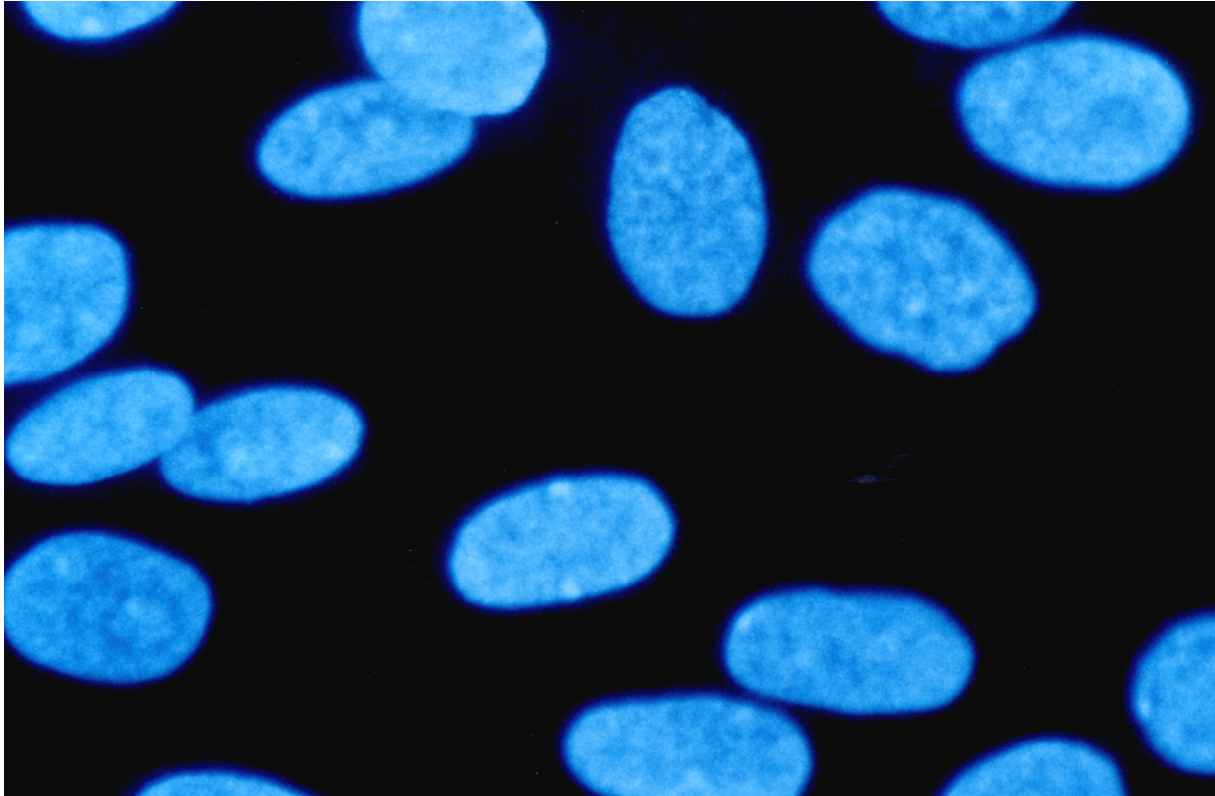


Figure 35: Histochemical label of DAPI to recognize nuclei within MDCK cells.

Alone the number of cultured cells exhibiting adhesion and adherence to a selected material does not reflect an equal distribution on a two-dimensional surface or within a three-dimensional matrix. This aspect is especially important for cells within scaffolds exhibiting an outer and inner surface. To obtain more information, cells were cultured on various materials for different periods of time. Then the cells were labelled with a fluorescent nuclear marker such as DAPI to determine the number of cells in combination with the spatial distribution of cells using a laser scanning microscope (Fig. 35). Applying a morphometric program the exact distribution of cells and the cell density in relation to the surface of the biomaterial can be visualized and calculated.

**[Search: cell culture adhesion adherence affinity]**

**Self-study: Read about adherence of cells in culture**

## **6.2. Adherence meets differentiation and dedifferentiation**

It was shown that cells do not retain their original morphology after isolation and at the start of culture but exhibit a more or less round shape (Fig. 36). After a while the cells show some adhesion on the bottom of a dish, then the round phenotype is altering with proceeding time into a flat cell type. One may argue that a loss of morphology is not dramatic for the performance of the culture experiment. However, the problem is that not only the morphological appearance but also important physiological and biochemical functions are altering during culture. This process is called dedifferentiation (Fig. 36).

Since all of the cellular functions are perfectly established within the organism, the isolation procedure and the environmental conditions during performance of culture must be responsible for the progress of dedifferentiation. The problem of dedifferentiation is not important as long as cell divisions are required. However, when tissue or organ specific features are needed in culture experiments, the problem of dedifferentiation will play an essential role for the success of the ongoing experiments. Thus, to prevent dedifferentiation and to gain redifferentiation the strategy in the culture experiments must be to offer always a most adapted environment.

It is obvious that for example cultured hepatocytes missing detoxification enzymes cannot be used for drug testing. Experiments further revealed that renal cells in culture frequently do not react as expected after application of a hormone. Reasons for decreased or missing hormonal response might be that the number of hormone receptors in cultured cells is decreased or that the receptors are not any more expressed due to dedifferentiation. It may also happen that the intracellular signal cascades are not any more linked to the transduction mechanism. In this case critically one has to analyze if the mechanism of transduction is destroyed due to the suboptimal culture conditions causing in turn dedifferentiation.

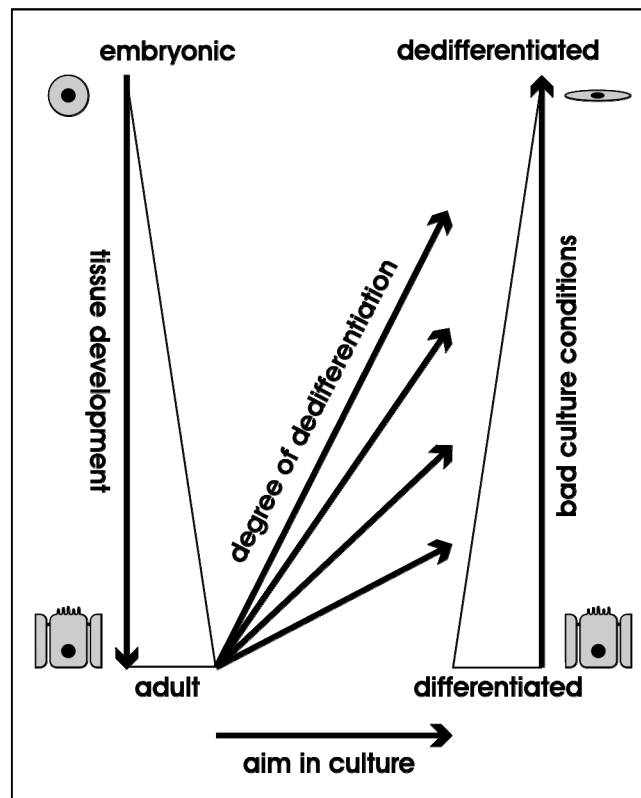


Figure 36: Schematic view to the process of differentiation during tissue development and dedifferentiation under culture conditions. The degree of dedifferentiation depends on the culture environment.

Not a single culture model is known, where the process of dedifferentiation does not play a role. Many factors are not known causing dedifferentiation of cells. To prevent the progress of dedifferentiation, the anti-parallel lining mechanism of differentiation must be improved under culture conditions (Fig. 36). This can be reached by offering an adapted fluid environment and by mimicking the natural extracellular matrix. Experiences in the last years showed that the application of such advanced culture techniques stimulates the degree of differentiation by reducing in parallel the process of dedifferentiation. However, up to date it is not elaborated in how far a dedifferentiated cell is able to a complete reversal development back into the differentiated type with its original morphology and specific functional features found within specialized tissues.

**[Search: cell culture differentiation dedifferentiation]**

**Self-study: Get updated about cell differentiation and dedifferentiation**

## 7. Cells meet materials

It is well known that a culture dish contains a transparent bottom so that cells can be easily visualized without staining under life conditions by a conventional inverse microscope equipped with phase contrast objectives. However, in many cases cells are cultured in combination with non transparent biomaterials such as filters, substrates, supports or scaffolds. In this case the cells have to be labelled by a fluorescent marker so that their distribution can be easily determined by a fluorescence microscope.

### 7.1. Visualization of cells on non transparent surfaces

Although the surrounding of cells is not transparent, the detection of cells can be performed within shortest time. For example, for identification of cells fluorescent dyes can be used, which aggregate with DNA so that a fluorescent nucleus shows the actual position of the cell. Typical dyes are propidiumiodide, 4-6-diamidino-2-phenylidol-di-hydrochloride (DAPI) or bisbenzimidazole. This labelling method is so sensitive that on non transparent materials a single cell or the arrangement of few cells can be recognized.

The protocol for the detection of cells growing on a selected biomaterial is very simple:

The selected specimens are fixed for 10 minutes in ice cold 70% ethanol, washed twice for 2 minutes in phosphate buffered saline (PBS). In the next step propidiumiodide (0.1 - 1 µg/ml) diluted in PBS is added for 2 minutes. The specimens have to be incubated in the dark so that the dye is not bleaching. Finally, the specimens are washed twice in PBS. Analysis is made under the fluorescence microscope using UV-excitation. Applying the described simple technique the nuclei of cells appear in a light colour (Fig.37).



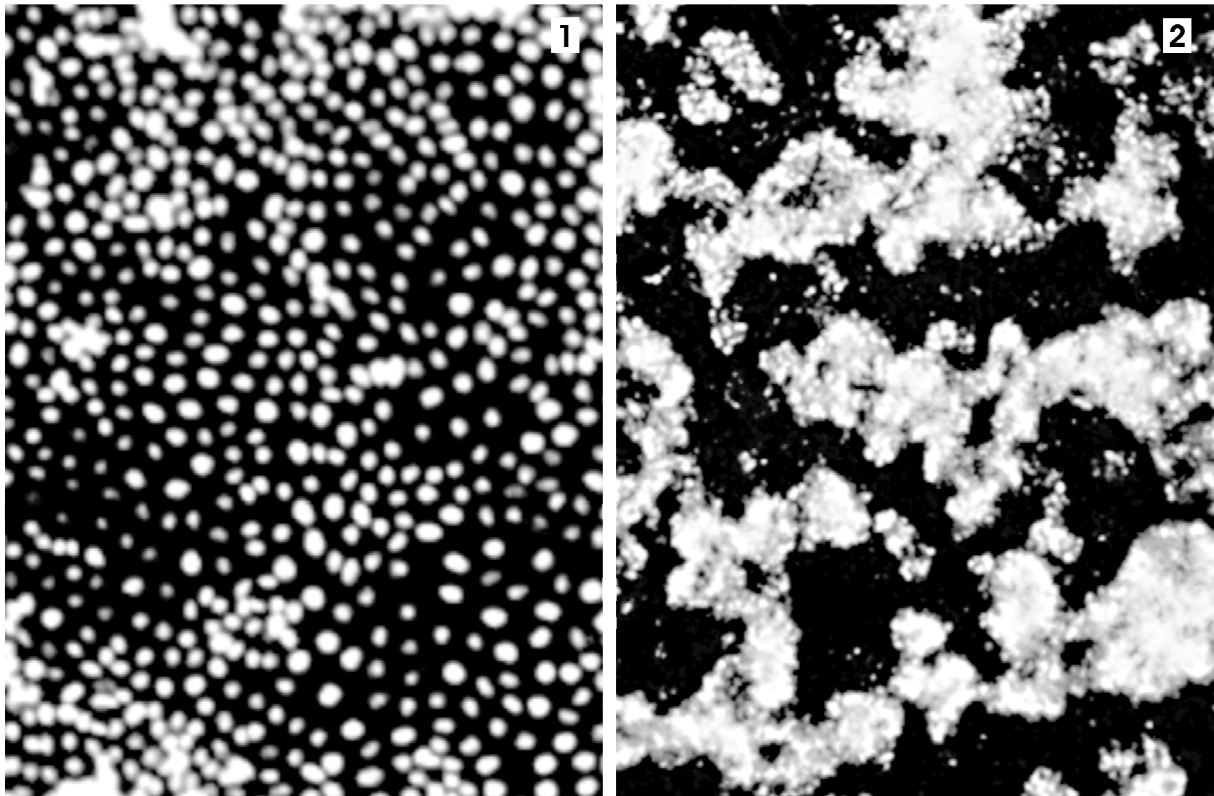


Figure 37: Fluorescence microscopy of nuclei after label with propidiumiodide. MDCK cells are growing on a suitable (1) and an unsuitable surface (2). The attractive surface shows more or less equal distribution of cells (1), while the unsuitable material exhibits intense cluster formation (2).

The described label technique shows for example that the adhesion of cells on an attractive biomaterial takes only few hours, while an unattractive material does not reveal adherent cells after days. Further comparing two different biomaterials illustrates that cells react in an unexpectedly sensitive manner. While the growth of cells on an attractive substrate results in an equal distribution of cells (Fig. 37.1), the unattractive material shows intense cluster formation of cells (Fig. 37.2). This example depicts that the contact to neighbouring cells becomes more attractive than the contact to the selected substrate.

**[search: cell culture dapi propidiumiodide]**

**Self-study: Inform yourself about fluorescent cell markers**

## **7.2. Polish of polystyrene surface**

Most of the currently available culture dishes are made out of polystyrene. It is important to mention that this material does not occur in the organism. Moreover, from the biophysical point of view polystyrene exhibits hydrophobic features and appears by the first cell biological view not to be suitable for cell culture experiments.

Missing or minor adhesion of cells is also true for freshly injection molded culture dishes. For that reason the surface of the dish has to be modified after the injection molding process so that more hydrophilic features arise stimulating in turn the adhesion of cells.

The change from a hydrophobic into a hydrophilic surface is performed by a plasma respectively corona treatment. During this process high electric voltage in the presence of an atmospheric gas modifies the inner surface of the culture dish. In an actual protocol the treatment is performed under a partial vacuum and a defined gas atmosphere so that under high voltage controlled plasma is created. As a result the hydrophobic surface is promoting yet perfect adhesion of many cell types on the polystyrene surface at the bottom of a culture dish.

Further it was shown that incorporation of nitrogen and the addition of cations to polystyrene positively influence the adhesion and spreading of cells. Following this strategy Primaria® culture dishes for the adhesion of individually selected cells are produced. When these specific dishes are applied in culture experiments, improved adhesion, adherence and proliferation was obtained with endothelial cells, embryonic neurons and hepatocytes.

**[search: cell culture dish polystyrene]**

**Self-study: Get updated about cell adhesion on polystyrene**

### 7.3. Modulation of adhesion

The quality of cell adhesion on the polystyrene of a culture dish does not only depend on a plasma treatment but is also influenced by the chemical purity of the polystyrene used for injection molding. The unique phenomenon of a more or less intense cell adhesion can be demonstrated in cultured dishes with specifically treated bottoms.

It is known for example by many experiences, how long time is needed until an individual cell type is growing in form of a confluent monolayer on the bottom of a polystyrene dish. When a part of the bottom is coated by a polymer such as polyethylene (PE), the number of adherent cells at this site is drastically reduced so that a confluent monolayer will not establish (Fig. 38). This example clearly shows how sensitive cells can react, when they contact a surface appearing in their opinion less suitable for adhesion. Further it is frequently observed that exposure of cells to a less suitable substrate is not leading to an equal distribution but to a separate aggregation of cells. For many classical two-dimensional culture experiments this behaviour of cells can be seen as a disadvantage. However, aggregation of cells is an important presupposition, when innovative three-dimensional cultures are performed.

Beside polystyrene (PS) other materials such as polycarbonate (PC) or polymethylmethacrylate (pMMA) are known to exhibit excellent features for cell attachment and growth (Fig. 38). Up to date it is not known, why cells show adherence to these materials although no molecular similarity exists to the natural extracellular matrix. It is most probable that not a single molecule but a whole series of biophysical parameters influence attachment, adhesion, migration and long lasting adherence. Experiments with fibroblasts for example show that the surface energy has an important influence on the distribution of cells on a culture substrate.

Obviously an interaction exists between the negatively charged plasma membrane of the cell and the surface of the selected material. For example, the growth of fibroblasts on

polyethylene (PE), polystyrene (PS), polycarbonate (PC), polymethylmethacrylate (pMMA) and glass appears very different (Fig. 38). According to this result it would make more sense to use for optimal cell adhesion not polystyrene but polycarbonate, polymethylmethacrylate or glass. Only few companies for example Laxbro® are producing dishes, who are made out of polycarbonate. However, due to optimal performance, ease of use and optical clear surface polystyrene became the most frequently used material for production of cell culture dishes and flasks.

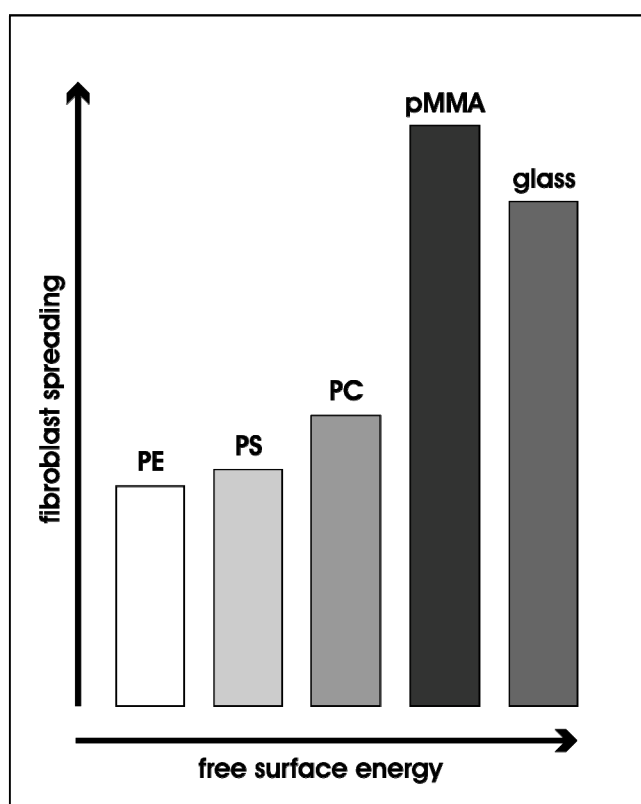


Figure 38: Schematic illustration of growth of cultured fibroblasts on polyethylene (PE), polystyrene (PS), polycarbonate (PC), polymethylmethacrylate (pMMA) and glass (G) surfaces. The spreading of fibroblasts obviously depends on further but not clearly specified features of the free surface energy.

When metal surfaces are tested with cell cultures, the problem arises that the electric conductivity stimulates redox reactions resulting in the denaturation of proteins leading in turn to an irreversible damage of cells. Further the spatial distribution of cells depends on microstructural features of the metal surface. A polished and plane surface will result in a

different cell distribution than a surface with various sizes of pores or roughness of the material.

All of the experiences with cells seeded on different materials show that a perfect material for every type of cell does not exist. The data further exhibit that every cell type has specific requirements. Thus, for each cell type a specific material containing features for an optimal adhesion has to be selected. A material most suitable for hepatocytes is probably not usable for insulin producing cells. For cells of the connective tissue such a material is completely unsuitable.

Finally, during development of an innovative surface for a biomaterial an optimal adhesion of cells cannot be theoretically predicted. For that reason cell culture experiments with many different material specimens have to be performed so that finally a candidate arises, which illustrates optimal features for adhesion.

During progress of the experimental work one can recognize how stable and how sensitive cells react with expected differentiation or unexpected dedifferentiation. A major problem in this coherence is that on the one hand seeded cells can show a high affinity to the material but on the other hand they can exhibit a low degree of tissue specific features. In consequence, the cells remain in a more or less immature state. Such a selected material would be worth to be used in further investigations.

The Corning Synthemax Surface® is an innovative synthetic surface coated onto multiple well plates that mimics aspects of natural cell environment. The special surface supports expansion of stem/progenitor cells in their undifferentiated state and enables differentiation.

**[search: cell culture polystyrene polycarbonate]**

**Self-study: Inform yourself about cells in contact with biomaterials**

## 8. Improvements for cell adhesion

It has been shown that a variety of cells is able to adhere on the bottom of a culture dish made of polyethylene, polystyrene, polycarbonate, polymethylmethacrylate or glass (Fig. 38). This reaction is curious, since these materials do not occur in the organism and they do not show any similarities to protein sequences found in the extracellular matrix (ECM). However, it is also known that a variety of cell types does not optimally adhere at the surface of a culture dish. In contrast, working with a different group of cells such as stem/progenitor cells, adhesion to the dish has to be prevented. As an alternative for the dish made out of polystyrene stem/progenitor cells and tumour spheroids can be kept for example in Non-Adherent Culture Dishes® (NACD).

Moreover, some sorts of cells like more dishes made of polystyrene, while others prefer polycarbonate or like to adhere on glass. It was also frequently observed that cells optimally adhere within polystyrene dishes made by a company X, while they do not show adhesion within dishes produced by a company Y, although both kinds of dishes were made of chemically pure polystyrene. Most probably a difference in the plasma respectively corona treatment is causing the different quality of adhesion.

In general, many cell lines do not react so sensitive, when they are kept in a culture dish. However, other cell lines and especially freshly isolated cells derived from tissues or organs feel very much irritated when they are exposed to polystyrene dishes reacting with minor adhesion and missing proliferation so that they die after a short period of culture time. In consequence, for these kinds of cells the surface at the bottom of the selected culture dish has to be modified. The challenge is to improve the polystyrene so that the surface resembles as most as possible the extracellular matrix. This modification can be performed by coating the polystyrene with serum, extracellular matrix proteins or analogous molecules.

A selection of coated dishes is commercially available, but a coating can also easily be produced in the own laboratory.

**[Search: cell culture polystyrene]**

**Self-study: Get updated about modifications of polystyrene in culture dishes**

## **8.1. Coating substrates with molecules mimicking ECM**

As shown before extracellular matrix proteins are regulating in the organism a wide range of cell functions such as migration, proliferation, adhesion, adherence and differentiation but also survival. When the problem in culture exists that cells do not show a widespread adhesion or differentiation, coating of the dish may help (Fig. 39.1).

The simplest form of coating can be performed with pure human or foetal calf serum, since for example circulating fibronectin is contained. In consequence, with a pipette serum is filled into the dish so that the complete surface of the bottom is covered. Then the dish has to be dried over night by evaporation under the sterile bench. The next morning a barely visible coat of adsorbed proteins is covering the bottom of the dish. For use the dish is carefully filled with medium including cells (Fig. 39.2).

Collagens, fibronectin, laminin, merosin, chondronectin, vitronectin, tenascin, hyaluronic acid with its numerous derivatives and other molecules of the extracellular matrix are commercially available so that they can be applied for the coating of cell culture plastic ware in the laboratory. First of all, the individual extracellular matrix protein is ordered. According to the suggestions of the supplier the protein is solubilised in a physiological buffer. Then the protein solution is transferred by a pipette into the dish so that the complete surface of the bottom is wetted. Over night the specimen is dried by evaporation under the sterile bench.

After drying a barely visible coat of extracellular matrix proteins is covering the bottom of the dish.

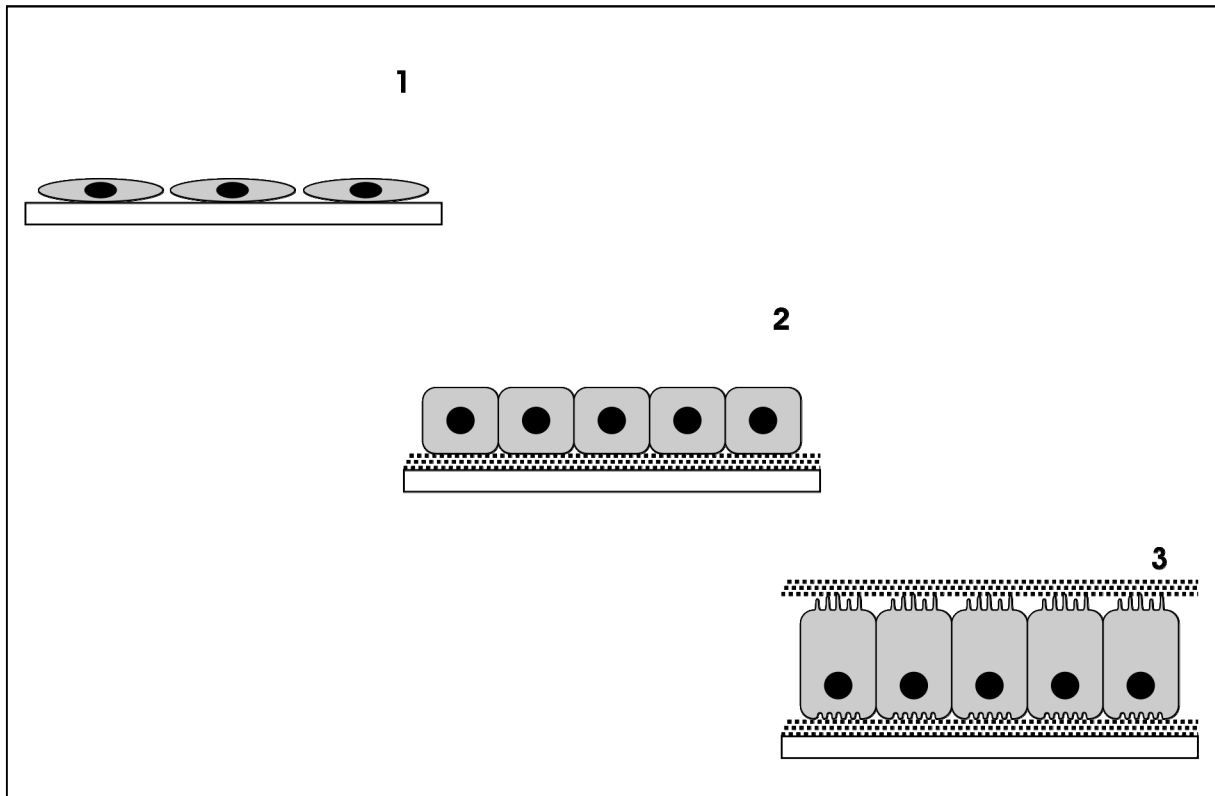


Figure 39: Schematic Illustration depicts development of hepatocytes on the bottom of a dish (1), on a bottom coated with collagen (2) and between coatings on the base and top (3). While low polarization is found at the bottom of the culture dish (1), increased differentiation is detected after coating with collagen (2). Sandwich coating leads to perfect functional differentiation (3).

The low solubility of some of the extracellular matrix proteins can be improved by increasing the NaCl concentration in the used buffer solution or by shifting the pH into the more acid range. In consequence, before application of cells the dish has to be carefully rinsed with a physiological saline buffered solution contained in the later used culture medium. Moreover, for coating it is not necessary to use intact proteins of the extracellular matrix. Excellent results were obtained by coating with molecular fragments of the extracellular matrix such as poly-d-lysine or polyornithine.



For optimal cell adhesion nowadays a great choice of plastic ware is commercially available containing different molecules of the extracellular matrix. However, not in all cases a comparable quality of coating is assured resulting probably in inconstant adhesion. The reason is that the origin of extracellular matrix is frequently uncertain and consequently the process of isolation is not certified. Further the industrial procedure for coating is technically difficult to perform, since proteins of the extracellular matrix are barely soluble so that they do not remain in equal concentration in solution. For that reason it is no easy to coat with always the identical concentration and the thickness of the protein layer at the surface of the dish may vary.

Industrially coated cell culture plastic ware contains for example collagen type I, II, III, IV, V and VI, fibronectin, laminin, merosin, vitronectin, chondronectin and tenascin. Frequently also poly-L-lysine and poly-L-ornithine are used for coating. It is evident that all of the coatings can be made for example in combination with fibronectin and laminin. Further, Extracel® is a semi-synthetic hydrogel composed of hyaluronic acid and denatured collagen. The hydrogel can be tailored to include matricellular proteins and growth factors.

Finally, Matrigel® or other analogous materials can be used for coating of cell culture ware. The material consists of a heterogenous protein composition consisting of extracellular matrix proteins and is synthesized by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells. The mixture resembles the complex extracellular matrix environment detected in many tissues. A common laboratory procedure is to pipette small volumes of Matrigel® at 6 °C onto a culture dish. When the specimens are incubated at 37°C, the contained proteins polymerize by forming a spider net at the bottom of the dish. The coating is effective for the attachment and differentiation of both normal and transformed anchorage dependent cell types.

An excellent alternative to Matrigel® is Cultrex® human derived Basement Membrane Extract (BME). The material contains extracellular matrix proteins purified from the human placenta. It is free from tumour or animal specific proteins. The extract is further tested for sterility and appears to be free from 8 relevant human pathogens. Further the extract is functionally tested for promoting and maintaining an improved phenotype in a variety of cell types.

FASTCell® Meta-Keratins are innovative coating products that make use of human keratin biomaterials, which can be utilized in place of tissue culture coatings such as collagen or basement membrane extract. Available as a lyophilized powder for reconstitution as a coating material, Meta-Keratins are purified, sterile and free of any animal materials. Meta-Keratins contain structural features such as binding domains that are recognized by integrin receptors promoting cell adhesion, differentiation and physiological function.

A further possibility of coating is to start the cell culture with adhering cells and then to make a sandwich overlay for example with collagen or agarose containing culture medium (Fig. 39.3). First of all, cells are transferred by a pipette onto the bottom of a culture dish. After adhesion of cells is observed, the culture medium is carefully drawn off by a pipette so that cells remain wetted in the dish. For coating a collagen or 0.5 to 1% agarose solution containing culture medium is warmed up to 39° C. Then the collagen or agarose solution is transferred within a warm pipette to the cells to covered them with a thin layer. In the same way cells can be coated by fibrin glue or a mixture of extracellular matrix proteins. This special overlay technique allows the cells to develop at the bottom of the dish or to detach and to spread into the covering extracellular coat material.

Finally, exact pattern of extracellular matrix proteins can be printed with the Nano eNabler® system on selected surfaces. The apparatus is suitable as a multifunctional patterning platform for dispensing extremely small volumes of extracellular matrix protein in the range of

attoliters to femtoliters onto a variety of substrates. The Nano eNabler<sup>®</sup> system prints spots or lines from 1 to 60 µm and has a stage resolution of 20 nm. Such patterns are of special interest to investigate the outgrowth of axons and dendrites or to analyze migration of cells during wound healing.

**[Search: cell culture polystyrene coating collagen]**

**Self-study: Get updated about coatings in cell culture dishes**

## **8.2. Adaption of coating for an individual cell type**

The coating of culture dishes can be very specifically adapted for the development of an individual cell type. In this coherence it has to be mentioned that not only the coating but also an individual culture medium and distinct growth factors play essential roles. Some examples are given here.

Smooth muscle cells can be excellently cultured, when the dishes are coated with collagen type I or fibronectin or both. To obtain a high degree of differentiation, the cells have to be kept in a medium containing Tumour Necrosis Factor  $\alpha$  (TNF- $\alpha$ ), Insulin like Growth Factor I (IGF-I), Platelet Derived Growth Factor (PDGF), Fibroblast Growth Factor (FGF) and Epithelial Growth Factor (EGF).

The multiplication of Foetal Bovine Heart Endothelial (FBHE) cells can be performed in culture dishes coated with collagen type I. Cells are kept for example in MCDB 131 medium containing Foetal Calf Serum (FCS), hydrocortisone and heparin. Another possibility is to apply collagen type I discs from Viscofan Bioengineering<sup>®</sup> to adhere for example Saos-2 cells, neurons or cardiomyocytes. In this case production is performed according recommendations for safety of the American Society for Testing and Materials (ASTM F 2212-02).

For the development of epithelial cells from the respiratory system culture dishes are used coated with collagen type IV and fibronectin. Depending on the species different kinds of media can be used for culture.

For an optimal differentiation of hepatocytes culture dishes can be used, which are coated either with Matrigel® or Cultrex® human derived basement membrane extract. It has been shown that excellent culture results were obtained with Williams E medium containing a cocktail of insulin, transferrin, selen, dexamethasone and EGF.

An optimal development of neural cells can be reached when coating is performed with collagen type IV, laminin, fibronectin and Matrigel® or in a combination with laminin, poly-d-lysine and poly-L-ornithine. In this set of experiments specific supplements must be added to the culture medium supporting differentiation of neural cells.

Flexcell® culture dishes provide the user with enhanced cell adhesion to investigate growth and differentiation. This specific type of culture plate was designed for use within a Flexercell® strain unit. The apparatus permits application of static or variable mechanical deformation to the flexible bottom of Flexcell® culture dishes. The surfaces of dishes are covalently bonded with collagen type I or elastin or are charged with a positive or negative surface. For example, in collagen type I coated dishes excellent development of osteoblasts, chondrocytes, synovial fibroblasts, tendon fibroblasts, ligament fibroblasts, endothelial cells and smooth, striated and cardiac myocytes is achieved.

**[Search: cell culture coating collagen]**

**Self-study: Inform yourself about collagen coating in culture dishes**

### 8.3. Adhesion on biological materials

To stimulate the development of cultured cells also fibroblasts producing extracellular matrix such as 3T3 cells can be used. First of all, a monolayer of 3T3 cells is established at the bottom of the dish. Then the medium is drawn off with a pipette so that the specimen can be dried under the sterile atmosphere of a bench. To eliminate cellular proteins, Triton X-100, ammoniumhydroxide or other detergents are used. Then the dish is rinsed several times with Phosphate Buffered Saline (PBS) solution. Finally, on the dish remains a newly synthesized extracellular matrix, which is favourable for the adhesion and development of a multitude of cell types.

Beside proteins isolated out of the extracellular matrix a series of intact but stripped matrices of different tissues and organs can be used to optimize the culture of anchorage dependent cells. Such cell free matrices can be obtained by biochemical extraction with detergents such as Triton X-100, desoxycholate or partial enzymatic digestion by collagenase. The extraction procedure can take several days. Then the specimens are rinsed several times in PBS and sterilized under ultraviolet (UV) light. The obtained ECM represents a spider net so that cells will adhere on the matrix and in the space between the synthesized fibers.

Using ultrasound isolated ECM specimens can be destroyed into small particles, which are suspended then in serum. The resulting mixture is transferred by a pipette onto the bottom of a culture dish. Then the specimens are dried over night under the bench. The next morning cells within medium can be added to the preparation promoting adhesion, adherence, migration, proliferation and differentiation.

A further substrate isolated out of a natural organ is Small Intestine Submucosa (SIS). For isolation favourably the gut of pigs is used. The mucosa containing the Lamina epithelialis, Lamina propria and Lamina muscularis mucosae is stripped off at the lumen. From the outer side of the gut the Tunica muscularis is isolated. The remaining Tela submucosa is used for

further preparation. The cellular material is treated by proteinases and detergents. Finally, the remaining reticular extracellular matrix is sterilized. Actual experiments show that the isolated SIS can be used in a variety of three-dimensional culture experiments exhibiting improved adhesion, adherence, proliferation and differentiation of cells. Most interesting is the regeneration of intestine epithelia within SIS, since it is used in the clinical environment for the repair of intestine. Yet a variety of materials such as CuffPatch<sup>®</sup>, Surgisis<sup>®</sup>, Durasis<sup>®</sup> or Stratasis<sup>®</sup> is on the market. Further acellular matrices from other tissues are available.

Beside the classical collagens other biological materials can be applied, which do not consist of amino acids but are based on polysaccharides such as dextran, starch or gellan. The exoskeleton of crustaceans such as crabs or shrimps is an excellent substrate for the culture of adherent cells. The isolated polymer chitosan is closely related to cellulose. It promotes cell development, inhibits bacterial growth and prevents inflammation. Chitosan is able to store humidity, binds a series of proteins and is degradable. A multitude of cell types was shown to adhere and develop on chitosan coatings.

Numerous experiment have shown that a single sort of molecule or an individual material does not exhibit the optimal adhesion and differentiation for the selected cell type. For that reason composite materials such as poly ( $\epsilon$ -caprolacton-co-D,L-lactide)/silk were synthesized to increase the adhesion of connective tissue cells for cartilage and bone regeneration.

It is understandable that not all of the multiple biomaterials can be mentioned in this coherence. However an insight was given that a variety of possibilities is available to promote adhesion, adherence and development of cells in combination with selected biomaterials.

**[Search: cell culture decellularized matrix, cell culture small intestinal submucosa]**

**Self-study: Read about adhesion of cells on extracellular matrix such as SIS or silk**

## 9. Cells contact biomedical materials

Beside the natural extracellular matrices of human and animal origin a variety of technical materials exists, which are tested by the help of cell cultures to evaluate their biocompatibility.

For each of the materials it has to be considered that cells react extremely sensitive when they come in contact with an individual surface. Since many of these materials are used in the bright field of biomedicine and in the clinical environment very carefully it has to be investigated if cells show close adherence or if a refusal is recognized. In contrast to in vivo implant experiments on animals culture set ups provide a useful tool to investigate cell and matrix interactions with alloplastic material surfaces in detail.

Processes such as cell adhesion, adherence, proliferation, differentiation and dedifferentiation can be analyzed in detail with cells in culture. In this case the cells are acting as extremely sensitive biosensors. They indicate without any doubts the difference between bad, mediocre, good and excellent adhesion, when they come in contact with the surface of a selected biomaterial.

All of these data reflect fruitful hints and result in important perspectives, since a close contact of cells is urgently needed and must be in the interest of all the patients. None of them accepts the implantation of a vessel prosthesis showing bad adhesion for endothelial cells but promoting coagulation of blood. Moreover, each patient trusts in the mechanical stability between bone and titanium used as dental or orthopaedic implant. With other words, no one likes to have a squeaking hip.

**[search: cell culture biomaterial test]**

**Self-study: Get updated about cell culture with biomaterials**

## 9.1. Kinds of materials

The presently used biomaterials consist either on homogenous or on composed materials:

- **Polymers** are made out of numerous pure chemical substances.
- **Composite materials** including **ceramics** exhibit biological and chemical features.
- **Metals and alloys** contain stainless steel or titanium and other materials

The different biomaterials are available in form of foils, membranes, three-dimensional fleece structures, discs or blocks. Superstructures can be generated by seeding cells on matrices, which are rolled, folded or grooved during further experimentation. A further possibility is to offer three-dimensional polymers in form of fleeces with different molecular composition. The space between fleece fibers offers an internal surface for cell seeding and tissue development including a perfect exchange of fluid providing nutrition and respiratory gas.

According to a variety of commercially available materials and matrices one could believe that up to date for each individual cell type and for each specialized tissue a perfect material respectively structured matrix is available promoting adhesion and adherence followed by an optimal differentiation. However, at present many of the used materials exhibit a mediocre attraction for the cells resulting in a suboptimal quality and utility. In so far it will take still decades until the biomedical materials are optimized. This import subject was neglected for a too long period of time and was consequently not sufficiently considered during production.

Problematic is further that the cell biological characteristics of a newly synthesized biomaterial cannot be predicted. For that reason intense research with individual cells on new biomaterials has to be performed so that positive and negative cell biological reactions can be recognized.



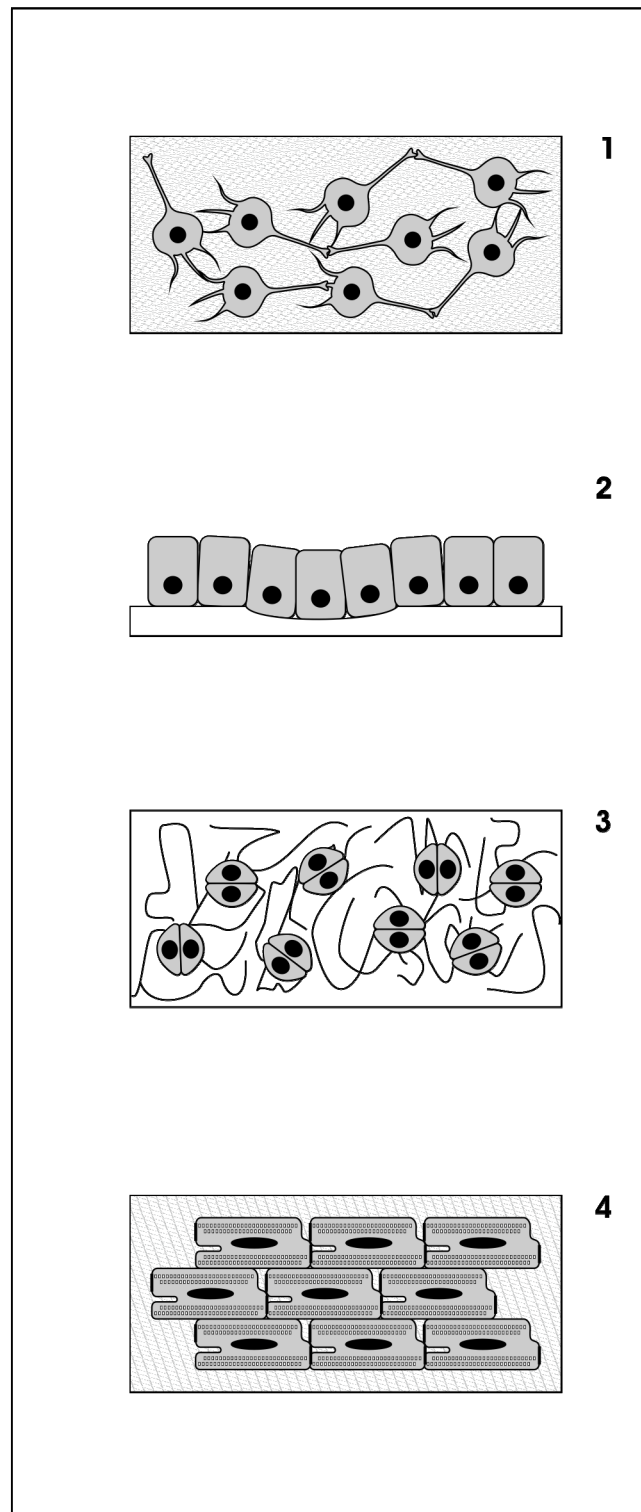


Figure 40: Schematic illustration shows development of tissues on biomaterials. In neural tissue the biomaterial has to support bridging of dendrites and axons (1). In epithelia the surfaces of selected biomaterials must be suitable so that cells can seed (2). In connective tissue the internal space within a biomaterial is for settlement of cells of special importance (3). In muscular tissue the applied biomaterial has to consist of flexible and highly elastic material so that multiple contractions will support maintenance of cells (4).

The spatial structure of a biomaterial has to follow the specific demands of an individual cell type and a specialized tissue so that first in culture and later in the organism typical features can develop in an interactive manner.

In artificial neural tissue the individual biomaterials have to support bridging of dendrites and axons (Fig. 40.1). Further the individual biomaterial must guide the unidirectional growth of the dendrites and axons so that connections over synapses with other neurons over a wide distance become possible. In the case of epithelia the surfaces of selected biomaterials must be suitable that cells can seed (Fig. 40.2). The adherent cells have to stay in close contact to the surface so that they resist mechanical, hydraulic and rheological influences. Regarding connective tissue the internal space within a biomaterial is for settlement of cells of special importance (Fig. 40.3). Here the biomaterial must promote in combination with cells the synthesis of extremely big amounts of extracellular matrix as known from the generation of cartilage, bone or tendon. Regarding muscle tissue the applied biomaterial has to consist of flexible and highly elastic material so that multiple contractions in combination with excellent biocompatibility will support survival of cells (Fig. 40.4).

The different examples show that each individual cell type and each specialized tissue have special demands on the required biomaterial. For that reason a single special matrix for all types of cells does not exist. In conclusion, only very individual biomaterials will promote optimal adhesion, adherence and development of cells in specialized tissues so that functional characteristics can arise and maintained over long periods of time. It can be assumed that each cell type needs its individual biomaterial.

**[Search: cells contact biomaterials]**

**Self-study: Inform yourself about different kinds of biomaterials**

## 9.2. Variety of polymers

The natural extracellular matrix (ECM) consists of proteins and glycosaminoglycans forming spider nets or fleece like structures supporting the mechanical stability and cell biological interactions of cells. However, regarding presently performed cell and tissue culture experiments in combination with innovative biomaterials both of these requirements are not sufficiently obtained. Up to date only a relatively small selection of available biomaterials can be applied as a widespread and technical substitute for the natural extracellular matrix.

The commonly used polymers are based on different chemical substances. To evaluate the cell biological reactions of cells coming in contact with those materials, different kinds of culture tests have to be made. The multiple reactions of cells will indicate how far the materials support adhesion and adherence over prolonged periods of time. Further the materials must be biocompatible so that they can be used in a patient. Especially in experiments within the clinical environment it is analyzed how far cells selectively adhere on a biomaterial and in which period of time a perfect adherence is followed by a specific differentiation. An optimal biomaterial could be recognized by the signal that cells selectively adhere within shortest time so that they remain bound over weeks with intense adherence. In contrast, an incomplete adherence is reflecting a low affinity between material and cells. It signals that the selected biomaterial is not suitable for the individual cell type.

Frequently applied biomaterials in biomedicine are polytetrafluorethylene (PTFE), polyurethane (PUR), polyethylene (PE), polyethyleneterephthalate (PET), polydimethylsiloxane (PDMS), polysulfone (PSU), polymethylmethacrylate (pMMA), polyethylen-covinylacetate (PECA), poly(L)-lactide acid (PLA), polystyrene (PS), poly-2-hydroxyethylmethacrylate (pHEMA), polyacrylonitrile (PAN), polyamide (PA), polypropylen (PP) and polyvinylchlorid (PVC).

The mentioned polymers including polystyrene found in culture dishes do not occur within the organism and all of these materials do not exhibit any similarities to proteins, amino acid sequences or other molecular structures contained in extracellular matrix (ECM). In consequence, up to date it remains obscure, which molecular mechanism makes the more or less intense contacts of cultured cells to these biomaterials.

**[Search: biomaterial polymer composition]**

**Self-study: Get updated about cell culture with polymers**

### **9.3. Smooth polymers**

A further important parameter for cell adhesion and adherence is the capability of polymers to bind solutes. Experiences show that an optimal adhesion and adherence of cells can be obtained with polymers showing not a high and not a low but a mediocre wettability. Most of the polymers additionally exhibit that the cells optimally adhere, when the surfaces was in contact with serum before culture. It may happen that fibronectin contained in serum is bound to the surface of the polymer. The bound fibronectin in turn can contact and activate receptors at the cell surface stimulating in consequence adhesion and adherence. An increased adherence can also be obtained by coating the polymer for example with the extracellular matrix protein vitronectin. Further it was recognized that not only extracellular matrix proteins but also various physico-chemical properties of the biomaterial surface influence adhesion and adherence of cultured cells.

Not only the adhesion but also the proliferation of cells can be influenced by proteins bound to the surface of biomaterials. Such proteins may derive from contacts with serum or by the autocrine secretion of adherent cells. To improve the situation for the binding of cells, surface coating with complete serum or with purified serum compounds such as fibronectin was

performed in the laboratory. Analysis of cell growth shows that coating with fibronectin strongly increases mitosis followed by a spreading of cells. The reaction is mediated by the RGD-sequence (Arg-Gly-Asp) contained in the fibronectin molecule. Instead of the intact molecule also the tripeptide RGD (Arg-Gly-Asp) can be bound to the polymer surface. Onto this peptide sequence cell surface proteins will bind in the same way as it is known from the intact fibronectin molecule contained in the natural ECM. Since this signal sequence is not only contained in fibronectin but also in collagens, laminin, tenascin, vitronectin and thrombospondin, multiple cell types react after contact with increased adherence and spreading.

Increased adherence of cells can be obtained, when for example different polymer materials such as PTFE, PET, PA and PUR are coated by the RGD peptide sequence. A similar improvement of cell adhesion, mitosis and spreading can be observed, when the coating is performed with polylysine, polyornithine, lactose or N-acetylglucosamine. For improved spreading of cells also bi-functional molecular groups such as glycolipids, oligopeptides and oligosaccharides can be absorbed onto polymer materials. Such molecules can be adsorbed by dipping or by plotting the surface of the selected biomaterial. Modifying the strategy of coating individual micropaths can be designed by etching or microgrooving on biomaterials so that for example outgrowth of dendrites and axons in adherent neurons is stimulated or migration respectively orientation of tendocytes and myoblasts is guided.

Also biophysical methods can be applied to improve the surface of polymers for adhesion, adherence, spreading and development of cultured cells. Reasonable adhesion of cells was observed on polymers exhibiting a positively charged surface. An increased synthesis of collagen was obtained with fibroblasts growing on a hydrophobic surface. Culture dishes made of polystyrene exhibited stimulated growth rates, when the surface was radiated or treated with sulphuric acid. Excellent results were obtained, when the polystyrene was modified by adding hydroxy (-OH) and oxygen (-O) groups.

Application of microlithographic methods showed that areas can be generated, where cells exhibit a minor respectively an improved adhesion. One could assume that an increased adhesion leads in turn to a stimulated rate of cell mitosis. However, immature hepatocytes elucidated on macrostructured surfaces an increased proliferation, while they exhibited in microstructured areas a decreased rate of cell divisions but an increased rate of albumin secretion.

**[Search: polymer RGD]**

**Self-study: Get updated about smart polymers in cell culture**

## **9.4. Biodegradable polymers**

In many cell biological experiments and biomedical applications it is aimed to seed cells on a polymer, which is biodegradable so that it can be replaced by newly synthesized extracellular matrix. The group comprises synthetic bioresorbable polymers, natural bioresorbable polymers and bioresorbable ceramics. Bioresorbable materials are frequently used for a wide range of medical applications from drug delivery to fracture fixation and regeneration of cartilage. These materials remain in the organism for weeks, month or even years.

Thus, biodegradable polymers exhibit molecular characteristics degrading during time in the culture medium or after an implantation. The molecular sites of degradation are recognized by the neighbouring cells replacing it in the ideal case during long periods of time by own newly synthesized extracellular matrix (ECM).

Since many years homo- or heteropolymers consisting of poly-L-lactide (PL), polyglycolide (PG) or polylactide-co-glycolide (PLG) are applied in combination with cells of connective

tissue. Some of these materials are used to produce scaffolds for tissue engineering for the regeneration of cartilage and bone.

A frequently used biodegradable polymer is for example PL and PG. Both materials belong to aliphatic polyester including poly( $\alpha$ -hydroxyacids) and are produced in bioreactors by bacteria. Degradation of the material occurs in culture mainly by the surrounding cells in a hydrolytic process. During production of the PG-PL-copolymers the physical and chemical properties can be individually adjusted by variation of the lactide and glycolic compounds. Also the time of degradation is different and can be triggered according to the individual needs. A pure PG fiber is for example completely degrading within seven weeks, while in a PL fiber 10% of degradation is found after six month.

A primary task of a biodegradable polymer is that cells can be seeded within a mechanically stable environment. After this primary function the polymer starts to degrade by different mechanisms such as hydrolysis, enzymatic digestion and dissociation of the three-dimensional polymer complex. According to the molecular composition this process takes either a shorter or a longer period of time.

By variation of the molecular composition the process of degradation can be adapted to the experimental or medical needs. Further, when a polymer with an optimal composition is implanted, the products of degradation are reaching the biological fluid circuit so that they are metabolized and eliminated. It has to be considered that the molecular size of the metabolites has to be as small as possible, so that elimination by the related organs such as the kidneys becomes possible without damage.

When biodegradable polymers are used in cell culture experiments, the degradation products can be detected in increasing concentrations in form of lactic or butyric acid. When these

polymers are tested under the static environment of a dish, two different things have to be considered:

- The degradation of the polymer does not occur on each molecule and on each site of the polymer, but is restricted to individual but not predictable spots.
- At the sites of degradation an increasing number of monomers is liberated so that for example the concentration of lactic or butyric acid becomes rather high in the surrounding culture medium.

Due to these reactions cells are exposed to a high danger that they are damaged during development by the alteration of pH shifting into the acidic range. When the metabolites are liberated over long periods of time and in increasing concentrations into the culture medium, at a certain point of time the physiological tolerance is at the end.

In consequence, a systematic damage of cells will occur when the concentration of metabolites further increases. Most frequently this case is found, when culture experiments with biodegradable polymers are performed in the static environment of a classical culture dish. To surmount the situation perfusion culture in suitable microreactors and with always fresh medium provision must be performed for the constant elimination of harmful metabolic products.

**[Search: polymer biodegradable]**

**Self-study: Inform yourself about biodegradable polymers**



## 9.5. Metals and alloys

The influence of microsurface topography of metal implant materials on cell adhesion, metabolism and tissue development has been widely reported. Features such as roughness, chemistry and surface energy play an essential role, since the sum of these actions influence in turn cell and material interactions.

Because of its suitable physical properties and excellent biocompatibility pure titanium is used as a widespread implant material in various dental and orthopaedic operations. Many in vitro experiments for example with Saos-2 cells, MC<sub>3</sub>T<sub>3</sub>-E<sub>1</sub> cells and osteoblast progenitor cells have shown that the implant surface influences the rate of proliferation, differentiation, synthesis of alkaline phosphatase, extracellular matrix and growth factors. Cells cultured on a rough surface exhibited features of differentiated osteocytes, while a smooth surface was leading to more immature cells. However, the variety of tested titanium disks sizes and the process of manufacturing as well the differences in cell culture protocols made it difficult to compare the results of various groups.

A metal can be used in pure form or it can be combined with other metals such as iron and aluminium to form an alloy. Other features are contained in stainless steel, which is an alloy of iron, carbon and chromium. Stainless steel is stronger than pure iron and more resistant to corrosion than regular steel. All of these metals exhibit very individual characteristics, when cells are cultured on their surfaces.

Unlike most metals, which do not integrate into the surrounding bone, titanium behaves more like a ceramic at the bone implant interface. Stainless steel screws for example become encapsulated by a fibrous membrane rich in inflammatory cells. In contrast, titanium induces a surface layer of calcium and phosphate binding directly to bone without evidence of a fibrous layer and with only minimal inflammatory response. Both examples show that osseointegration is not always complete. In any case the data exhibit that a lot of culture

experiments have to be made in future so that the composition and surface of implants is further improved.

**[Search: cell culture metal alloy]**

**Self-study: Inform yourself about cell culture and alloys**

## **9.6. External surface and internal volume**

For seeding of cells on a polymer its external surface and internal volume are important parameters. The surface of the material exhibits always a certain roughness resulting in a smooth or a more porous outer surface. Beside that many polymers and metals have an internal surface. The pores of the inner surface can have a small or a big diameter. Further the shape can be round, oval or irregular. Due to these various parameters the porosity of the polymer with its inner surface is of great importance.

Some of the parameters can be evaluated by measuring the mass of water absorption. First the dimensions of the polymer material with included water ( $V_{\text{TOTAL}}$ ) are measured.

After freeze drying the weight and the dimensions are determined of the polymer material ( $V_{\text{VOID}}$ ). In consequence, the porosity is defined as volume of void divided by the total volume mass:

$$\text{Porosity} = \frac{V_{\text{VOID}}}{V_{\text{TOTAL}}}$$

From a round polymer material the total volume is calculated from the measured dimensions:

$$V_{\text{TOTAL}} = \frac{\pi d^2}{4} \times h$$

In this case d is the diameter of a circular polymer material, while h is the thickness of the specimen.

The void volume ( $V_{\text{VOID}}$ ) of the polymer material can be calculated by measuring the absorbed water volume. This is done by dividing the water mass, which is the mass of the wet ( $m_w$ ) minus the mass of the dry ( $m_d$ ) polymer material by water density ( $\rho$ ):

$$V_{\text{VOID}} = \frac{(m_w - m_d)}{\rho}$$

All of these parameters have to be determined so that the space for cell seeding and available internal volume for culture medium providing nutrition and respiratory gas can be calculated.

**[Search: cell culture metal internal surface]**

**Self-study: Read about internal and external surface of biomaterials**

## 10. Pattern of adherent cells in culture

Experiments with anchorage dependent cells in culture are frequently performed in the field of actual biomedicine. The applications comprise the multiplication of cells in classical dishes, the search of biocompatible materials for implantation, the selection of biodegradable scaffolds for tissue engineering, the screening of newly developed drugs and the registration of toxic influences derived from industrial or environmental pollution. Most of these experiments are started with continuous cell lines and then succeeded by primary cells, before trials on animals are performed. The experiments are made to obtain a broad spectrum of information comprising cell survival, proliferation, distribution, differentiation, dedifferentiation, metabolism and apoptosis. In many cases they have to be performed in dishes by the help of special coating so that the adhesion sites are improved promoting in turn to develop functionality of cells. It is obvious that not single but a series of parallel experiments has to be performed, until the results can be compared with experienced parameters defined by a Golden Standard. Depending on the special demands in all cases an equal two-dimensional or coordinated spatial distribution of cells plays an essential role.

Considering the mentioned fields in biomedicine it is obvious that culture experiments with anchorage dependent cells are performed on very different materials exhibiting an outer and an inner surface. Not in all cases an even and confluent growth of cells can be expected. For that reason the pattern of growth has to be controlled. To visualize cells on individually selected materials a broad spectrum of techniques have to be applied ranging between conventional microscopy and innovative imaging. Growth of cells on transparent materials can be visualized by inverse microscopy equipped with phase contrast optics, while cells on non transparent surfaces have to be detected principally by fluorescence techniques. Depending on a flat polished surface, a porous material or a spatially structured scaffold one has individually to decide about the suitable imaging system.

## 10.1. Cell growth on flat surfaces

To obtain information about the growth pattern of cells, individually selected biomaterials are excised and sterilized so that they can be placed at the bottom of a dish. Then cells are transferred by a pipette together with culture medium into the dish. After a certain period of time the specimens are controlled by an inverse microscope equipped with phase contrast optics.

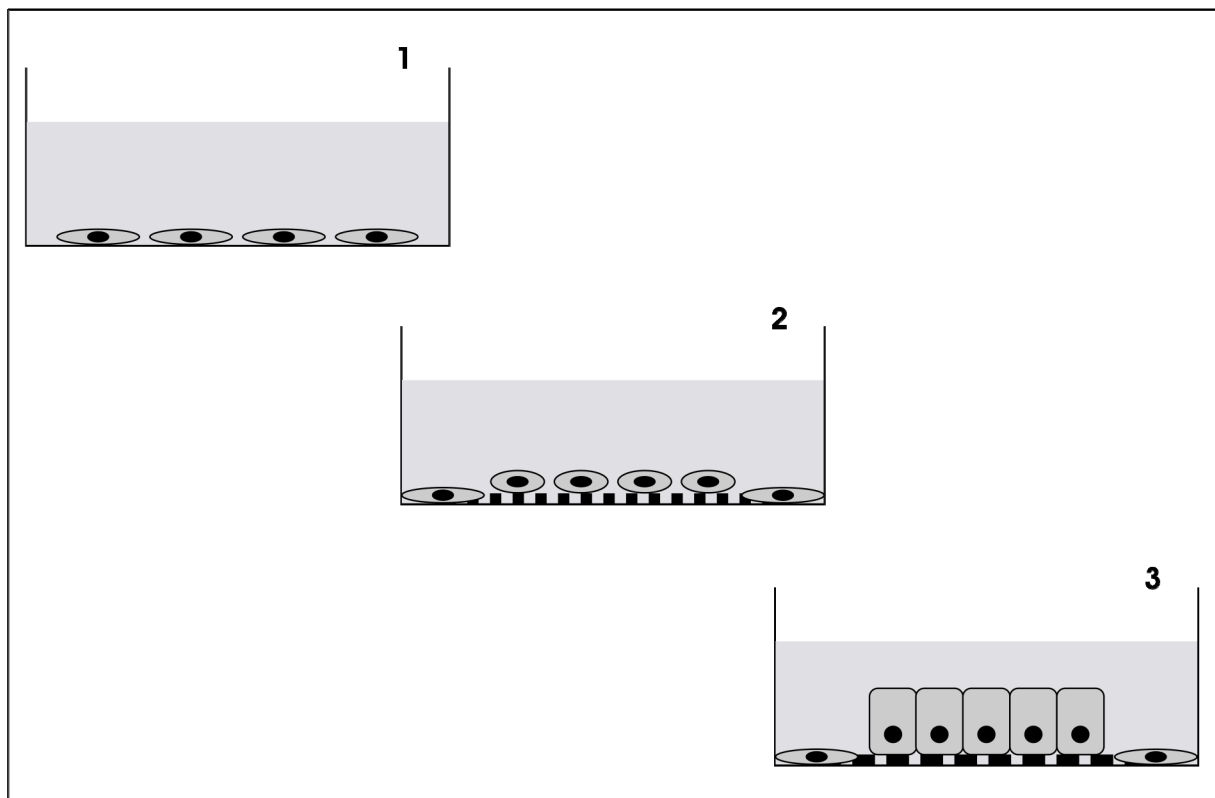


Figure 41: Schematic illustration of cells growing on the bottom of a culture dish (1), on a biomaterial A (2) or on a biomaterial B (3). The shape of adherent cells can be flat, when they are growing on the bottom of a dish (1). Depending on the offered substrate the shape of cells can be oval on the biomaterial A and by contrast flat on the bottom of the dish (2). On a biomaterial B the cells can show a cuboidal shape and again a flat form at the bottom (3).

When no biomaterials are used, it can be observed that cells are adhering to the bottom of the culture dish (Fig. 41.1). When a biomaterial A is offered, cells migrate over the surface to develop an oval shape, while remaining cells stay in flat form at the bottom of the dish (Fig.

41.2). In the case a biomaterial B is administered, cells can form an isoprismatic shape, while the rest exhibits a flat form at the bottom of the polystyrene dish (Fig. 41.3).

However, in many cases the growth of cells has to be tested not on transparent but on non transparent biomaterials. To visualize the pattern of growing cells the cultured specimens are best fixed in 70% ethanol to label them with a fluorescent nuclear dye such as propidium iodide or DAPI. Yet the specimens can be investigated with a fluorescence microscope (Fig. 42). The two-dimensional or spatial distribution of labelled nuclei reflects the position of growing cells. This method is so sensitive that the occurrence of a single cell can be detected without any doubts by this method. In so far the distribution of cells adhering on individual biomaterials can be used to obtain a first impression about positive or negative aspects of cell anchorage and related aspects of the applied biomaterial.

When culture experiments are performed with different biomaterials, it is surprising to observe to which degree surface features are influencing the pattern of cellular growth and distribution. To illustrate these incredibly sensible reactions, in a set of experiments always the same amount of MDCK cells was cultured in contact with selected materials. After three days the culture was stopped by fixation in 70% ethanol to label the nuclei of cells (Fig. 42).

Regarding six different biomaterials six different pattern of cell growth can be detected. Application of biomaterial 1 shows close contact to numerous cells growing on its surface (Fig. 42.1). In contrast, biomaterial 6 depicts a discontinuous growth of cells resulting in formation of clusters, cysts and domes (Fig. 42.6). Such a biomaterial is not suitable for seeding of MDCK cells, since it does not promote optimal adhesion on each site and does not support formation of single layered and polarized epithelium. Also biomaterial 2 (Fig. 42.2), biomaterial 3 (Fig. 42.3), biomaterial 4 (Fig. 42.4) and biomaterial 5 (Fig. 42.5) cannot be used for further applications, since not a confluent growth of cells but formation of

irregular cell rows (Fig. 42.3 and 42.5) or formation of clusters (Fig. 42.2 and 42.4) is observed.

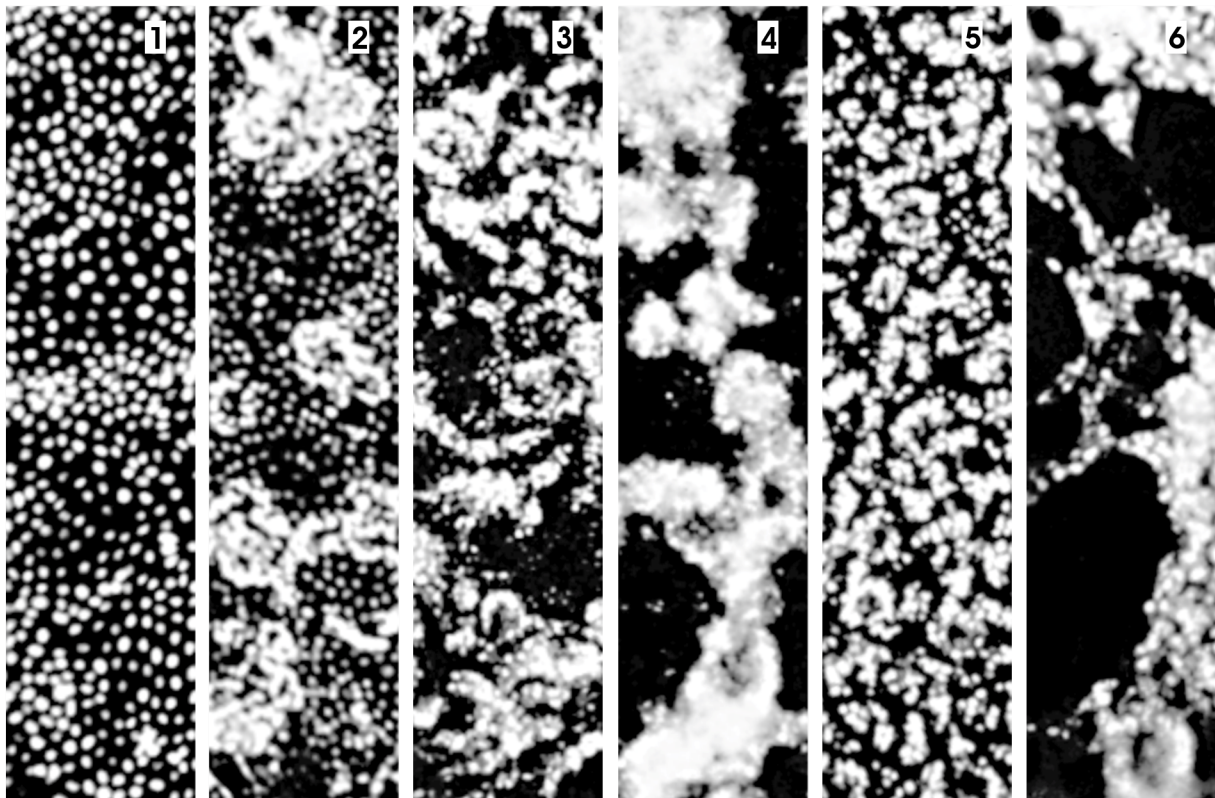


Figure 42: Growth pattern of MDCK cells on six different biomaterials after 3 days of culture. Label of cell nuclei was performed with propidium iodide. Perfect confluent growth of cells is observed on biomaterial 1 (1), while atypical growth in form of cysts is found with biomaterial 6 (6). Use of other biomaterials (3, 5) shows growth in form of irregular cell rows or cell clusters (2,4). The cells react so sensitive that the contact on a biomaterial results in an individual pattern of cell distribution.

The pattern of growth on a selected biomaterial always has to be compared with a well known and accepted Golden Standard. It means that under clearly defined experimental conditions such a Golden Standard has to be created so that the participating scientific community can accept it. Only the comparison with a certified material will make it possible to recognize in how far a newly tested material is less or more suitable than the Golden Standard.

**[search: cell culture flat growth pattern]**

**Self-study: Get updated about biomaterials in cell culture**

## 10.2. Growth on external and internal surfaces

Using in culture flat materials such as foils or filters in the most cases a two-dimensional growth of cells on the surface can be expected (Fig. 43.1).

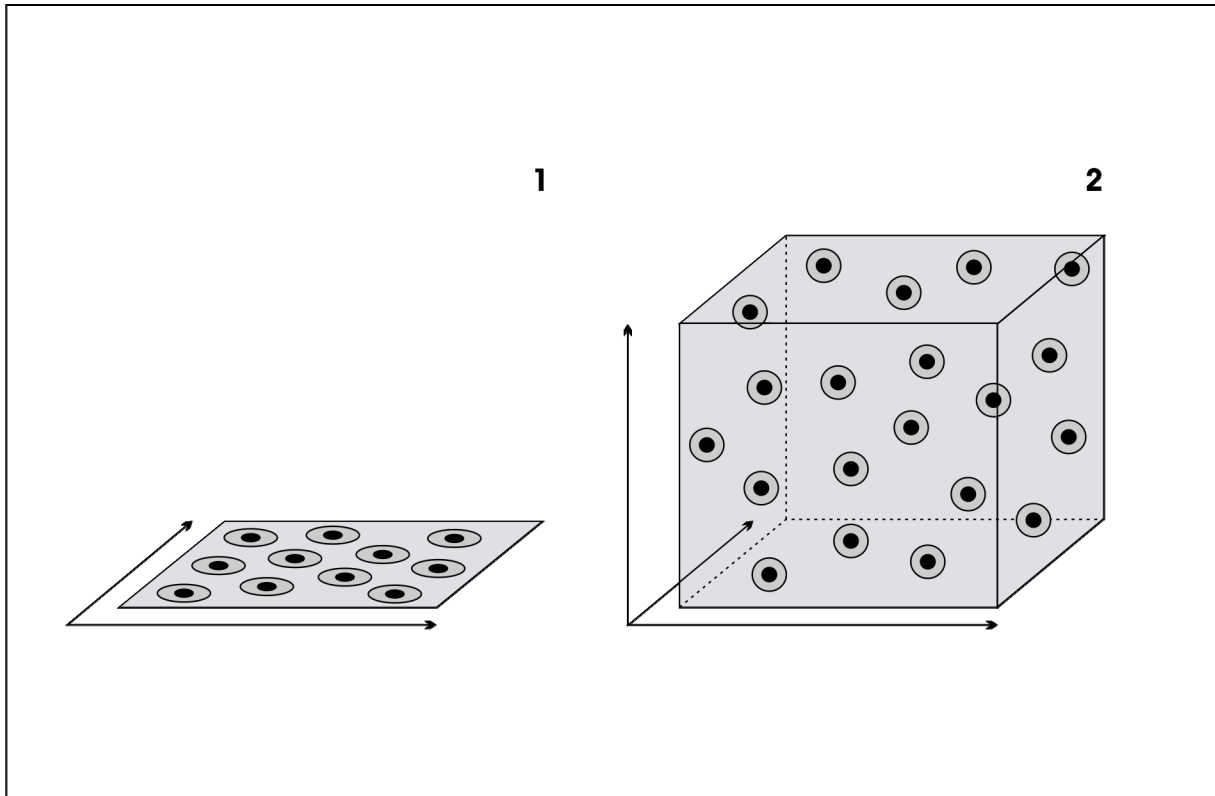


Figure 43: Schematic illustration demonstrates cells growing in two dimensions in form of a flat monolayer (1) and in three dimensions within selected biomaterials (2). The biomaterial in two-dimensional culture will not limit provision with medium and respiratory gas (1), while three-dimensional extension hinders exchange of nutrition and diffusion of respiratory gas but stimulates spatial development (2).

However, when the used material contains a rough surface with protrusions, depressions or even large pores, both the outer surface but also the contained inner surface is accessible for the cell growth within a three-dimensional environment. In so far not only the outer but also the inner surface of a tested material could be attractive for cell adhesion (Fig. 43.2).

However, the situation is more complex. In the case the outer surface is extended and the inner one contains only microporous cavities, the cells will grow as a monolayer only on the



outer surface. In contrast, when the roughness and the pores sizes of the biomaterial increase, cells will grow on the outside as well as inside to cover the internal surface. In this case both either a monolayer or several layers of cells can establish within the pores. By the increase of cell communication within a spider net like fashion finally tissue like structures or even organoids can arise. However, due to an increasing thickness and spatial orientation of cells in parallel unstirred layers of fluid can arise hampering provision with nutrition and respiratory gas. In this case perfusion of always fresh medium has to be made to avoid cell death by necrosis.

When cells are cultured in contact with a spatially structured polymer containing an inner and an outer surface, the quality of adhesion and the development of cell features in various depths of the polymer have a special meaning. Neurons must have the chance to develop multiple dendrites and an axon (Fig. 44.1). In consequence, the applied biomaterial must support the development of dendrites and axons in a spatial manner so that a functional network between multiple neurons can arise. The quality of the biomaterial determines if epithelial cells are equally distributing on the outer surface (Fig. 44.2). In contrast, for cells derived from connective tissue such as chondrocytes a homogenous distribution on the inner and outer surface is of special importance so that later at all sites a mechanically burden extracellular matrix is built up (Fig. 44.3). When cells are seeding on focal sites, in turn only here an extracellular matrix is synthesized, while the area between the cell groups does not exhibit extracellular matrix. In consequence, only an inhomogeneous and mechanically non burden extracellular matrix will develop.

Especially for myoblasts it is important that the available space is effectively seeded so that a close functional cell in cooperation with the applied biomaterial is built up (Fig. 44.4). When multiple myoblasts are fusing to become a functional muscle fiber, this process must occur in close cooperation with an elastic biomaterial.

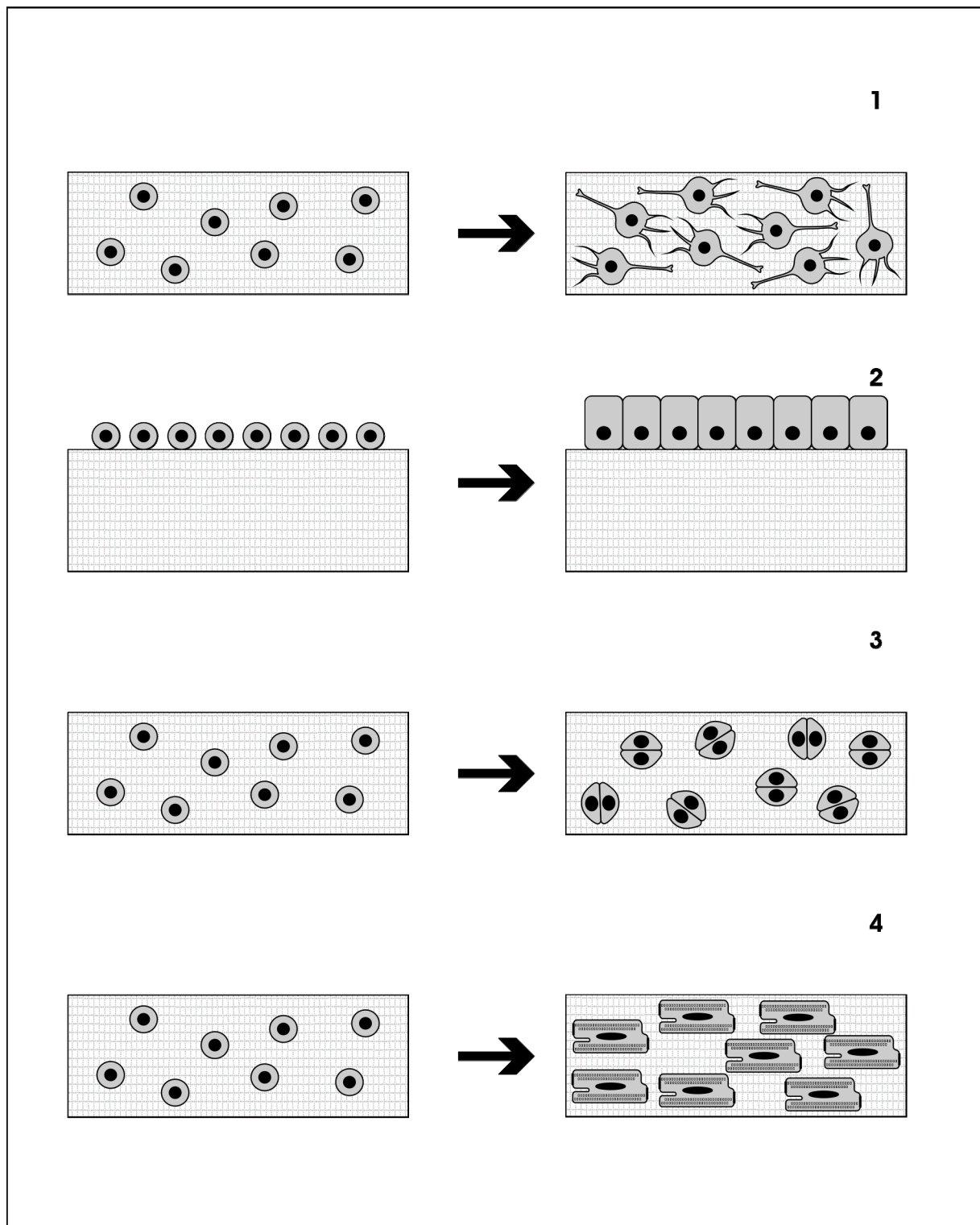


Figure 44: Spatial properties of a biomaterial are influencing developmental features of neurons (1), epithelial cells (2), connective tissue cells (3) and muscle cells (4). Schematic illustration depicts situation of immature cells (left side) and differentiated cells within maturing tissues (right side).

In summary, not only a perfect adhesion of cells to an applied biomaterial but also its spatial features included in an outer and inner surface influence the development of cell biological characteristics of cells to a high degree (Fig. 44). Presupposition for an optimal cell biological development is therefore that the individually selected biomaterial is strongly supporting the development of specialized cell features in analogy to the extracellular matrix. For that reason it has to be carefully elaborated, which kind of net, filter, scaffold, matrix or other biomaterials is more or less suitable for the culture in combination with an individual type of cells. In the meantime a variety of materials was investigated in combination with cultured cells. Surprisingly, it was elaborated that correct theoretical prognoses for a newly developed biomaterial cannot be drawn from these experiments. In consequence, for each newly developed biomaterial a new set of experiments with individual cells has to be performed so that advantages and disadvantages of a biomaterial can be recognized.

**[search: cell culture spatial growth pattern]**

**Self-study: Read about three-dimensional cell culture**

### **10.3. Spreading of cells**

When experiments in combination with an attractive biomaterial are performed, after a few days it can be recognized that the total surface is covered more or less by adherent cells. This widely spreading cells demonstrate on the one hand adhesion to material surfaces, on the other hand they show cell to cell contacts and exhibit intense proliferation. This multifunctional process of cultured cells is called spreading. It is obvious that the occurring cells show very different physiological functions. The spectrum comprises different phases of the cell division, interphase, starting differentiation, dedifferentiation and redifferentiation. Most interesting is that during all these phases the cells stay in close contact with the bottom of the culture dish (Fig. 45).

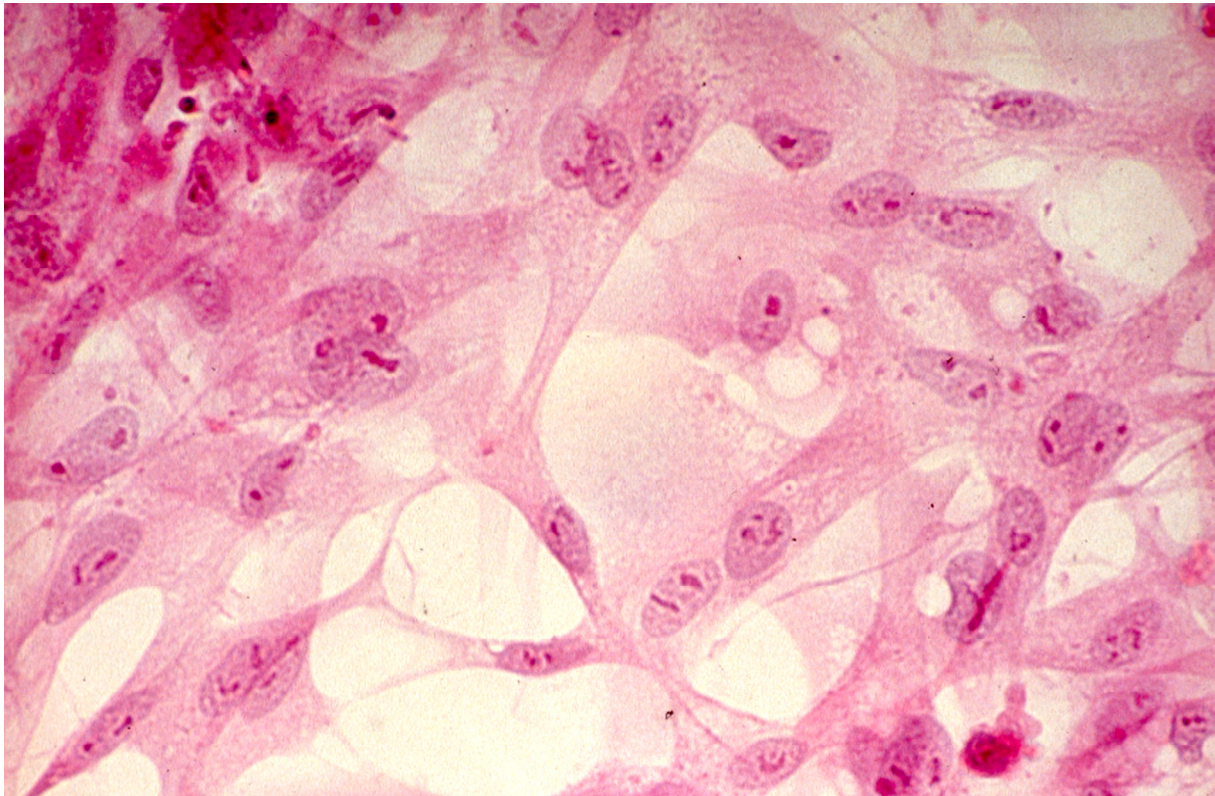


Figure 45: Spreading of adherent fibroblasts at the bottom of a culture dish. Multiple mitotic figures can be recognized.

The spreading of cells is caused by a series of influences. Beside an attractive material surface the spreading of cells is promoted by mitogen stimuli contained in the serum of offered culture media. Most interestingly, the stimulation can be rather unspecific or can be induced by specific growth factors. Especially the fluid environment appears to stimulate the spreading of cells. For example, alteration of the electrolyte composition of the culture medium is leading to a change in osmolarity. The new environmental situation is registered by the cells reacting in turn by biochemical cascades within the cytoplasm. The stimulus is transduced by Extracellular signal Regulated Kinases (ERK) and Mitogen Activated Protein kinases (MAP). In a complex reaction cascade these enzymes can trigger mitosis, adhesion or length of interphase and postmitotic phase. The Wnt/beta-catenin pathways in turn activate morphological and transcriptional events involved in cell spreading. In consequence, when cells are contacting a material, a culture medium and/or a growth factor, the resulting sum of reactions is influencing the processes in mitosis and the period of the interphase.

These reactions in turn evoke signals for cell dedifferentiation or redifferentiation. By these mechanisms the cells are controlling the contacts to neighbouring cells and the interactions to extracellular matrix. When new influences are registered deriving from growth factors, the fluid environment, the cell to cell contacts or adhesion sites the intracellular parameters for spreading are adjusted by new.

In the interior of cells these multiple informations are obviously mediated by the help of the ERK system triggering processes related to synthesis of nucleotides, gene expression or protein synthesis. The downstream processes are regulated by MAP kinases. One of the key enzymes for DNA respectively RNA synthesis is Carbamyl Phosphate Synthase II (CPS II). For example, application of Epidermal Growth Factor (EGF) stimulated ERK and MAP kinases to transfer phosphate groups to CPS II. This step of phosphorylation can be raised by the administration of Phospho Ribose di-Phosphate (PRPP) leading to an increased synthesis of nucleotides followed by an elevated activity of transcription. The signal of cellular MAP kinases is leading then to an increase in gene expression by the activation of Rapid Response Genes (RRG) within minutes. This effect is leading in turn to an activation of transcription factors and the phosphorylation of histone proteins. The result is a change in configuration on the histones opening thereby specific gene regions on DNA for the transcription of RNA. At the same time protein synthesis is activated at the rough endoplasmic reticulum by Mnk1 and the translation factor eIF-4E.

The ERK system and MAP kinases are further regulating the cell cycle over Cyclin Dependent protein Kinases (CDK). First the cyclin D1 protein and his partner CDK4 are activated leading to the formation of a complex. When this complex is phosphorylated, the cell can start with the mitotic cycle. In a further step the transcription factor E2F is activated leading to an increased transcription of genes triggering in turn replication of DNA and cell division.

However, in reality the cell biological interactions during spreading of cells are much more complicated than written here. For that reason actual literature must be read so that more information can be obtained. Nevertheless regarding activity of the ERK system and MAP kinases clearly shows that very different extracellular influences can have an effect on the spreading of cells.

The external stimuli are heterogeneous and comprise morphogens, growth factors, hormones, adhesion sites, information motives on extracellular matrix, osmolarity, hydraulic pressure and rheological stress. Each of the various influences, the resulting sum or part of its sum can lead in turn to an activation or even inhibition of mitosis, migration, adhesion, differentiation, dedifferentiation and redifferentiation of cells.

**[search: cell culture spreading]**

**Self-study: Inform yourself about proliferation of cells in culture**

## 11. Improving adhesion parameters

As shown before different aims exist to perform cell culture experiments. One of the strategies is to obtain an effective proliferation of cells so that within shortest time a maximum of biological material can be harvested. Since in this category of experiments an increasing number of cells stands in the foreground, specialized tissue functions including typical morphology are not aimed.

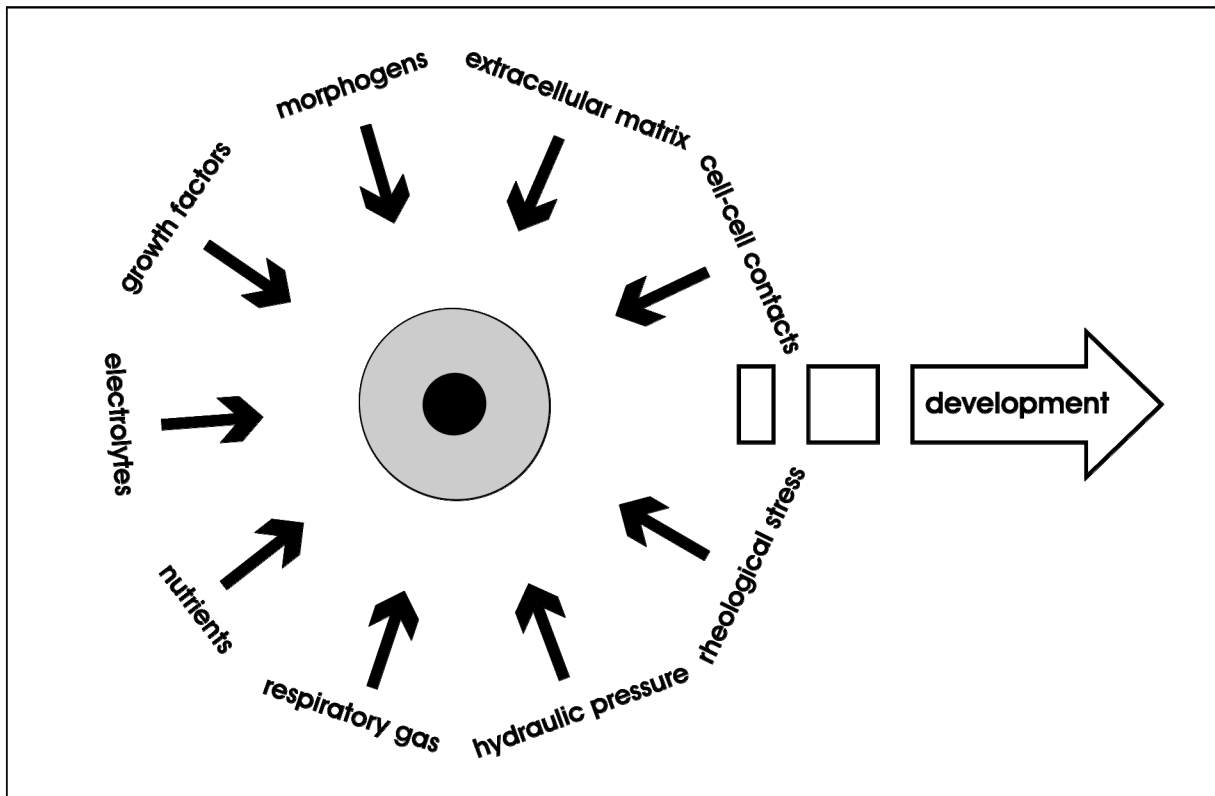


Figure 46: Illustration depicts multiple parameters influencing specific development under in vitro conditions. Beside cell to cell contacts and adhesion to extracellular matrix the cell biological development depends on suitable morphogens, growth factors, electrolytes, nutrients, respiratory gas, hydraulic pressure and rheological stress.

However, when culture experiments in the field of biomaterial research, tissue engineering or drug delivery are performed, highly differentiated cell features are required. It is obvious that this urgently needed quality of differentiation cannot be produced with proliferating cells kept

on the bottom of a classical culture dish. The adhesion on non-porous polystyrene does not mimic due to several reasons the natural environment. To surmount the situation and to generate highly differentiated cells, the structural environment within a culture dish has to be improved by an adaption to the individual needs of specialized cells within tissues.

The development of cells and specialized tissues can be influenced by various parameters (Fig. 46). To overcome the limitations of minor differentiation cell to cell contacts and the adhesion to the extracellular matrix have to be promoted by an improved structural environment within the dish. Further powerful morphogens and growth factors have to be applied so that a perfect degree of cellular differentiation can arise. This developmental process must be supported by the administration of tissue specific culture media with improved electrolyte composition, nutrition and adapted rates of respiratory gas. In addition, naturally occurring influences such as hydraulic pressure or rheological stress must act on cells so that they are able to up-regulate specific characteristics. Finally, a constant provision with nutrition and a permanent elimination of metabolites must be given so that a high degree of cell biological differentiation can arise and is maintained in the cultures.

**[Search: cell culture influence development]**

**Self-study: Get updated about factors influencing differentiation in cell culture**

### **11.1. Anchorage on spherical supports**

To improve the structural environment for cultured cells, different techniques are available. To offer a substitute for the plane bottom of a culture dish (Fig. 47.1), particles with a structured surface such as microbeads respectively microspheres can be offered (Fig. 47.2-3). The microbeads consist of a variety of materials. They can be solid so that they exhibit a



smooth outer surface. Other kinds of microbeads are porous resulting in an inner and outer surface for anchorage of cells. Further the materials can be coated by extracellular matrix molecules or its binding sites such as poly-l-lysine or they can be engineered that they contain drug delivery systems for a controlled local liberation of growth factors, morphogens or drugs. In so far the microbeads have beside an outer also an inner surface. Culture in combination with microbeads is either performed within a dish (Fig. 47.2) or in a stirred spinner flask (Fig. 47.3).

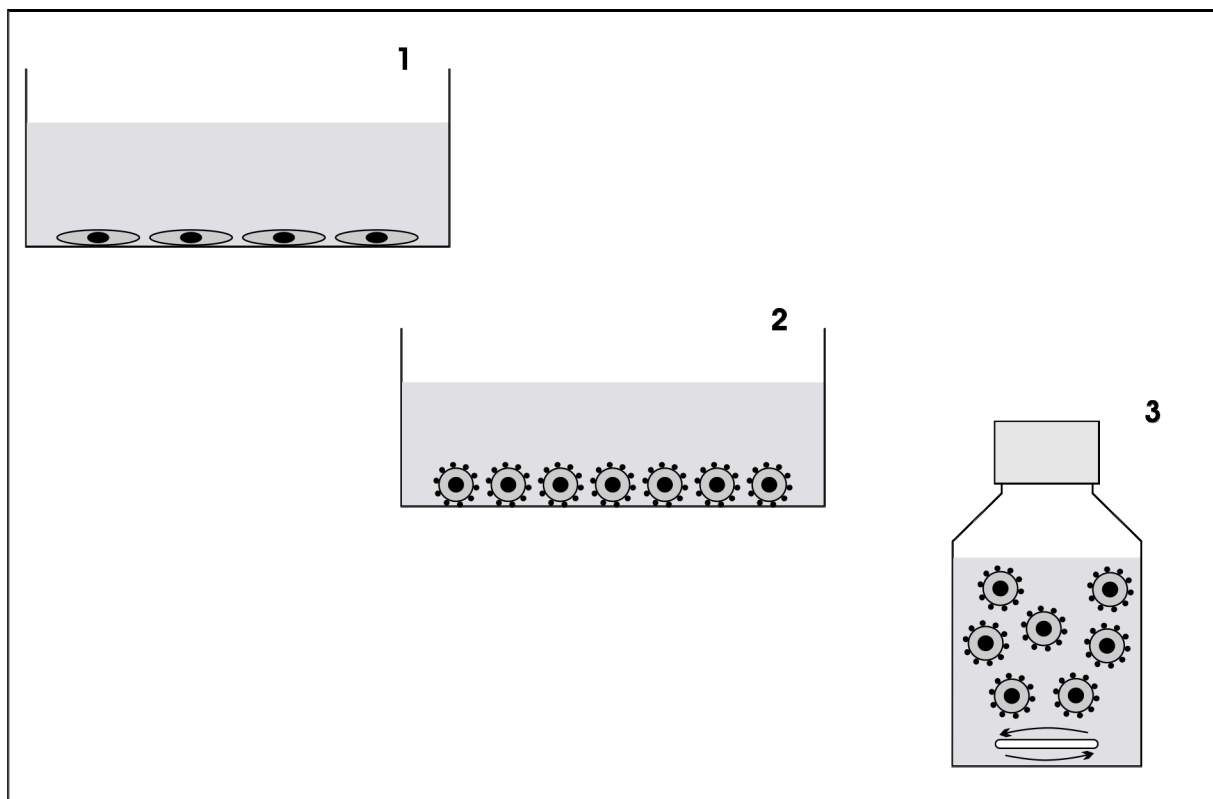


Figure 47: Illustration shows adherent cells at the bottom of a culture dish (1), seeding of cells on microbeads in static culture (2) and anchored on microbeads in a stirred spinner flask (3).

In the meantime a series of primary liver cells and cell lines were cultured on microbeads or microspheres to offer an improved substrate for cell adhesion and development. For example, hepatocytes require attachment to solid anchors for survival and proliferation before they form cellular aggregates with enhanced functionality. HepG2 or HHY41 human cell lines were cultured in alginate beads for more than 20 days. During this time production

of albumin, prothrombin, fibrinogen,  $\alpha$ -1-acidic glycoprotein and  $\alpha$ -1-antitrypsin was maintained. The synthesis was between 300 and 1100 % more as compared to conventional monolayer cultures at the same cell number per unit medium volume. Also features of detoxification such as ethoxyresorufin de-ethylase activity, androstenedione metabolism and urea synthesis from arginine was several times increased. Electron microscopy further revealed that spherical colonies of cuboidal cells arised exhibiting cell to cell contacts via desmosomes and tight junctional complexes. Abundant microvilli and cytoplasmic appearance suggested transcriptionally active hepatocytes. In so far the microbeads supported adhesion, proliferation and spontaneous formation of hepatocellular aggregates exhibiting intense physiological features.

Sertoli cells have been described as the nurse cells of the testis whose primary functions are to maintain epithelial polarity, to provide essential growth factors and to create an appropriate environment for development of germinal and stem/progenitor cells. However, the greatest challenge at present is that it is difficult to obtain sufficient Sertoli cells of normal physiological function for cell transplantation and biomedicine, largely due to traditional static culture parameters in a dish. In consequence, the process of culture was optimized by using microcarrier bead technology in static culture and in flasks with a rotating spinner.

Data obtained in these studies demonstrate the potential of microbead culture, since a strongly increased rate of physiological activity in Sertoli cells was registered. Thus, microbead culture provides an easily scalable approach to culture highly differentiated anchorage dependent cells, providing a much higher surface area to volume ratio compared with flat layer culture in a dish.

**[Search: cell culture microbeads]**

**Self-study: Read about spheroids and microbeads in cell culture**

## 11.2. Flat supports with an inner and outer surface

Many cell culture experiments are made by the assumption that monolayers in a culture dish reflect the physiology situation of specialized tissues. However, this is not true. More and more it is recognized that the flat and rigid polystyrene, polycarbonate or glass bottoms of currently available culture dishes are not representatives of the environment found within an organism. In consequence, cell to cell communication, mechanical and biochemical cues and specific tissue like architecture are frequently not developed or even lost under such simplified culture conditions.

To illustrate the drastic alterations of cells cultured in a dish, special attention is dedicated further to other specialized cells such as epithelia. Within the organism the epithelial cells are polarized so that specific functions are expressed either at the luminal or at the basal side (Fig. 48.1). Normally the epithelia are growing in a gradient environment, where different media (fluid/fluid or fluid/air) occur at the luminal and basal sides.

However, when the polarized epithelial cells are isolated, the cells gain an untypical round shape (Fig. 48.2). In this stage it can be seen that all features of polarization are lost. Within the culture dish the cells are exposed to a single medium. The situation for the cells is not improved, when they show primary attachment and then adhesion (Fig. 48.3) to the bottom of a culture dish. While provision with medium is sufficient at the upper side of cells, at the lower side the contact with polystyrene hinders a constant provision with fresh medium. It appears that the attraction to polystyrene leads to an untypical flattening of cells, where tissue specific differentiation is expressed in many cases only to a minor degree (Fig. 48.4).

Considering these facts here it is not said that adhesion to polystyrene is the only parameter influencing cells to undergo such morphological and physiological alterations by dedifferentiation during culture.

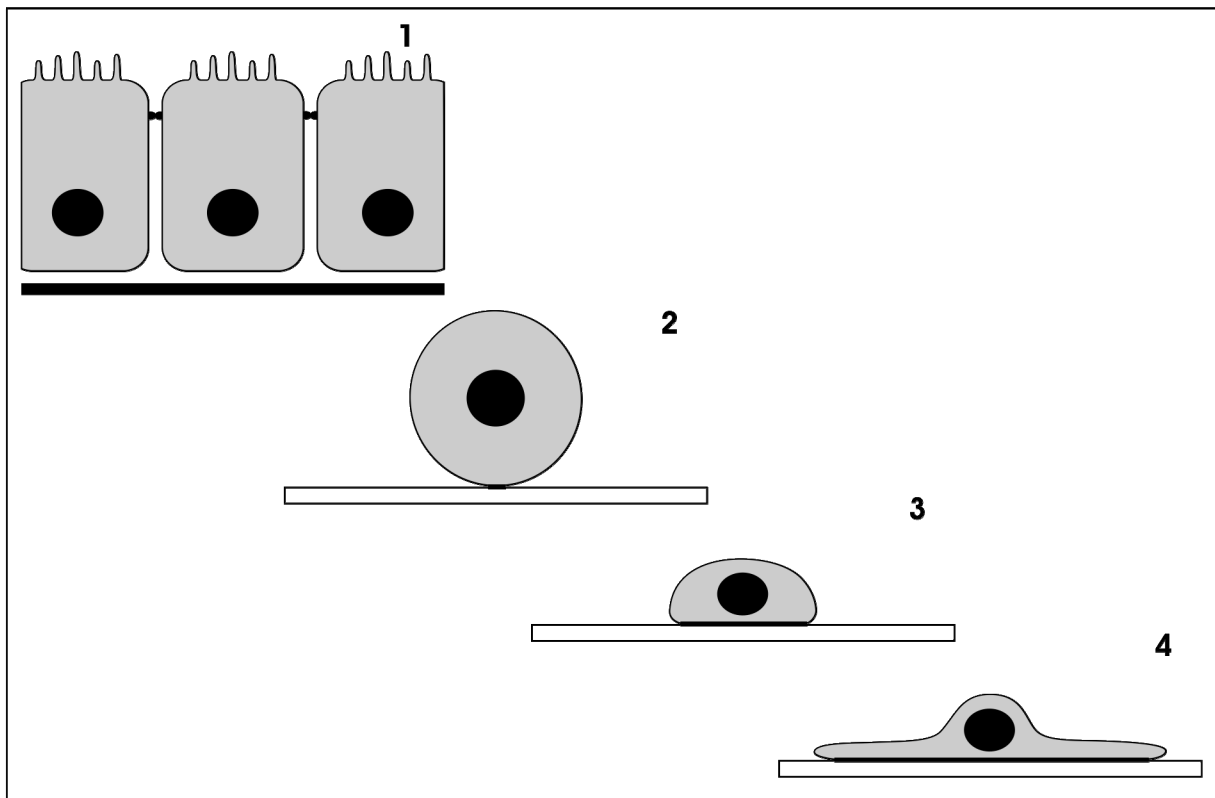


Figure 48: Illustration shows atypical development of cells in culture. Cells within an epithelium show polarized features (1). In contrast, after isolation and during primary attachment they exhibit a round shape (2). During adhesion (3) and adherence (4) the cells remain flat so that an untypical cell form is maintained during long periods of culture.

To offer an alternative to the bottom of a dish (Fig. 49.1) and in analogy to microbeads respectively microcarriers a further possibility for a higher surface area to volume ratio exists namely the culture of cells for example within Fibracell discs® (Fig. 49.2). The microporous carriers consist of a 50 to 50 mixture of polyester and polypropylene with dimensions of 6 mm diameter and 2 mm in thickness. For example, primary liver cells have a limited capacity to divide. To stimulate these cells to divide and to differentiate successful culture was performed within Fibracell discs®.

In consequence, a substitute for the basal lamina or the surrounding extracellular matrix has to be offered for cells so that better cell migration, adhesion, adherence and polar differentiation can occur. Such a semi-spatial microenvironment can be simulated by the growth of cells on fiber meshes (Fig. 49.2) or within fleeces (Fig. 49.3).

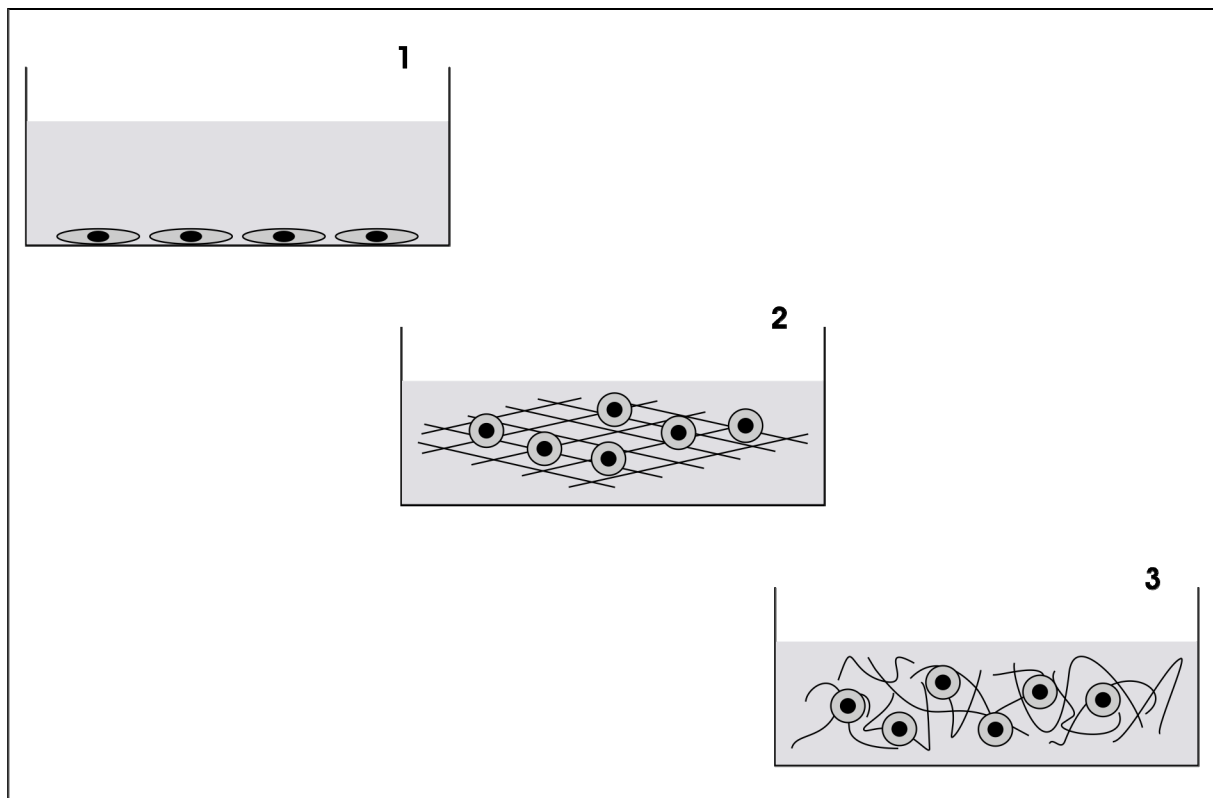


Figure 49: Schematic illustration of cells cultured on the bottom of a dish (1), on a fiber mesh (2) and within a fleece (3).

Depending on the offered material the cells use first the surface of the fiber material for adhesion and proliferation. In a second step the gaps to neighbouring cells are closed by the synthesis of extracellular matrix proteins. Finally the spreading epithelial cells have a mechanical contact to the underlying fibers and the space between the fibers to built up further contacts to neighbours. This assembly is leading on the one hand to close epithelial layers covering the complete fiber mesh, but on the other hand it results in a more or less fragile mechanical situation so that parts of the cell layer can be damaged easily during manipulation.

Further Alvetex<sup>®</sup> 3-D scaffold mesh is suitable for animal and human cell growth and tissue formation. It has been tested with different cell types including liver, skin, cancer and stem cells. The white membrane like material is placed at the bottom of a dish to adhere cells. The

highly porous architecture supports migration and spatial growth of cells. It is compatible with many cellular and molecular assays to test arising functionality under culture conditions.

When hepatocarcinoma (HepG2) cells are used in culture as a model for studying drug toxicity, important markers such as cytochrome p450, albumin and urea metabolism must be expressed. Numerous investigations have shown that this quality of cells cannot be obtained in classical dishes. To surmount the situation special polystyrene (PS) and polycaprolactone (PLC) scaffolds were designed.

Thus, the 3D Biotek 3D Insert<sup>®</sup> scaffolds contain cross-linked fibers with 150 µm in diameter and 200 µm spacing. Experiments demonstrated that this network appears as an ideal environment for the spatial development of HepG2 cells with more differentiated features.

Electrospun nanofiber substrates are available for improved cell and tissue cultures. Substrates from Nanofiber Solutions<sup>®</sup> mimic specific human in vivo environment. As a result, more accurately the effects of chemical compounds on cell behaviour can be measured. This is especially true as researchers attempt to model and measure metastasis from the primary tumour.

Finally, Nanex<sup>®</sup> hematopoietic stem cell expansion system is a nanofiber-based fleece scaffold that mimics the human stem cell niche. An expansion of human cord blood, bone marrow and mobilized peripheral blood derived Hematopoietic Stem Cells (HSCs) has been performed using Nanex<sup>®</sup> expansion technology. For example, during a 10 day culture period in serum containing medium over 200 fold expansion can be achieved.

**[Search: cell culture fiber mesh]**

**Self-study: Inform yourself about support materials for cells in culture**

### 11.3. Filter as cell support

To offer epithelial cells a constant mechanical contact in a semi-spatial environment, they are cultured on a filter. The culture of cells on a filter is a suitable arrangement to offer later a tissue specific provision with medium at the upper and lower sides. The plane filter surface simulates the basal lamina, while a cylinder glued on the filter separates the media at the luminal and basal side. Specifically for improved polarization and differentiation of epithelial cells such filter supports are used. Nowadays inserts with different types of filters and individual coating by extracellular matrix proteins are commercially available. However, in individual cases specific filters for cell culture have to be mounted in the laboratory.

The mounting of a filter insert used in a 24 well plate sounds complicated but is rather simple. First of all a conduit made of polycarbonate with an inner diameter of 10 mm and an outer diameter of 13 mm is cut in cylinder pieces to a length of 5 mm. Then a filter with an individual pore size not smaller than 0.2  $\mu\text{m}$  is selected. It can be made out of polycarbonate, nitrocellulose, aluminium oxide or polyethylenterephthalate. For the present experiments a diameter of 13 mm is used. Many kinds of filters are commercially available in this size or are excised by a punching tool.

In the next step the filter has to be glued on the polycarbonate cylinder (Fig. 50). First of all the excised filter is placed on one opening of the plastic cylinder. For preparing the glue for example a small amount of nitrocellulose is dissolved in a small volume of a solvent such as acetone or xylene. In a first step a glass capillary is taken to draw some solvent containing dissolved nitrocellulose. Then the opening of the capillary (Fig. 50.1) is brought for a short moment in contact with the edge of the filter resting on top of the polycarbonate cylinder (Fig. 50.2). As a result during contact some of the solvent is drawn into the slit between the filter and the plastic cylinder. This procedure is repeated several times by turning the cylinder clockwise. After evaporation of the solvent the glue is fixing the filter on the polycarbonate cylinder (Fig. 50.3).

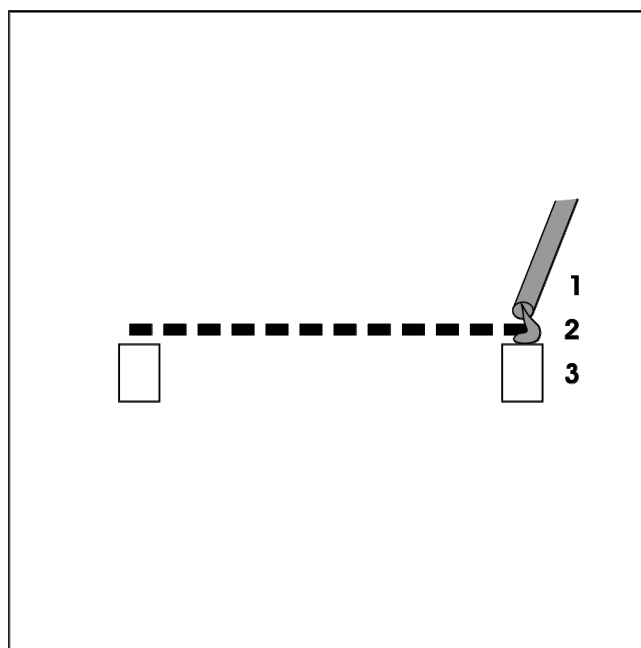


Figure 50: Schematic illustration to glue a filter on a conduit. First a glass capillary is taken to draw some solvent containing dissolved nitrocellulose. Then the opening of the capillary (1) is brought for a short moment in contact with the edge of the filter resting on top of the polycarbonate ring (2). As a result during contact some of the solvent is drawn into the slit between the filter and the ring. This procedure is repeated several times by turning the cylinder clockwise. After evaporation of the solvent the glue is fixing the filter on the polycarbonate cylinder (3).

Before culture the polycarbonate cylinder with the filter glued on top has to be sterilized. Depending on the filter material, the polycarbonate cylinder and the kind of glue a suitable way of sterilization has to be found. Very comfortable is disinfection in a solution of formaline (4%) for 10 minutes. After disinfection the filter assembly is rinsed several times with phosphate buffered saline.

For culture of cells the filter assembly is placed in a dish. Commercially available filter inserts are standing or are hanging in a well of a 24well culture plate (Fig. 51). The cells are transferred by a pipette within a drop of medium onto the surface of the filter (Fig. 51.1), then medium is filled in until the meniscus of the fluid is moistening the filter surface (Fig. 51.2). This simple procedure is seeding cells only on the surface of the filter (Fig. 51.3) by



preventing at the same time adhesion on the inner surface of the polycarbonate cylinder. When cells are showing adhesion, more culture medium can be filled into the dish.

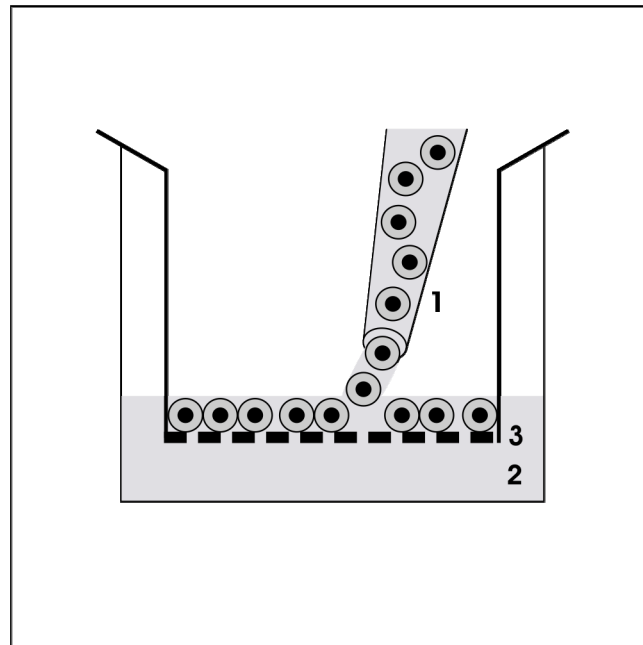


Figure 51: Seeding cells within a filter insert. The cells are transferred by a pipette within a drop of medium onto the surface of the filter (1), then the medium is filled in until the meniscus of the fluid is moistening the filter surface (2). This simple procedure is seeding cells only on the surface of the filter (3) by preventing at the same time adhesion on the inner surface of the polycarbonate cylinder.

Correct provision of cells with nutrition is made by replacing medium as well within the cylinder as on the dish (Fig. 51.2). During culture the filter is mimicking advantages of a preformed basal lamina for the growth of epithelial cells. When a suitable culture medium is used, the cells will proliferate to form a confluent monolayer at the surface of the filter.

Applying for example Millicell® or ThinCert® culture inserts the adhesion on the filter in combination with the density of cells stimulates formation of contacts between neighbouring cells leading to an arising polarization of the epithelium. This in turn supports during culture a functional seal resulting in a separation of the compartments within the lumen of the polycarbonate cylinder and the compartment at the bottom of the culture dish.

The epithelial cells adhering to the filter have a limited contact between both fluid compartments over the pores of the filter. Applying this culture technique either one sort of medium or different kinds of media can be offered in the upper and lower compartment.

**[Search: cell culture on a filter]**

**Self-study: Read about culture of cells on filter materials**

## **11.4. Experiments with filter inserts**

When epithelial cells are adhering during culture at the bottom of a dish, the provision with medium is optimal at the upper side, while restricted at the basal side of cells (Fig. 52.1). The situation for epithelial cells can be improved, when culture is performed within filter inserts, since the luminal and basal sides are equally provided with nutrition and respiratory gas (Fig. 52.2). For experiments with a filter insert as well primary epithelial cells as epithelial cell lines such as MDCK, CaCo-2 or Calu-3 cells can be used. These and others can be ordered from the American Type Culture Collection (ATCC, Rockville, MD, USA).

For example, MDCK cells derived from canine kidney resemble cells from the distal nephron segments. CaCo-2 cells represent a human colon carcinoma cell line, which is frequently used as an experimental cell model for barrier function within the intestine. Most interestingly, this model is accepted by the Food and Drug Administration (FDA) in the United States of America. Under certified conditions it is possible to investigate the influence of newly developed drugs on intestinal adsorption. Moreover, the Calu-3 cell line derives from human bronchotracheal gland cells representing a model of respiratory epithelia. It is evident that on such renal, intestinal and pulmonal cells a variety of experiments such as the uptake or metabolism of newly developed drugs, proteins, DNA- and RNA-constructs can be investigated.

MDCK cells are normally growing in form of untypically flat monolayer cells at the bottom of a culture dish (Fig. 52.1). However, when the cells are kept on the surface of a filter, the flat phenotype is transforming into isoprismatic cells (Fig. 52.2). The surprising change in growth and differentiation is resulting in an epithelial barrier. While the subculture of flat MDCK cells is performed in a normal proliferation medium, for the culture on filters a special medium has to be applied containing 10 to 20% native or heat inactivated foetal calf serum. Only within this medium MDCK cells are developing and maintaining features of a sealing epithelial barrier.

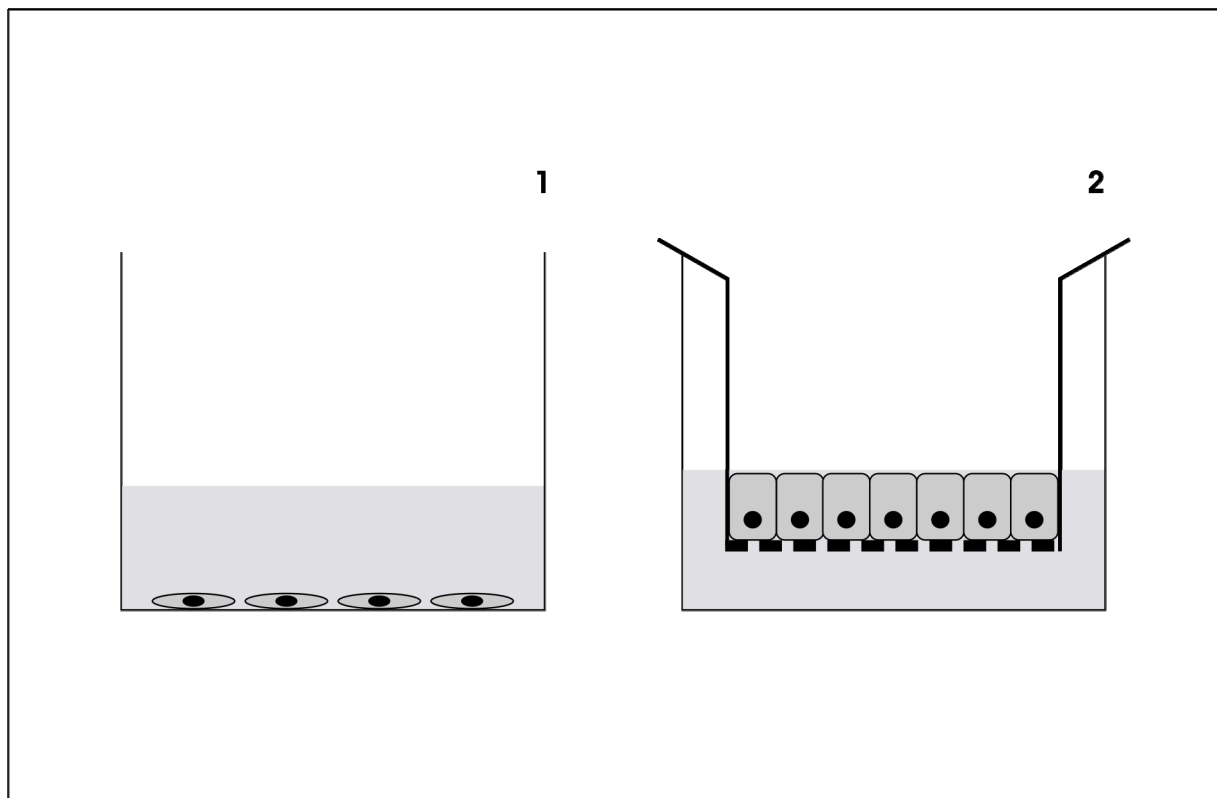


Figure 52: Schematic illustration shows epithelial cells growing on the bottom of a dish (1) and on the porous substrate of a filter insert (2). During culture it can be recognized that epithelial cells form an untypical flat monolayer within the classical dish (1) but show intense polarization on the filter (2).

It is of special interest if features of transport are developed in cultured MDCK cells. Independent from an immunohistochemical detection of tight junction proteins it has to be

elaborated by electrophysiological methods if really a functional barrier has been established. In the typical case the Trans-Epithelial Electric Resistance (TEER) has to be determined by a recorder and two electrodes, which contact each the basal and luminal culture medium. The recording shows that it takes 7 to 10 days, until a TEER greater than  $100 \text{ Ohm} \times \text{cm}^{-2}$  is established. However, only a TEER over  $500 \text{ Ohm} \times \text{cm}^{-2}$  is signalling an intact barrier. It is important to note that a physiological TEER is not automatically obtained, but depends to a high degree on the culture conditions including optimal filter materials and suitable serum specimens in applied culture medium.

Thus, tight epithelial cell layers exhibit high electric resistance, while low electric resistance correlates with a high permeability. Consequently, the obtained data can be used to compare or to monitor the establishment of cell to cell contacts forming the barrier. However, analyzing the properties of an epithelial cell layer is not limited to measure the electric resistance but can be completed by recording the electric capacity ( $C_{cl}$ ). This parameter indicates development of cell biological features in the epithelial cell layer such as formation of a brushborder by microvilli. Both transepithelial electric resistance and capacity inform about the complex impedance ( $Z$ ) of a cell layer, which can be measured electronically.

Finally, placing electrodes in the luminal and basal compartment and applying a small AC voltage ( $V_{ac}$ ), the electric impedance of the epithelial cell layer can be defined. This value reflects features of the epithelial cell layer including the culture medium between the electrodes. Corresponding mathematical models can be applied in order to extract parameters, which mirror the epithelial barrier functions. An advantage of all electric measures is that they show physiological functions and that they do not require a fluorescent or radioactive tracer.

However, beside the electrophysiological measurement of TEER the sealing of an epithelial barrier can be determined by the help of radioactively labelled mannitol. Thereby one has to

notice that mannitol is not taken up by the cells, but can pass the epithelial barrier over the paracellular shunt between the lateral plasma membranes. In consequence, for determination of the tightness of an epithelium a defined small amount of radioactive mannitol is applied to the luminal culture medium. After a hour it is measured, how much radioactivity is found in the basal culture medium. In the case the cultured epithelium is leaky, a lot of radioactivity will be reaching through the paracellular shunt to the basal side of the culture medium. However, in the case that less than 1% of administered radioactivity is detected in the basal compartment, an intact barrier appears to be established. In the case more than 1% is found at the basal side of the epithelium, it has to be investigated if the tight junctions are fully developed if a sufficiently confluent monolayer is established or if an edge damage between cells and the inner side of the filter insert is the reason that too much radioactivity will penetrating in an uncontrolled manner the barrier.

Finally it has to be considered that the luminal and basal compartments of the filter insert exhibit a static milieu and that they differ in their volume. This situation leads to a relatively quick exchange of culture medium between the basal and luminal compartment through the epithelial barrier and the filter pores. Since the fluid volume in the luminal compartment is much smaller than in the basal compartment after a certain time the fluid gradient cannot be maintained from the epithelia due to mixing of fluid.

**[Search: cell culture filter inserts]**

**Self-study: Get updated about cell culture with filter inserts**

## **11.5. Co-culture experiments with filter inserts**

Beside the improved adherence to a basal lamina substitute the culture of epithelial cells on a filter has the further advantage that co-culture experiments can be performed. By this

experimental set up it is possible to investigate numerous cell biological interactions between different types of cells and tissues.

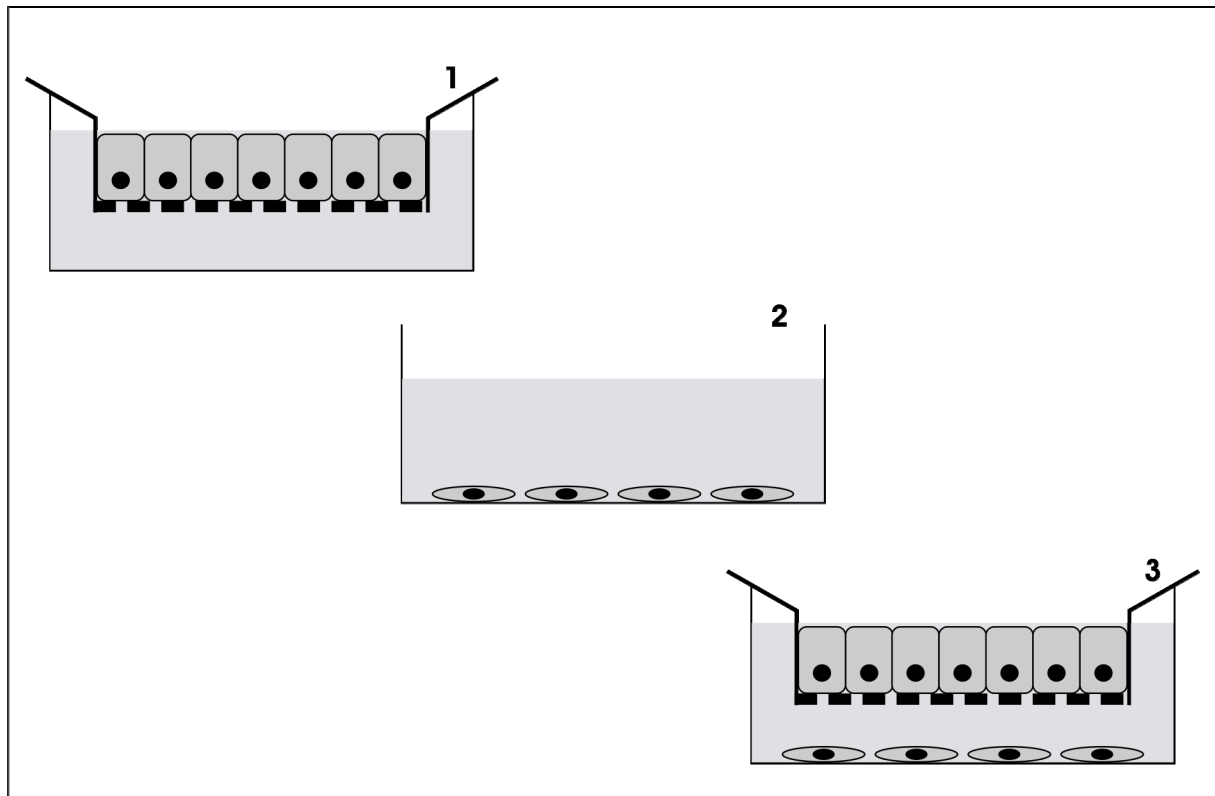


Figure 53: Schematic illustration of co-culture experiments by the help of filter inserts. in a first set of experiments epithelial cells are seeded within a filter insert (1). In a second set of experiments fibroblasts are cultured on the bottom of a classical dish (2). When both cell types become adherent and confluent, the filter insert is transferred to the dish with fibroblasts (3).

For example, to investigate the influence between the lamina propria and an epithelium during development, in a first set of experiments epithelial cells are seeded within a filter insert (Fig. 53.1). In a second set fibroblasts are cultured on the bottom of a classical dish (Fig. 53.2). When both cell types become adherent, the filter insert is transferred to the dish with fibroblasts (Fig. 53.3). Following this strategy the interaction of both cell types can now be investigated over an extended period of time. It can be analyzed in how far diffusion of molecules is promoting cell growth and differentiation. Successful experiments were further performed with endothelial cells in combination with smooth muscle cells, to analyze

stimulating and inhibiting effects on the formation of an artery. However, disadvantage of this method is the spatial separation of both cell types by the filter assembly, since close tissue integration cannot be investigated by this experimental set up.

**[Search: cell culture filter co-culture]**

**Self-study: Inform yourself about co-culture experiments with filter inserts**

## **11.6. Culture at the interface between fluid and air**

When culture dishes are filled with medium, in the most cases not the identical but a varying volume of medium is transferred with a pipette to the dish. However, variations in the height of the meniscus indicate that different volumes of medium are present in the individual dishes. In consequence, not the same but varying amount of nutrition is available. In addition, the volume for the waste metabolites differs. Finally, contained oxygen has to diffuse through the fluid column down to the bottom of the culture dish to reach the adherent cells. The variations in the height of the fluid column do influence the diffusion so that the available amount of respiratory gas is not constant. The same influence is observed not only at the bottom of classical dishes but also within filter inserts. The availability of nutrition and the diffusion of respiratory gas strongly depend on the height of the fluid column. In consequence, for exact comparison of parallel experimental set ups always the same volume of culture medium has to be added to the individual dishes respectively filter inserts.

To facilitate the diffusion process for respiratory gas, a minimal amount of fluid within a droplet can be offered so that the medium is just covering the cells cultured on a filter. This strategy highly improves the diffusion of respiratory gas, since it shortens the distance of permeation. However, the small volume of medium drastically decreases the provision with nutrition due to a lack of fluid volume. For that reason an optimum has to be elaborated

considering on the one hand the distance of oxygen diffusion and on the other hand the available amount of nutrition.

To improve both provision with medium and respiratory gas, cells and small pieces respectively slices of tissue can be cultured at the interface between a fluid and a gas atmosphere within a Trowell container. Excellent results were obtained with cells from lung, brain, liver, kidney and pancreas.

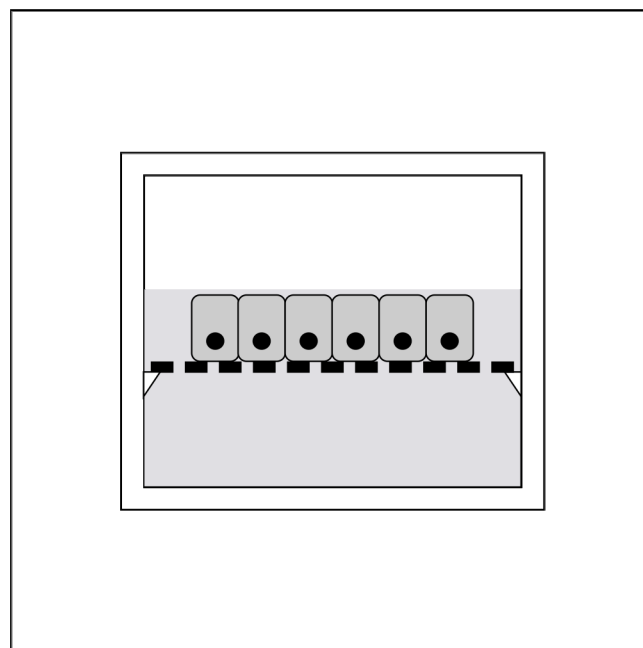


Figure 54: Schematic illustration of a Trowell container. The adherent cells are growing on a filter. Optimal diffusion of respiratory gas is given, since the medium is just covering the cells.

Trowell has originally detected that cells under development have a higher requirement for oxygen than adult cells. To offer a better respiratory gas provision, a container with a lower and higher compartment was constructed (Fig. 54). Both compartments were separated by a perforated plate and a filter. In the lower compartment a newly invented culture medium T8 was filled in, until the fluid is moistening the perforated plate. Then a filter with adhering cells can be placed on the perforated plate. The upper compartment in the Trowell container is accessible to ventilate respiratory gas. The basal part contains a sufficient amount of culture medium so that nutrition is not a limiting factor. Up to date cells and pieces of tissue are



cultured in modified Trowell containers including improved filter materials. The relatively big amount of culture medium in the lower compartment sufficiently provides the cells with nutrition for the whole period of culture.

Nowadays experiments in Trowell containers are seldom performed. Instead filter inserts are used. In such membrane cultures the apical epithelial side is just covered by a thin liquid film. The so called Air Liquid Interface (ALI) cultures are now state of the art for the culture of airway, gastric and intestine epithelial cells. The apical medium volume is minimized to a thin film supporting diffusion of oxygen and uptake of nutrition.

**[Search: cell culture Trowell]**

**Self-study: Get updated about cell culture at the air liquid interface**

## **11.7. Imaging cells on spatial materials**

Beside transparent supports cells are cultured on a variety of non transparent biomaterials. It is obvious that the distribution of cells cannot be analyzed by classical microscopical methods. Instead histochemical label techniques in combination with sophisticated microscopical analysis has to be applied. An exact quantitative and qualitative analysis of cells adhering on a non transparent material requires an excellent signal to noise ratio, optical sectioning capability, large field of view, good spatial resolution, fast image stack recording rates and a low sample fluorophore excitation level.

Confocal fluorescence microscopy represents the commercially available standard imaging of relatively thick specimens. However, as in conventional wide field epifluorescence, in confocal imaging the excitation light illuminates the entire object and extends photobleaching and phototoxic effects in all planes. Two photon and multiphoton microscopy seem to be

more attractive when observing large specimens due to their twofold penetration depth compared with confocal microscopy. Although multiphoton microscopy is regarded as the technique of choice for imaging three-dimensional samples, it allows only a low resolution and requires large light intensities causing bleaching in the focal plane.

Alternatives are Optical Coherence Tomography (OCT) and Optical Projection Tomography (OPT) for imaging cell layers, thick tissue slices, organs and tumour spheroids. However, OPT and OCT have limitations in term of spatial resolution and recording rate. Looking over all, the optimal technique for three-dimensional live imaging is currently not invented.

In consequence, in many cases one has to go back from very innovative to traditional techniques. In the case a scaffold material can be cut to ultrathin sections, then Transmission Electron Microscopy (TEM) can be performed to analyze the spatial growth of cells on internal surfaces. A further possibility for excellent cell imaging is the application of Scanning Electron Microscopy (SEM).

**[Search: cell culture imaging]**

**Self-study: Get updated about microscopy with cell cultures**

## 12. Proliferation versus differentiation

When cells are cultured, in the most cases they are provided with a suitable medium containing Foetal Bovine Serum (FCS) or growth factors. By the help of these additives the number of cells will strongly increase within shortest time. After a short period of time they become confluent. For subculture the cells are treated with trypsin or collagenase to obtain a suspension. After transfer of cells by a pipette into new dishes, a new confluent monolayer can be expected within few days. When they are controlled under the microscope numerous mitotic figures can be observed (Fig. 55). This is a clear hint that the culture environment appears by the first view optimal.

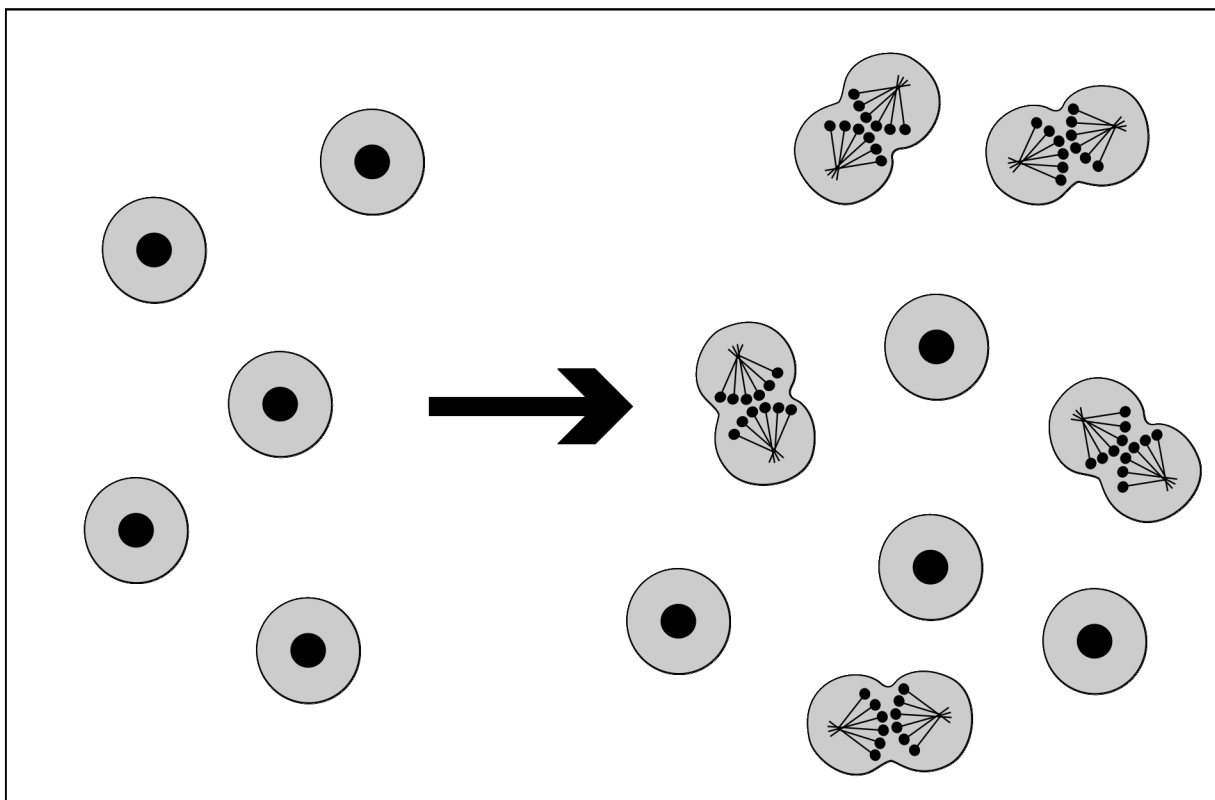


Figure 55: Illustration demonstrates the transition from isolated cells to proliferating cells after addition of a mitogenic stimulus. The increase in cell number can be recognized by numerous mitotic figures.

However, going back to the human or animal organisms and comparing cells of developing with adult tissue very different rates of proliferation can be seen. It is not surprising that a de-

veloping organ such as liver or kidney in a foetus exhibits much more cells in mitosis than an organ of an adult person. The same situation is obtained within a culture dish. Isolated cells from a foetus or a young person will exhibit much more mitotic activity than cells derived from an old person. Most probably this effect is due to different length of chromosomal telomeres.

Further it was found that cells from aged people cannot be sub-cultured in the same degree as the ones derived from young persons. This phenomenon might be one of the reasons that primary cells from an old person do not show spontaneous activity to become a continuous cell line. In so far the origin and the actual age of a cell might be a limiting factor to perform a successful culture.

**[Search: cell culture stimulation of mitosis]**

**Self-study: Inform yourself about proliferation of cells**

## **12.1. Phases of the cell cycle**

To obtain an idea about the actual rate of cell divisions one has to analyze the specialized tissues within the organism. In an adult person extremely few cell divisions are found in neural tissue, cartilage and heart muscle cells (Fig. 56). Minor rates of cell divisions occur in hepatocytes, parenchyma of the kidney, pancreas, glands of the stomach and intestine. High rates are found in the basal layer of the epidermis and in superficial cell layers of stomach, intestine and oral mucosa.

In contrast, a high degree of cell renewal is also found in the hematopoietic system and in tumour cells. Also in frequently used continuous cell lines an extremely short period of interphase is found lasting only for one to two days.

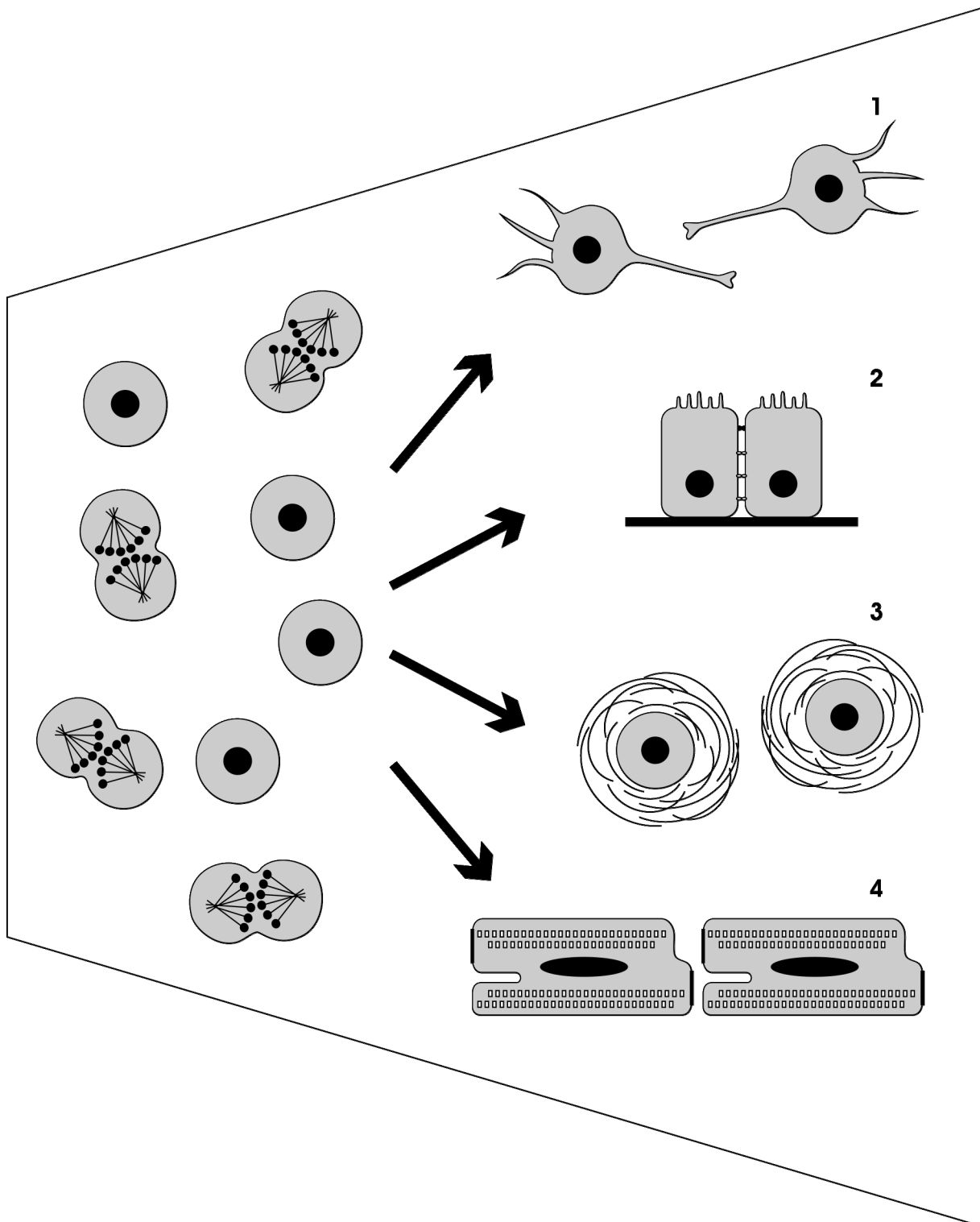


Figure 56: Illustration describes the development of dividing into postmitotic cells found in numerous specialized tissues. Arise of functional cell features is correlated with a decrease of cell divisions in neural tissue (1), epithelium (2), connective tissue (3) and muscular tissue (4).

In consequence, cells within various adult tissues remain for defined periods in the interphase of the cell cycle frequently also called  $G_1$  phase. This phase can persist in neurons, cardiomyocytes and chondrocytes for the whole adult life span, while it can persist in liver and kidney parenchyma over years. The extended interphase or  $G_1$  phase is often called  $G_0$  phase or stage of postmitosis (Fig. 57). In permanently regenerating tissue such as epidermis within the skin a relatively short  $G_1$  phase exists lasting for only several days.

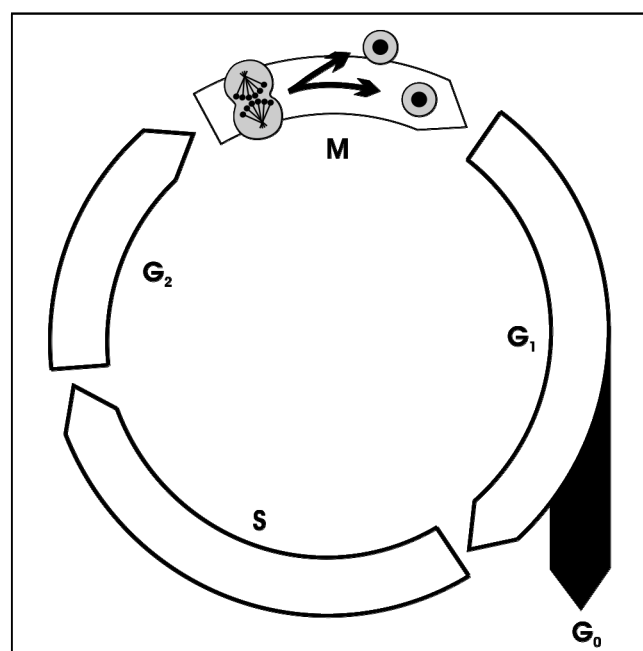


Figure 57: Illustration depicts the cell cycle consisting of mitosis and the succeeding interphase. In the  $G_0$  phase typical functional cell features are expressed in specialized tissues.

Up to date it is not known, which mechanism is steering the length of the  $G_1$  phase in the single specialized tissues (Fig. 57). With cultured cells it could be shown that very different stimuli such as application of foetal bovine serum, growth factors or alterations in the electrolyte environment promote transition from the  $G_1$  into the S phase to prepare mitosis.

However, one has to consider that the progress in cell division is certainly coordinated by a series of different mechanisms. A plausible signal for the transition of cells from the  $G_1$  into

the S phase is the activation of the protein Sic1. Normally Sic1 is blocked by a protein complex belonging to Cyclin Dependent Kinases such as CDK1.

As long these kinases are inhibited, cells cannot proceed to the S phase of the cell cycle. However, when Sic1 is several times phosphorylated, the entrance into the cell cycle is opened. At this time the protein complex SCF is associating with Sic1. As a result the Sic1 molecule is ubiquitinated, reaches the proteasome and is degraded. In turn the S phase of the cell cycle can start.

However, not only the increase in cell number by permanent cell cycling but also their capability to develop special features has an extra meaning, when culture experiments are made. The underestimated problem is that cells cycling permanently from one to the next division cannot be stimulated to develop at the same time specific features of differentiation. The take home message is that mitosis and differentiation cannot occur in parallel but appear after succeeding periods of time.

The succeeding periods of mitosis and interphase can be simulated in culture experiments with anchorage dependent cells. The procedure is very simple. When an increasing amount of cells is needed, medium containing serum or growth factors is applied. Maintaining this environment for the whole culture period the cells are cycling as quickly as possible from mitosis to the next.

By this treatment they do not have a chance to stay for a while in the interphase or G<sub>1</sub> phase. As a consequence, the cells remain to a high degree in an undifferentiated stage, since mitosis (cell division) and interphase or G<sub>1</sub> phase (functionality) cannot occur in parallel.

**[Search: cell culture cell cycle control]**

**Self-study: Read about stages in the life cycle of a cell**

## 12.2. Interphase replaces mitosis

To obtain a high degree of differentiation, in a first step multiplication of cells must be performed. In contrast, in a second step mitosis must be down-regulated, while the period of the interphase is extended as long as possible so that differentiation can start.

Due to these reasons culture experiments with highly differentiated cells undergo a period of high proliferation activity, down-regulation of mitotic activity and finally for functional differentiation (Fig. 58).

Not only in the culture dish, but also in the organism it can be observed that proliferation is followed by a period of differentiation. Up to date it is unknown by which mechanism these specific processes are controlled. For example, it is not known why enterocytes on the intestinal villi show an intense rate of proliferation so that the renewal takes place within few days.

In contrast, the basally granulated cells and lysozyme producing Paneth's cells in the intestinal crypts show an extremely slow renewal within years. Most interestingly, the illustrated high versus low rate of proliferation is controlled for example at a lateral cell border so that the epithelium on the villi is renewed within few days, while the cells in the intestinal crypts remain in a differentiated stage over years.

Surprising differences in the rate of proliferation are found during development of tissues. For example, chondroblasts and osteoblasts exhibit high rates of proliferation in the first phase of cartilage formation.

In contrast, when extracellular matrix has been synthesized, chondrocytes and osteocytes down-regulate proliferation so that they do not exhibit mitosis over years.



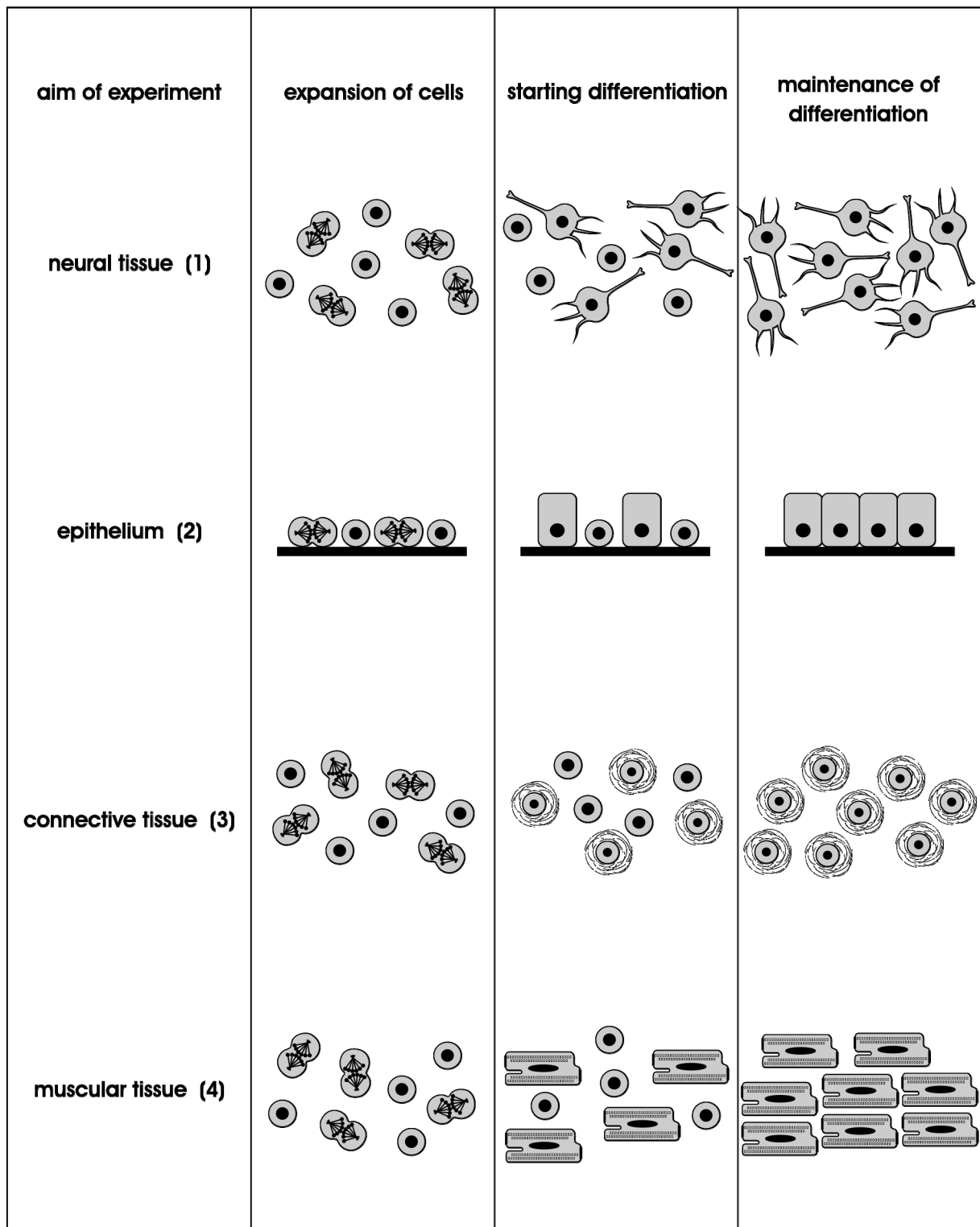


Figure 58: Illustration shows that the culture of anchorage dependent cells comprises a period of proliferation followed by starting and maintaining differentiation in neural tissue (1), epithelium (2), connective tissue (3) and muscular tissue (4).

In conclusion, when culture experiments with anchorage dependent cells are performed, the resulting efficiency cannot only be estimated according the acute rate of proliferation and the

spatial distribution of cells. When the cells are kept in a culture medium containing growth factors or foetal bovine serum a massive promotion is given to cycle as quickly as possible from mitosis to the next.

Due to the permanent mitotic cycling the cells do not have a chance to up-regulate functional features. One has to consider that a permanent mitotic cycling does not occur in the most specialized tissues of the organism. Further should be mentioned that the permanent presence of mitotic stimuli creates an atmosphere only found within embryonic, foetal and some juvenile phases of growth. In contrast, to simulate an atmosphere to up-regulate features of differentiation, a completely different environment has to be offered. In this developmental phase the mitogens have to be reduced and interstitial fluid environment has to be simulated.

**[search: cell culture cell cycle interphase]**

**Self-study: Get updated about functions in postmitosis**

### **12.3. Stepwise to more differentiation**

Culture experiments with anchorage dependent cells often show that typical morphological, physiological and biochemical features are down-regulated or are lost during culture by dedifferentiation. In such an atmosphere atypical proteins may be expressed leading in turn to barely interpretable results.

Thus, more and more it is recognized that a tissue specific frequency of mitotic divisions and a long lasting interphase can be simulated under advanced culture conditions. In consequence, at the start of the experiment one has to decide about the period of permanent

mitotic cycling and about the length of the succeeding interphase so that typical physiological features can be up-regulated (Fig. 59).

<b>culture technique</b>	<b>static culture</b>	<b>perfusion culture</b>	<b>perfusion culture</b>
<b>media</b>	<b>+ growth factors + foetal bovine serum</b>	<b>serum free</b>	<b>tissue adapted chemically defined</b>
<b>biophysical stimuli</b>	<b>none</b>	<b>slight</b>	<b>increased</b>
<b>hormonal stimuli</b>	<b>minor</b>	<b>important</b>	<b>important</b>
<b>degree of proliferation</b>	<b>quick mitotic cell cycle</b>	<b>decreased cell cycling</b>	<b>interphase / postmitosis</b>
<b>mitotic stress</b>	<b>high</b>	<b>decreased</b>	<b>low</b>
<b>degree of differentiation</b>	<b>missing</b>	<b>increasing</b>	<b>high</b>

Figure 59: Schedule for the culture of cells to obtain intense mitotic cycling, starting development and maintenance of functional features. According to the cell cycle three different phases have to be observed. One has to consider that mitosis and development cannot occur in parallel but arise in succeeding phases.

Keeping the natural cell cycle in mind a protocol for advanced culture consists of three different steps (Fig. 59).

### **Step 1:**

**Proliferation** - This step comprises the multiplication of cells so that the necessary cell mass can be harvested. The administered culture medium contains for permanent mitotic cycling either growth factors or foetal bovine serum or both.

**Step 2:**

Starting development - In this important experimental step mitosis is down-regulated so that differentiation can start. By the application of reduced serum concentration or by the use of serum free media the frequency of mitotic cycling is decreased so that the majority of cells can enter the interphase so that the process of physiological differentiation can start. Previously performed experiments demonstrate that this phase can take several days.

**Step 3:**

Maintenance of differentiation - In this phase morphogens and hormones are administered so that a specific differentiation is maintained. Further chemically defined media with adapted electrolytes are used so that the initiated differentiation is proceeding. This process needs in primary cells at least 10 days. During that time the functional differentiation remains up-regulated. According to this culture protocol cells and tissue can be maintained for many weeks without subculture.

Finally, it has to be considered that most of the commercially available culture media were originally made to promote the proliferation of cells and not to support their differentiation. In future improved culture media are needed resembling the interstitial fluid environment and maintaining better the degree of tissue specific differentiation. In principal a first medium for proliferation, a second medium for starting differentiation and a third medium for maintenance of differentiation is urgently needed.

**[search: cell culture cell cycle differentiation]**

**Self-study: Inform yourself about differentiation of cells in culture**

## 13. Limitations in static culture environment

When culture experiments with adherent cells are made, the aim is frequently to generate as much as possible typical features found in specialized tissues. Such a development depends on the one hand on an optimal anchorage of cells at a suitable substrate and on the other hand on different influences deriving from the fluid environment. Regarding a classical culture dish the adhesion can be improved by offering special coatings or modifications of the individually used material surface. The fluid environment can be adapted by the addition of serum, growth factors or hormones. However, in the static fluid environment of a dish many naturally occurring influences such as continuous provision with always fresh medium, hydraulic or rheological stress and exposure to medium gradients are missing.

**[search: cell culture static environment]**

**Self-study: Read about static environment within a culture dish**

### 13.1. Unstirred layers of fluid

When experiments are performed, it is frequently observed that at the start of a culture cells adhere to an offered substrate to form a monolayer. However, with ongoing time it is seen that the rate of mitosis decreases but a progress in differentiation cannot be noticed, although an optimal adhesion was offered for example by coating the dishes with extracellular matrix proteins. Since at the begin of culture a positive development is registered, the minor differentiation observed during ongoing time might be caused by a limited provision with nutrition, respiratory gas and environmental fluid stress.

An inadequate provision with culture medium under the static environment of a dish can be caused by several reasons. A reason for a minor development of cultured cells might be

provision with medium and respiratory gas at the contact site to a coating material and at the bottom of the dish (Fig. 60.1). Since cells closely adhere to these materials, the exchange of medium is restricted here hampering in turn the further development. For example, harmful metabolites are concentrated here, since the exchange of fluid does not occur. These metabolites can arise by secretion of cells, by degradation of the extracellular environment and by the accumulation of molecules derived from biodegradable scaffold material.

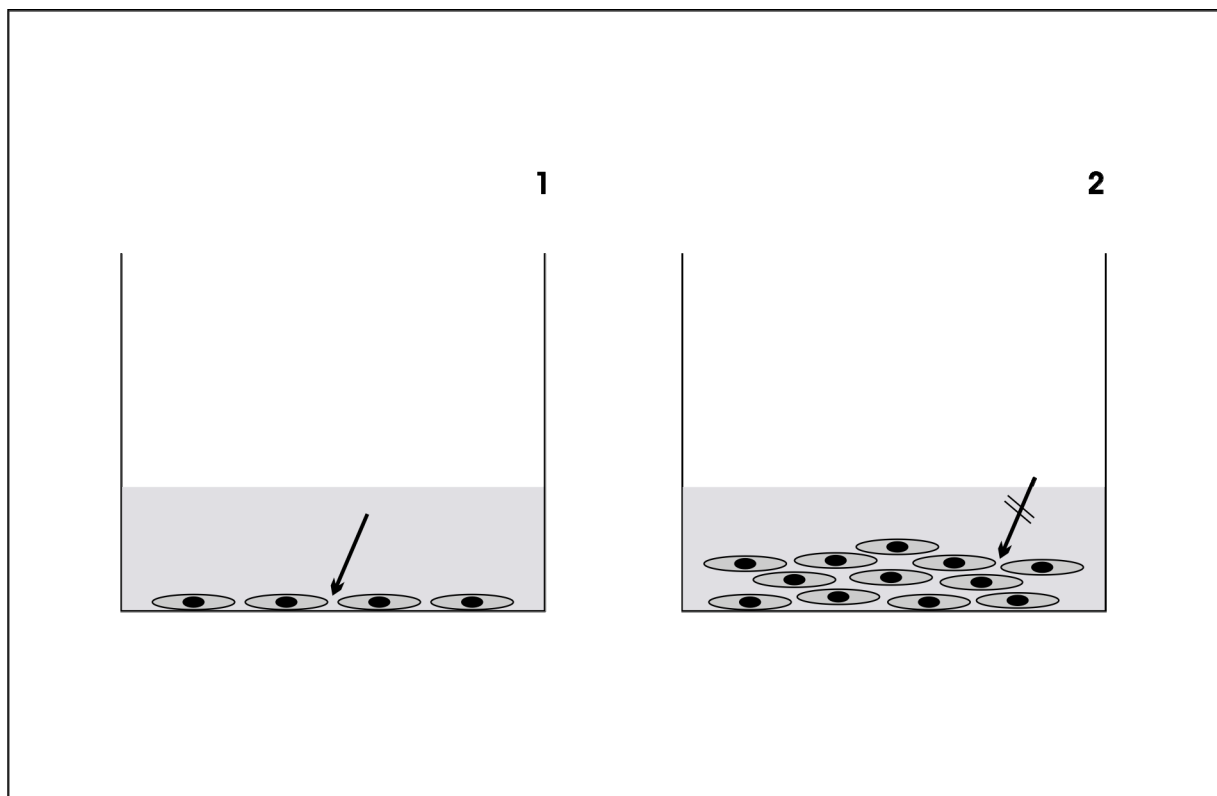


Figure 60: Provision of cells growing as a monolayer (1) or multilayer (2) with nutrition and respiratory gas. The supply of a monolayer (1) with nutrition and respiratory gas is possible at the upper side, while it is restricted at the contact site between the plasma membrane and the bottom of the dish. Problems with provision can arise in multilayered cells (2). Problematic zones for supply with nutrition and respiratory gas are spaces between cells, the contact to coating material and the bottom of the dish.

The situation is much more complicated, when several cell layers are established. In consequence, a minor admittance of medium is given by too narrow slits between cell neighbours hindering in turn exchange of nutrition and respiratory gas (Fig. 60.2). On the one hand fluid cannot reach all of the cells, on the other hand culture metabolites are not

eliminated. A narrow space hinders admittance of medium due to strong capillary forces so that a constant exchange of nutrition and respiratory gas cannot occur. This situation is finally leading to unstirred layers of fluid causing in turn fouling and necrosis of cells. It is obvious that cells under those environmental parameters are not able to develop.

**[search: cell culture static environment metabolism]**

**Self-study: Read about unstirred layers of fluid in cell culture experiments**

## **13.2. Alterations in nutrition**

The static fluid environment within a culture dish causes that cell metabolites and paracrine factors accumulate, while in parallel offer of nutrition decreases. At the start of a culture the situation is not problematic, since low concentrations of metabolites do not have a toxic influence on the growth of cells.

However, during increasing periods of time the concentration of metabolites raises resulting in a toxic effect on dividing cells and developing tissue. That is one of the reasons that in static culture always a relatively big volume of medium in relation to a low number of cells has to be added. In consequence, exchange of medium has to be made within short periods of time and as regular as possible.

Further it has to be considered that the static fluid environment within a dish is continuously altering its composition (Fig. 61). Part of the contained ingredients is degraded by cellular consumption and metabolism. Due to this reason the concentration of cellular metabolites continuously increases. When fresh medium is filled into a dish, it is obvious that the contained cells are exposed to an original and intact fluid environment (Fig. 61.1).

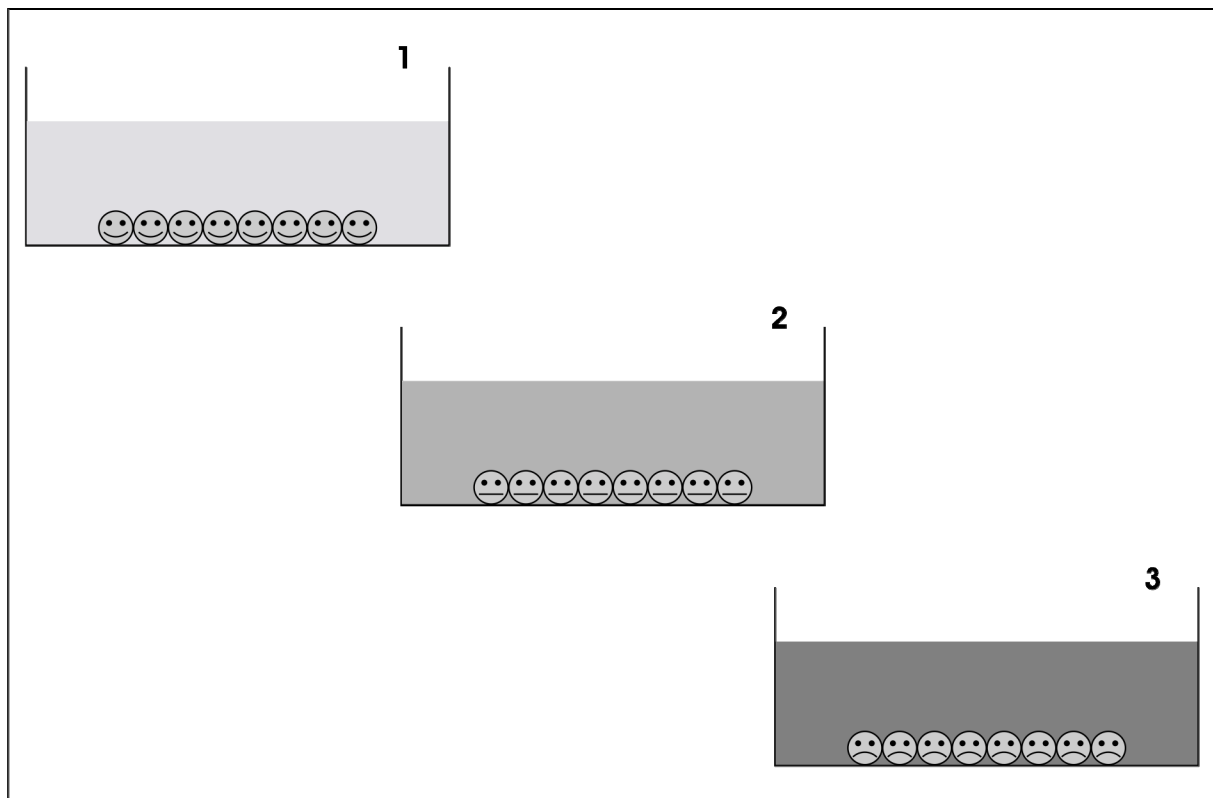


Figure 61: Schematic illustration of changes in cell activity within the static milieu of a culture dish. In fresh culture medium cells are exposed to an optimal environment (1). After a few hours alterations in the medium are found due to an increase of metabolites (2). Cells start to feel unhappy. Already after one day the influence of harmful metabolites can inhibit further development (3).

Already after a few hours the composition of the medium is continuously changing due to a decrease of nutrition, an increase of metabolites and an uncertain concentration of paracrine factors (Fig. 61.2). It must be mentioned that already after a few hours metabolized medium can inhibit development. After only one day the concentration of metabolites can reach such a high level that the multiplication of cells is stopped (Fig. 61.3). In consequence, an optimal fluid environment can only be obtained by feeding cells in sufficient amount as well as in regular and short periods of time. In all cases one has to consider that the volume of medium is big enough in relation to the mass of cells.

**[search: cell culture metabolite]**

**Self-study: Get updated about metabolic parameters in a culture dish**



## 14. Options for dynamic culture

When culture experiments are performed one should consider that each type of cell and each specialized tissue has individual requirements concerning nutrition, respiratory gas and environmental stress. When the situation is given that the static environment within a dish does not promote cell differentiation and development of tissues, considerations have to be made to improve the situation by a specific adaption of culture techniques.

It is understandable that such an adaption cannot be made within a classical dish due to the spatial and technical limitations. In consequence, improvement of the environment has to be made by an advanced housing and sophisticated microreactors promoting during culture the exchange of fluid, nutrition and respiratory gas. Moreover, in microreactors the environmental conditions can be adapted in single steps to individual physiological needs of cells supporting thereby the process of tissue specific development.

The environmental influences steering differentiation are manifold. An optimal site of anchorage is one of the basic requirements for cell development. Experimentally this can be reached by culturing cells in combination with extracellular matrix proteins or optimal biomaterials.

Further improvement can be made, when optimally adhering cells are exposed to a permanent flow of culture medium. This situation creates a naturally occurring rheological stress respectively hydraulic pressure.

**[search: dynamic cell culture]**

**Self-study: Read about advantages and disadvantages of dynamic cell culture**

## 14.1. Mass transfer in a spinner bottle

Keeping culture medium for adherent cells in permanent motion, a special housing has to be used. In this kind of experiments cells are seeded on a suitable substrate. This initial experimental step is made in the static environment of a classical dish. When the cells show adhesion, the substrate is fixed at one edge by a thread. Then it is mounted in a spinner bottle, where the cells are exposed to a permanent fluid motion (Fig. 62). The medium is set in rotation by a magnetic stirrer. This simple technique is producing a permanent fluid mass transfer promoting exchange of nutrition and respiratory gas and avoiding at the same time arise of unstirred layers of fluid. When this technique is applied, metabolites inside several cell layers or within the interior of a scaffold can be optimally eliminated.

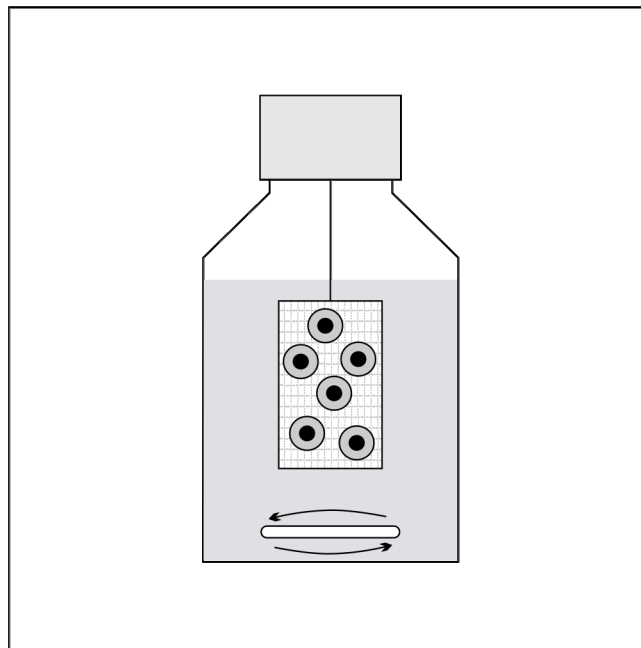


Figure 62: Culture of cells in a spinner bottle. A substrate with adherent cells is fixed at one edge by a thread hanging inside a spinner bottle. Fluid is permanently kept in motion by a magnetic stirrer.

Despite a big volume of medium the use of spinner bottle exhibits also disadvantages. No question, the culture medium can be kept in permanent motion. However, the metabolized medium is not permanently replaced against fresh one. For that reason an increasing amount of metabolites and a decreasing amount of nutrition is found during the course of a culture experiment. It is obvious that a constant quality of medium cannot be maintained by

this method. Finally, during culture the closure of the spinner bottle cannot be tightly closed, since an exchange of respiratory gas with the surrounding atmosphere must be given so that an uptake of oxygen and an equilibration of the pH can take place. During long term culture this might be disadvantageous, since the cultures are exposed to a high risk of infection.

Further the inner diameter of the spinner bottle is relatively small as compared to the total volume of culture medium. The small surface limits the exchange of respiratory gas and the adjustment of the pH within the medium. In consequence, corrections in respiratory gas content and pH occur within a long period of delay.

**[search: cell culture spinner bottle]**

**Self-study: Inform yourself about diffusion of nutrition and oxygen**

## **14.2. Microgravity in a rotating reactor**

A further possibility for dynamic culture of adherent cells and developing tissues is given in a rotating wall vessel bioreactor (Fig. 63). It consists of a wall vessel, whose hollow space is filled with medium and cells adhering on a suitable substrate. For the period of culture the vessel is mounted on an axle slowly rotated by a motor. The rotation is leading to a permanent motion of cells and a permanent mass transfer of medium. These effects are leading to a discontinuous microgravity.

Inside a rotating bioreactor cells are exposed to a permanent motion and a mass transfer of medium leading to dynamic culture conditions. In this case unstirred layers of fluid are minimized between adherent cells on a substrate. The exchange of fluid occurs via a gas permeable wall of the rotating culture chamber.

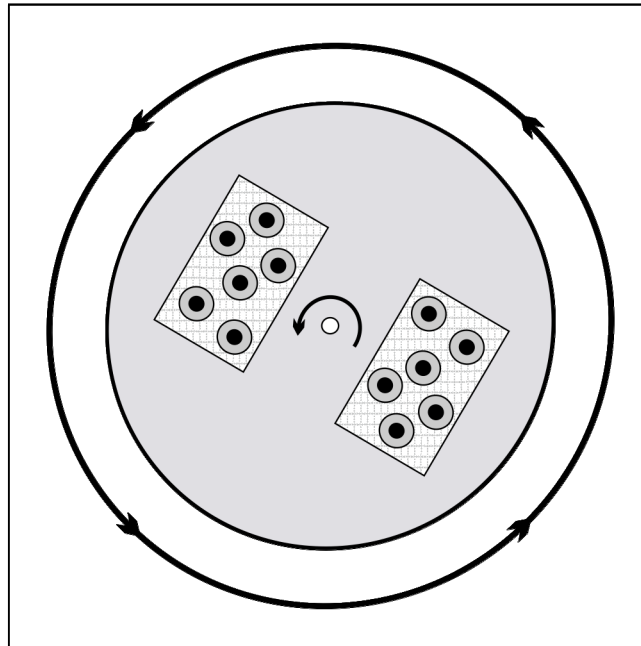


Figure 63: Culture of cells under dynamic conditions in a rotating wall vessel bioreactor. The vessel is mounted on a rotating axle. Inside the cells are exposed to a permanent motion and a mass transfer of fluid resulting in a dynamic culture environment.

However, applying this kind of culture a continuous provision with always fresh medium does not take place. For that reason metabolites are not continuously eliminated leading probably to damage of developing cells.

In an extended version the rotating wall vessel bioreactor can be connected to tubes so that a permanent provision with fresh medium becomes possible. Multiple successful culture experiments were performed applying this method.

**[search: cell culture rotating bioreactor]**

**Self-study: Get updated about microgravity in cell culture**

### 14.3. Exchange of medium via hollow fibers

Except epithelia and cartilage all other specialized tissue need an intact blood capillary system for a sufficient provision with nutrition and respiratory gas. In analogy, when several layers of cells are generated, an always constant diffusion for nutrition must be guaranteed during the whole period of culture.

By the help of a specific housing containing hollow fibers a capillary system can be mimicked to provide adherent cells with medium over long periods of time (Fig. 64). For example, single hollow fibers can be mounted in parallel into the housing. Then they are connected with thin tubes for the exchange of medium. The tubes are linked in turn to a peristaltic pump transporting medium to the interior and over an inlet in the housing to the outside of the hollow fiber. Most advantageous for the experimental design is that cells can be cultured as well on the inner side as on the outer side of the hollow fiber.

In the meantime different kinds of hollow fiber modules are commercially available. The hollow fiber itself can consist either of polysulfone, acrylic polymers and cellulose acetate. The modules are offered in different sizes and various numbers of inserted hollow fibers. In a special module they are orientated in form of bundles so that an arterial and venous transport path can be simulated.

In a hollow fiber module always fresh culture medium is transported to the cells (Fig. 64). Due to fluid mass transfer harmful metabolites do not accumulate in hollow fibers but are diluted by a permanent process of diffusion. When adherent cells are growing on the inner side of a hollow fiber, their extension is limited due to the diameter of the lumen. In contrast, when cells are growing on the outer side, broad extension can take place so that several cell layers will develop. By the continuous transport of culture medium constant environmental conditions for the contained cells can be created within a module. Convincing results were

obtained with hepatocytes maintained in differentiated form for weeks inside a hollow fiber module.

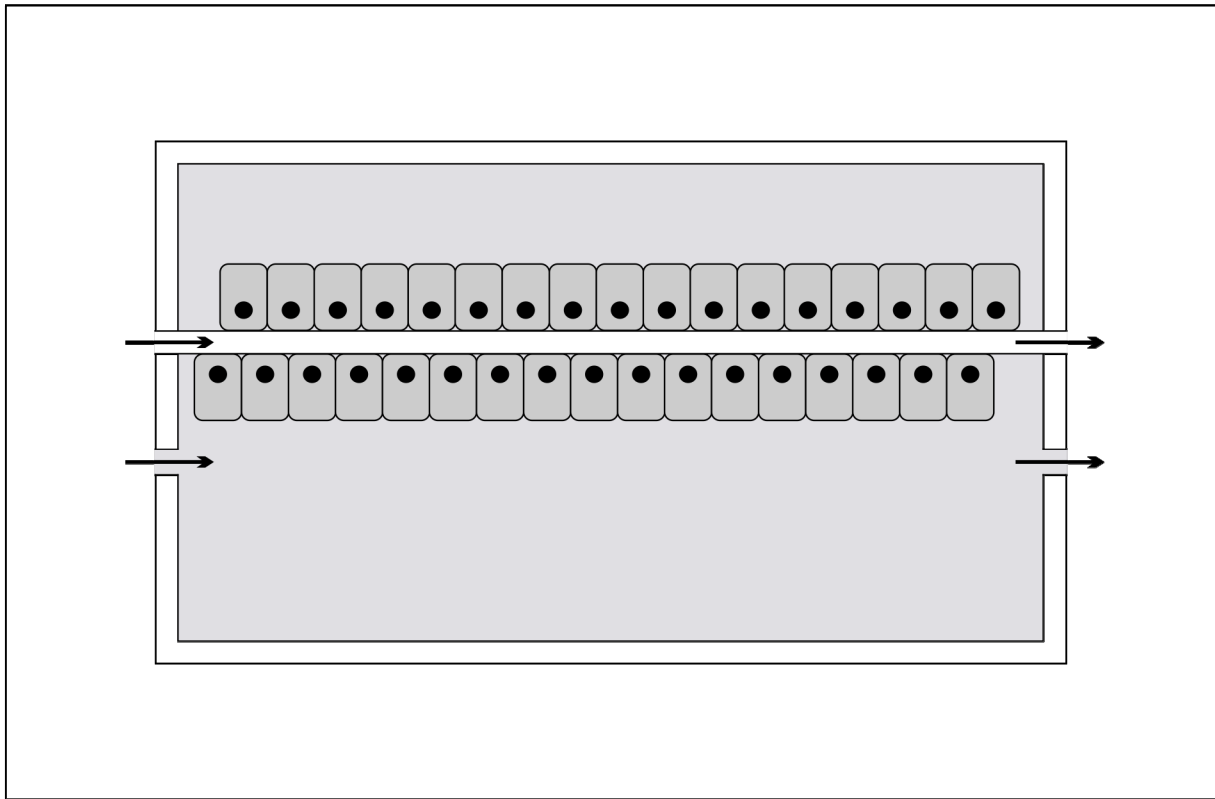


Figure 64: Schematic illustration depicts a hollow fiber module. It consists in the typical case of a housing and a bundle of integrated hollow fibers connected to tubes for the transport of culture medium. In the housing a further port for culture medium transport exists to reach the outer side of hollow fibers. Cells can grow either in the lumen of a fiber or on its outer surface.

When a hollow fiber membrane bioreactor module is used, the arising cell layers should not be thicker as 150  $\mu\text{m}$  and should always keep close neighbourhood to fibers. Regenerated Cellulose, Polysulfone, Polypropylene and Polyethylene fibers meet the requirements for the intense growth of a broad range of cell lines. The large surface area of fibers leads to increased cell concentrations. These kind of fibers are contained for example in CELLMAX<sup>®</sup> Hollow Fiber Bioreactors.

However, experiments demonstrate that cells are not always equally distributed within the hollow fiber module. Frequently sites are found, where cells are concentrated or barely

occur. In other cases sites are found, where more dead than living cells are found. However, it is very difficult to find the exact reason for the uneven distribution of cells. In the focus of research are apoptotic influences, necrotic lesions and unstirred layers of fluid resulting in turn in an insufficient provision with nutrition and respiratory gas. In each case the analysis of minor cell development is difficult to perform, since a hollow fiber cannot be taken out of a module without damage of cells on its surface and surrounding.

**[search: cell culture hollow fiber]**

**Self-study: Get updated about cell culture experiments with hollow fibers**

## **14.4. Perfusion cultures**

It is obvious that multiple cell layers and many specialized tissues are not developing in the static environment of a classical culture dish. To surmount the technical limitations, controlled mass transfer of culture medium has to be offered to the developing cells. A technical solution is the perfusion of medium inside a spatial environment (Fig. 65).

In the typical case chondrocytes or osteocytes are seeded on a porous but solid support respectively scaffold. When the cells have migrated into the pores, the support material is fixed between the base part and lid of a special housing (Fig. 65). Both base and lid are connected to tubes for the transport of medium.

During culture over weeks a peristaltic pump is transporting medium from the basal to the upper side of the housing. A big advantage is that always fresh culture medium is reaching the seeded cells, while harmful metabolites are constantly eliminated.

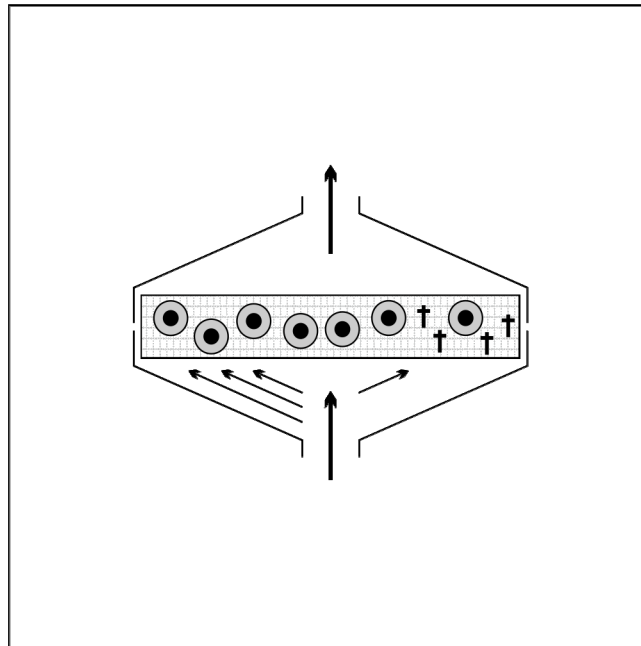


Figure 65: Schematic illustration of a questionable perfusion culture container. Cells are seeding on a substrate fixed between a lid and base part of a special housing. Culture medium is transported from the base to the lid of the housing. However, the flow of medium is not equal at all sites of the substrate.

However, problems arise by the inconstant direction of streaming fluid (Fig. 65). Not always the total cross sectioned area of the support is equally provided with fresh medium. In contrast, areas exist with intense provision of medium, while other sites receive less nutrition and respiratory gas resulting in an irregular occurrence of cells and an uneven synthesis of extracellular matrix.

**[search: cell culture perfusion]**

**Self-study: Inform yourself about perfusion culture techniques**

## 14.5. Perfusion housing with exact placement of cells

It has been shown in many experiments that cells kept in a perfusion culture container are exposed to a much better milieu than cells in the static environment of a classical dish. Performing perfusion culture mass transfer in form of medium transport is made from the



defined entrance to the exit site of a special perfusion container. Offering always fresh medium, a constant nutritional environment can be principally built up.

An important presupposition is to build up an optimal fluid stream. One of the defined aims is that the medium is not re-circulated. Thus, always fresh culture medium is reaching cells inside the perfusion container, while the metabolized medium is collected in a waste bottle.

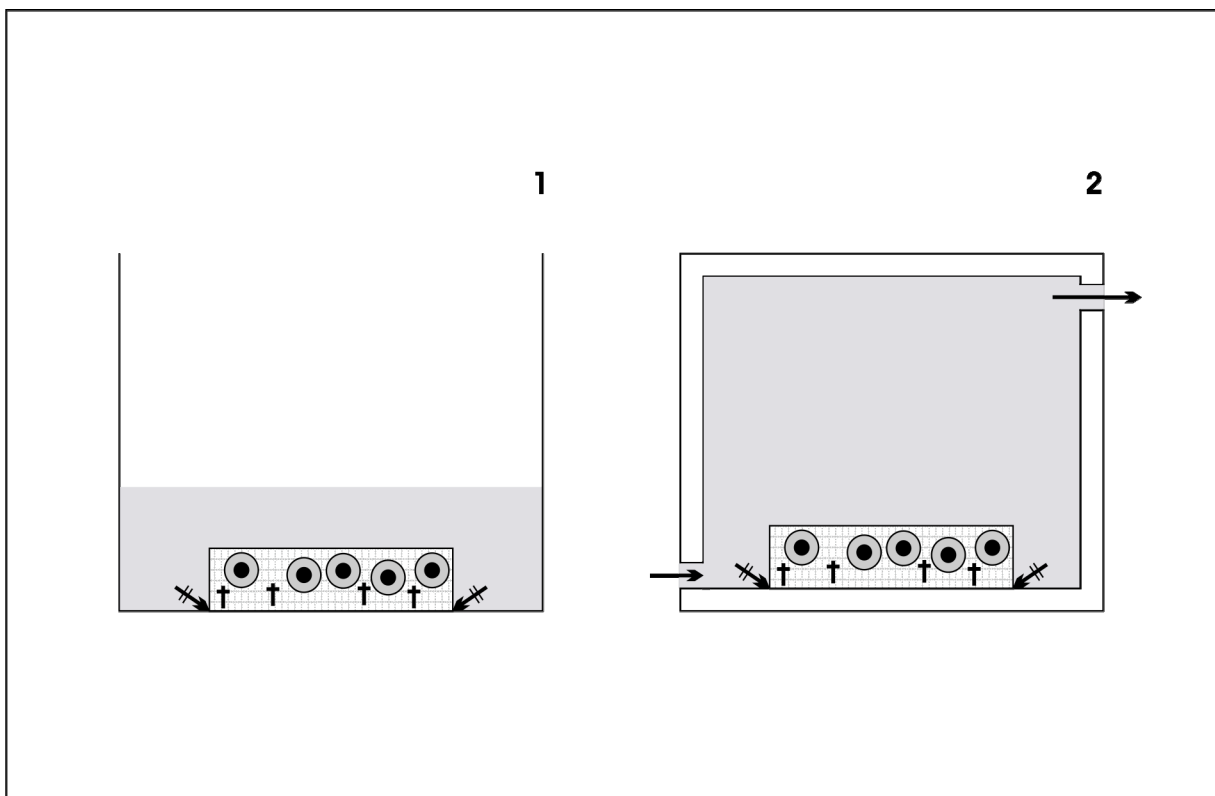


Figure 66: Illustration depicts unstirred layers of fluid, when a scaffold with contained cells is placed at the bottom of a culture dish (1) or at the base of a perfusion housing (2). The problematic zone is the contact between scaffold and bottom of the dish or perfusion housing. Exchange of fluid is lacking here.

A second presupposition is that the support with seeded cells is neither placed at the bottom of a dish (Fig. 66.1) nor at the base of a perfusion container (Fig. 66.2). In both cases the situation would lead to unstirred layers of fluid preventing an optimal provision of cells with nutrition and respiratory gas.

Instead an exact geometrical position inside a perfusion housing is required (Fig. 67). This aim is essential, since a contact of the support with the wall of the housing always will cause unstirred layers of fluid leading in turn to minor or missing development of cells.

In consequence, to avoid unstirred layers of fluid, supports with adherent cells are mounted best in a tissue carrier as described later. Then the carrier with adherent cells is placed in an exact geometrical position into a perfusion culture container (Fig. 67). The tissue carrier prevents on the one hand damage of cells and guarantees on the other hand an exact geometrical position inside the container so that transported culture medium can reach all sites with adherent cells.

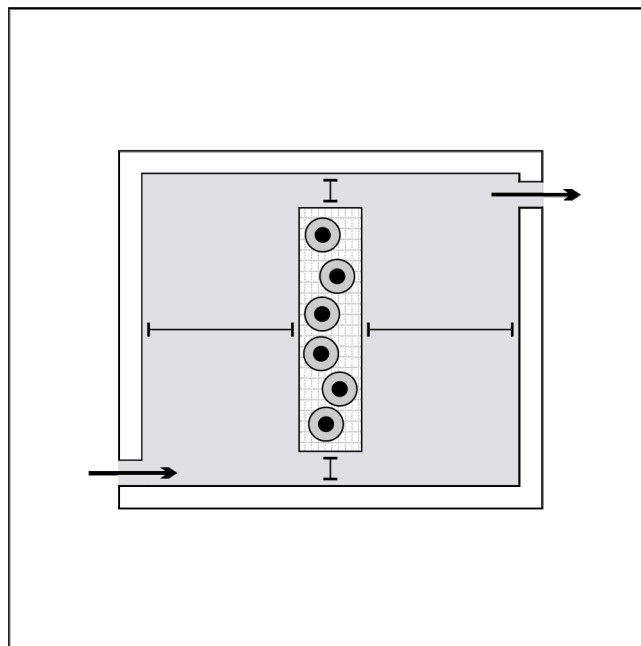


Figure 67: Illustration of a cell support fixed within a tissue carrier. The carrier is mounted in exact geometrical position inside a perfusion culture container so that all sites with seeding cells are provided with medium.

Regarding mass transfer in a spinner bottle (Fig. 62), a rotating wall vessel reactor (Fig. 63), a hollow fiber module (Fig. 64) and a perfusion housing (Fig. 65) all of the single techniques exhibit advantages and disadvantages for the culture of individual adherent cells.

No question, each of the demonstrated techniques was leading to interesting examples of application. All of them allow culture of adherent cells under permanent mass transfer. However, only the new version of the rotating wall vessel reactor (Fig. 63), the hollow fiber module (Fig. 64) and the perfusion housing (Fig. 65) makes transport of always fresh culture medium possible.

In contrast, administration of always fresh medium in combination with exact geometrical placement of cells and an even distribution of fluid is only possible with a perfusion housing including a tissue carrier (Fig. 67).

An elementary disadvantage of all of the demonstrated techniques is that they do not have a common technical denominator. It means that cells from a spinner bottle cannot be transferred without technical problems to a hollow fiber module. With other words cells cannot be transferred without technical break from a rotating wall vessel reactor to a spinner bottle. Thus, a technical bridge has to be created to transfer cells from a 24 well culture dish to a set of different microreactors so that experiments can be performed under advanced perfusion culture conditions. The challenging aim for the future is to simulate an environment with different but compatible technical culture modules reflecting the requirements within specialized tissues.

**[search: cell culture perfusion tissue carrier]**

**Self-study: Get updated about types of microreactors for perfusion culture**

## 15. Adaption of environment to specific needs

One of the defined aims for the culture of adherent cells is to reach a high degree of differentiation as observed in the specialized tissues occurring in the organism. To obtain such a quality, not only a perfect housing, but also environmental parameters such as optimal adhesion sites, a dynamic offer of nutrition and respiratory gas, continuous elimination of metabolites and tissue specific culture media have to be applied. In addition, suitable developmental stimuli should be administered allowing cells to go either from one division to the next or to develop functional features as found within specialized tissues. It is obvious that such multiple requirements cannot be found within a simple culture dish and cannot be reached by a single technique (Fig. 68.1).

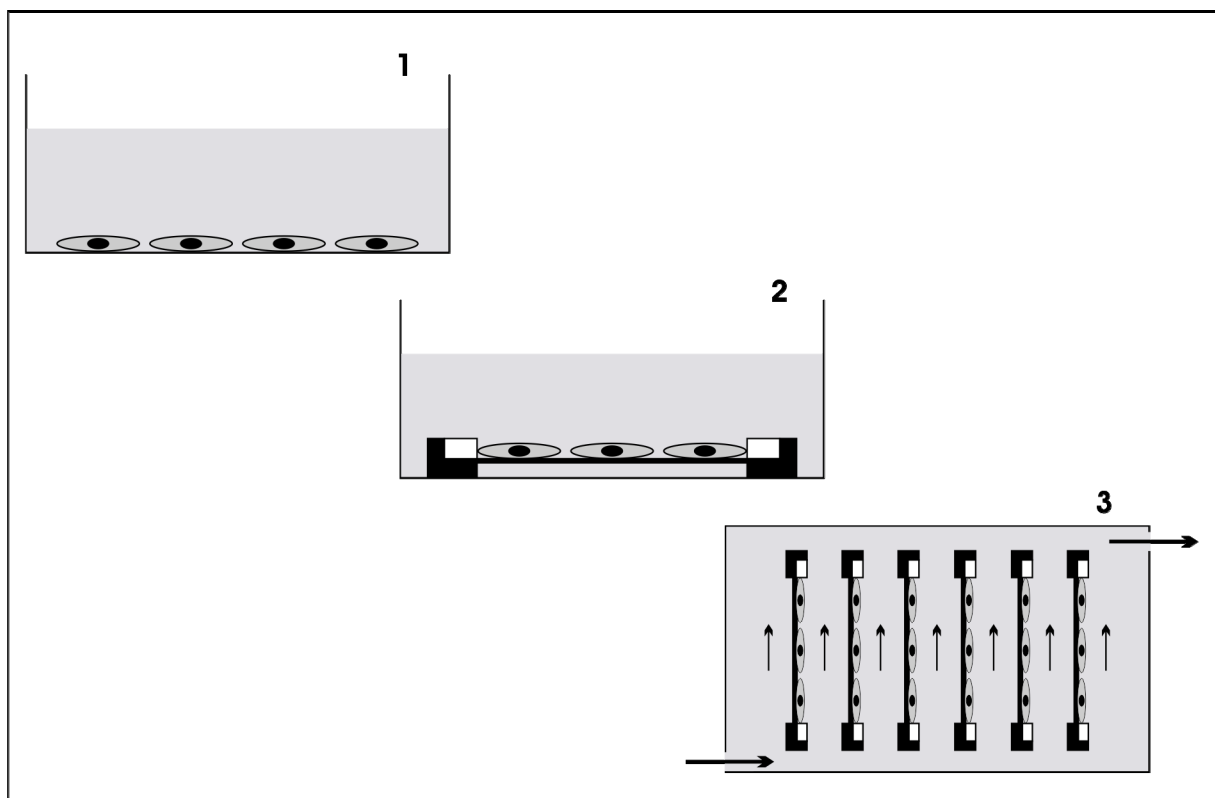


Figure 68: Schematic illustration of culture of cells at the bottom of a dish (1), in a tissue carrier with a selected adhesion substrate (2) and within a microreactor (3). While cells are exposed to static environment within a dish (1) and on a tissue carrier (2), cells are exposed to permanent fluid exchange in a microreactor (3).

Due to the requirements a modular culture system was developed that makes simulation of individual cell needs possible and allows adaption of culture environment according the surrounding of specialized tissues. The concept is to offer individually selected cell adhesion sites (Fig. 68.2). These substrates are mounted in a tissue carrier so that transfer of adherent cells can be made without damage from the static environment of a dish to specific microreactors (Fig. 68.3). Yet an environment can be created resembling the situation within single specialized tissues and individual organs.

The adaption of a typical environment for anchorage dependent cells means that in the most cases disposable parts are not available. Instead individual substrates, supports or scaffolds for optimal adhesion of cells have to be selected and punched out so that they can be placed in a tissue carrier. After seeding of cells in the static environment of a dish (Fig. 68.2) the tissue carriers have to be transferred then by forceps in a microreactor (Fig. 68.3). Since permanent fluid transport is performed, microreactors have to be connected over tubes with storage and waste bottles. Fluid has to be transported with a suitable peristaltic pump. Finally one has to decide if the perfusion culture experiments are performed in a CO<sub>2</sub>-incubator or under atmospheric air. Depending on the method a suitable biological buffer has to be added to the medium so that a correct pH is maintained over a culture period for several weeks or even months.

**[search: cell culture environment]**

**Self-study: Read about environment within a microreactor**

## **15.1. Selection of optimal adhesion sites**

Cells are able to show adhesion respectively attachment on many different material surfaces. However, only during proceeding culture it can be recognized if primary adhesion of cells is converted to close adherence. For the ongoing culture experiments this characteristic feature

is essential. Adherence has to be maintained so that on the one hand cells can develop individual features and on the other hand cells can stand fluid stress over long periods of time. Regarding these criteria it becomes obvious that the choice of commercially available materials is relatively little, when substrates for close cells adherence are needed.

Principally adherent cells can be cultures on a variety of materials. For example, flat filters, foils, nets, fleeces, sponges or even scaffolds can be used. Suitable filter materials for cell adhesion consist of polycarbonate, nitrocellulose, aluminiumoxide, polyethylenterephthalate, nylon or polyethylensulphone. If necessary the selected materials can be coated for improved adhesion by extracellular matrix proteins. Further either commercially available protein coats are ordered or individual coatings are made in the laboratory.

Most important is the size of the materials used in the individual experimental series. Very practical for the experiments are filters or other materials, when they are punched out to a diameter of 13 mm in the laboratory. In this size the substrates with adherent cells can be used in a 24-well culture plate. On the one hand this diameter can be used under static culture conditions and on the other hand it can be transferred to numerous microreactors working under perfusion with medium. Further many foils, filters, nets and sponges are commercially available in this 13 mm format.

**[search: cell culture adhesion site]**

**Self-study: Inform yourself about optimal biomaterials for cell culture experiments**

## **15.2. Punching out an adhesion substrate**

Many newly developed materials are delivered in big sheets or pieces so that they have to be cut or punched out with a sharp cylinder to a 13 mm format (Fig. 69). When a very thin material is punched out, it may happen during punching that the cut is not sharp so that the

specimens are not perfectly separated. In this case the quality of punching can be improved by placing a thick piece of white paper at the upper and lower side of the selected adhesion substrate. When the specimen is punched out, it results in an exact and perfect separation.

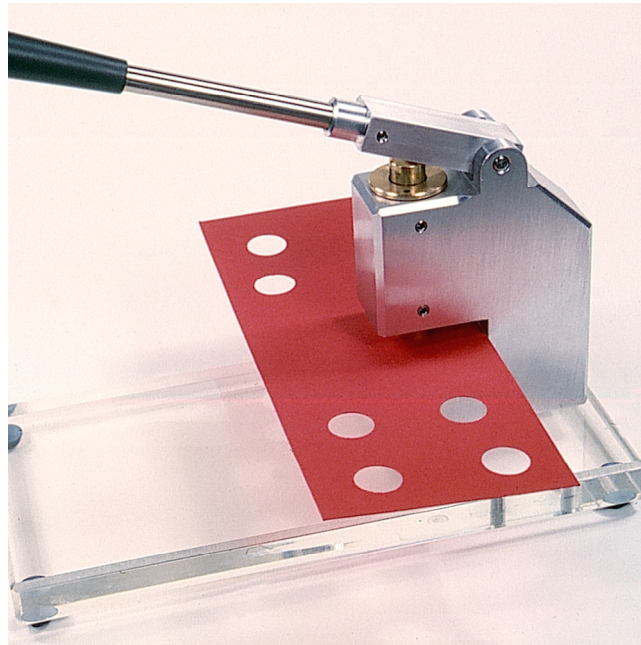


Figure 69: Illustration of a punching tool to separate specimens for cell improved adhesion with a diameter of 13 mm.

Beside foils or filters also fleeces or sponges as substrates for adherent cells and three-dimensional cultures can be applied. When such porous materials are selected as adhesion substrates, one has to consider that the internal pores size is not smaller than  $0.4\ \mu\text{m}$ , since capillary forces limit cell communication over protrusions and the exchange of nutrition and respiratory gas.

Most advantageous are transparent materials, since adherent cells can be easily visualized by conventional microscopy. When translucent or non-transparent materials are applied, adherent cells can be analyzed after staining them with fluorescent dyes. In this case the distribution of cells is analyzed by a fluorescence microscope.

**[search: cell culture adhesion substrate]**

**Self-study: Inform yourself about materials with optimal adhesion for cells**

### 15.3. Protection of adhesion substrate in a tissue carrier

Beside the bottom of a culture dish it was shown that adherent cells can be cultured on a variety of biomaterials such as foils, filters, fleeces or scaffolds. Most important for the selection of a substrate is that it optimally supports adhesion of cells resulting in intense proliferation and differentiation.

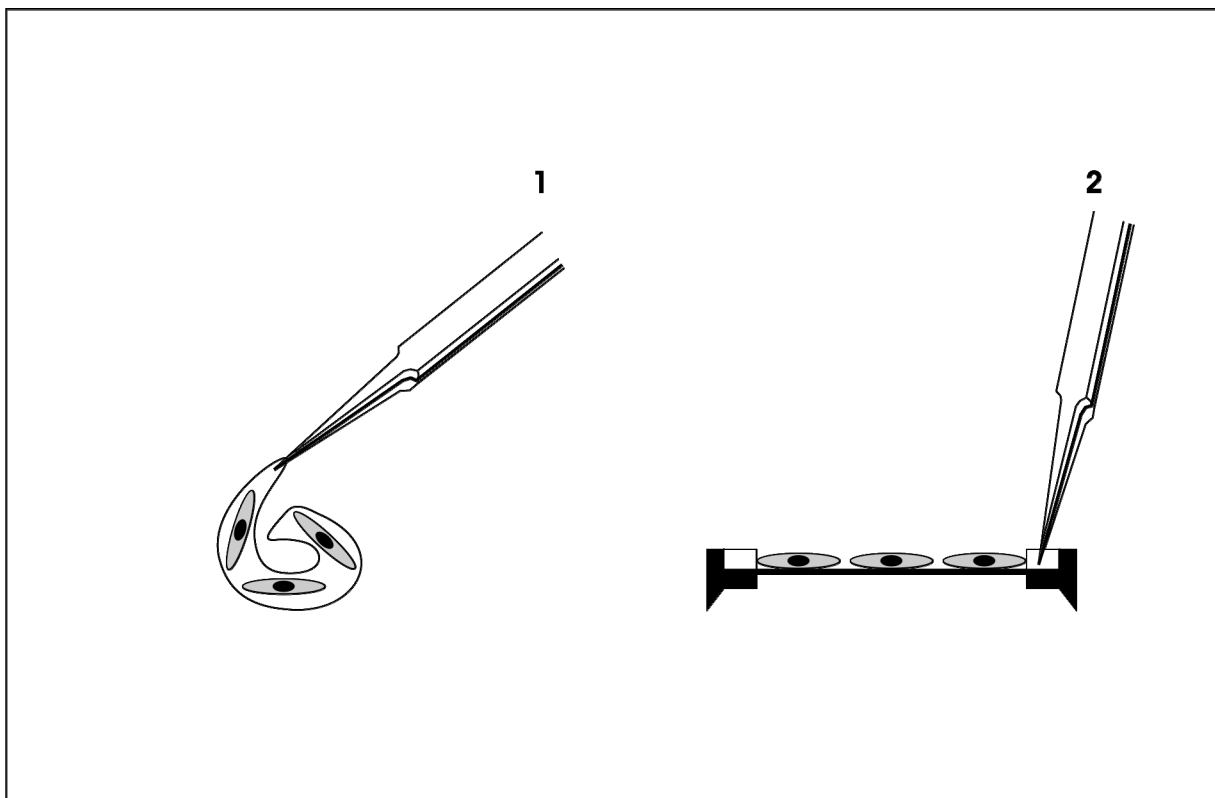


Figure 70: Transfer of adherent cells by forceps. A foil is curling up during transport with forceps so that adherent cells are damaged (1). In contrast, a foil within a tissue carrier is fixed so that cells are not damaged during transfer (2).

In a simple experimental version the selected substrate is placed on the bottom of a dish. Then cells are added within culture medium. After a few days cells are found to grow as well on the selected material as on the bottom of the dish. However, problematic is when a transfer of cells has to be made by forceps from the dish with static to a microreactor with dynamic environment. The thighs of the forceps and the curling of a filter will damage the adherent cells (Fig. 70.1).



To prevent damage of cells during culture and during transfer from one experimental set up to the next, the selected adhesion substrate is mounted in a tissue carrier (Fig. 70.2). Yet transfer can be made by a forceps without any damage of cells from the static to dynamic culture, to microscopic observation or to an electrophysiological set-up.

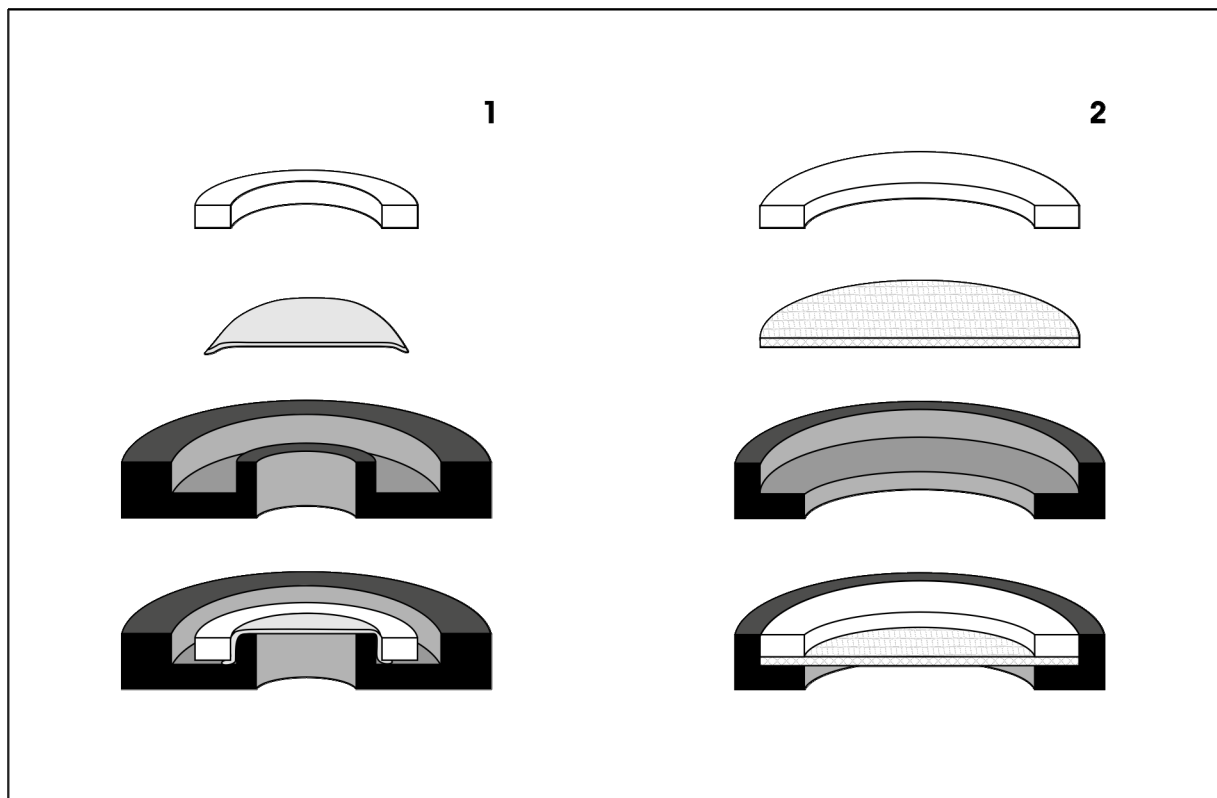


Figure 71: Schematic illustration depicts mounting of flexible (1) and rigid (2) materials for cell adhesion within a tissue carrier.

In consequence, flexible substrates for cell adhesion such as a thin sheet of collagen are mounted in a tissue carrier consisting of a specifically machined base cylinder and a span ring (Fig. 71.1, 72.1). Such flexible collagen sheets were isolated from submucosa of the small intestine or from urinary bladder. After placing the collagen sheet on the cylinder the span ring is pressed down. Yet the flexible adhesion substrate is fixed between the base cylinder and the span ring like the skin of a drum (Fig. 72.2). At the end of culture the tissue carrier with the mounted collagen sheet for example can be fixed in formalin or dismantled. The substrate is removed by lifting the span ring from the cylinder by a fine forceps.

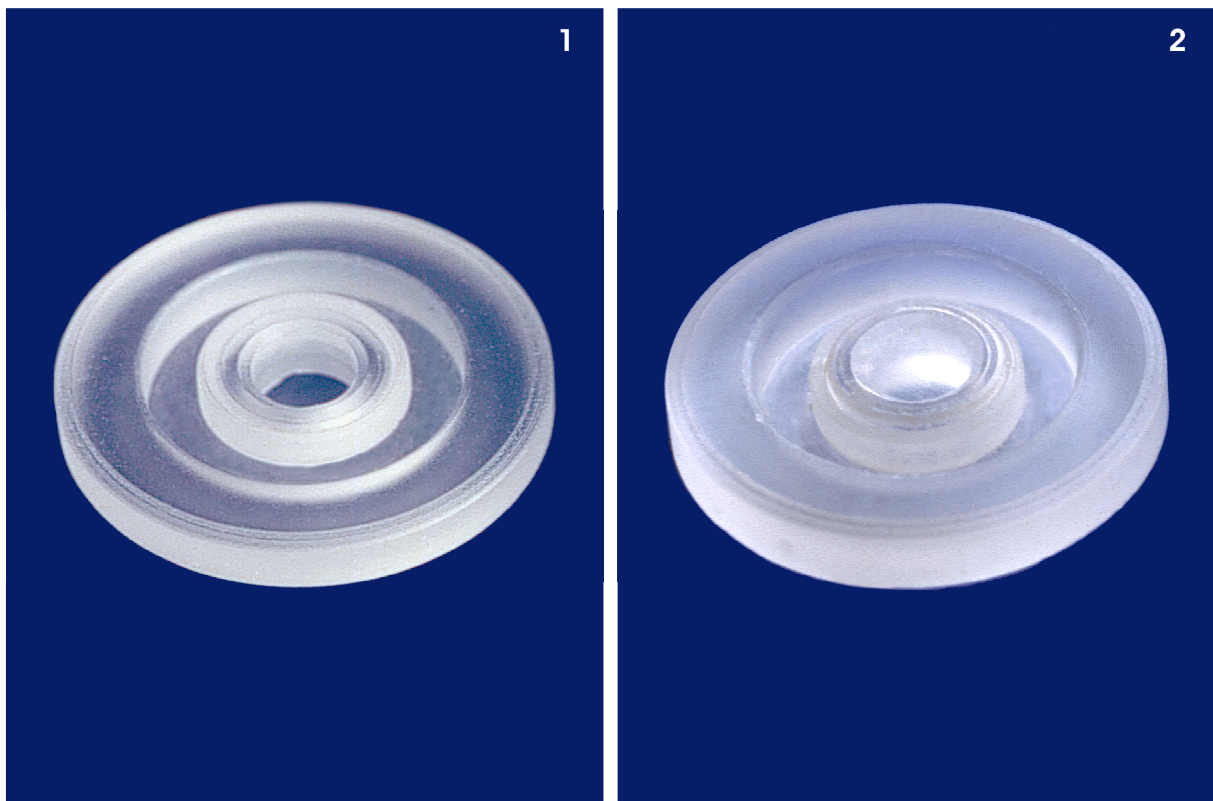


Figure 72: Photographic illustration demonstrates a tissue carrier in 13 mm format consisting of a base cylinder and a span ring (1). The mounted collagen sheet can be recognized as a shiny layer in the centre (2).

The seeding of cells on a collagen sheet (Fig. 71.1; 72.2; 73.1) can be performed before or after mounting on the tissue carrier. Most interesting results were obtained with cells on Small Intestine Submucosa (SIS) used for the regeneration of epithelia. In this special case the seeding is performed in a classical culture dish.

In contrast, rigid substrates for cell adhesion such as a filter or a foil are mounted in a tissue carrier consisting of a specifically machined base cylinder and a span ring (Fig. 71.2).

After placing the sheet on the base ring the span ring is pressed down. Yet the tissue carrier can be sterilized by different methods depending on the inserted substrates for cell adhesion.

After sterilization seeding of cells on a rigid substrate is performed in a classical culture dish (Fig. 71.2; 73.2). First the tissue carrier is placed into a 24well plate, then so much medium is filled in that the surface of the tissue carrier is wetted.

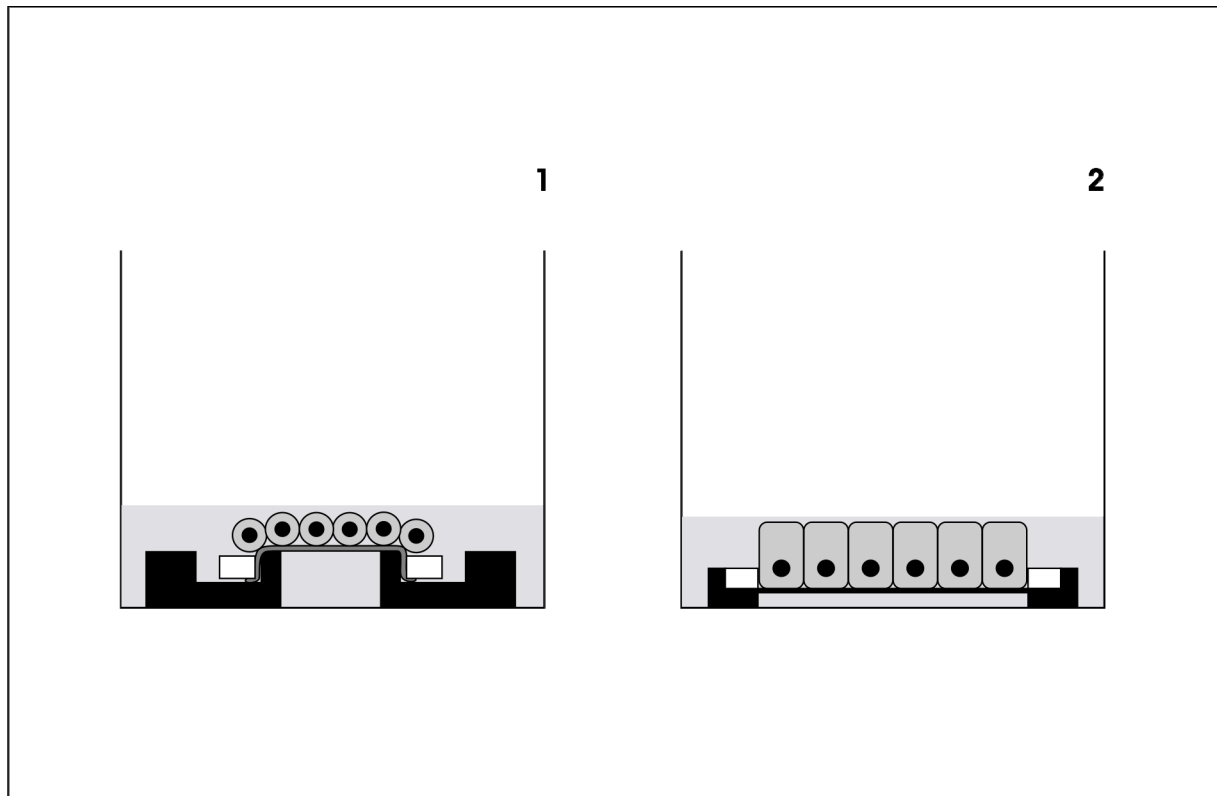


Figure 73: Schematic illustration demonstrates a tissue carrier in 13 mm format consisting of a base cylinder and a span ring within a culture dish (1). The flexible collagen sheet with adherent cells can be recognized in the centre. A rigid adhesion substrate for adherent cells is held between a base ring and a span ring (2). Both types of tissue carriers are used for cell seeding in a dish.

**[search: cell culture tissue carrier]**

**Self-study: Get updated about tissue carriers for cell culture experiments**

## 16. Handling a tissue carrier

For more rigid adhesion substrates such as foils, filters or nets an improved type of tissue carrier can be used. Also this type of tissue carrier consists of a base ring and a span ring. Between both of them the selected material for cell adhesion is mounted. For better understanding the single steps for exact mounting and dismantling a tissue carrier are described here.

### 16.1. Mounting an adhesion substrate

The advantage is that mounting of an adhesion substrate can be performed with individually selected specimens in the own laboratory.

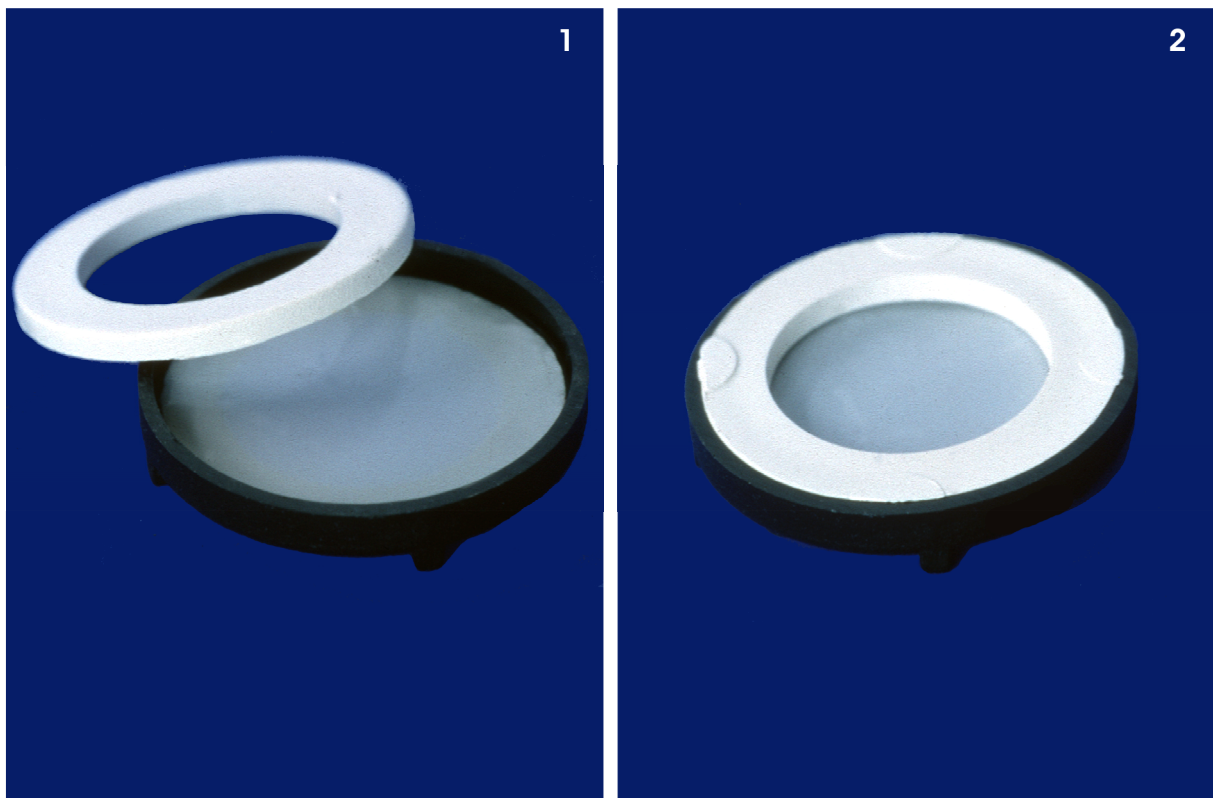


Figure 74: Mounting of a filter in a tissue carrier. For example, a filter with 13 mm in diameter is placed in a black base ring. Then the white span ring is pressed down. Small protrusions at the lower side of the base ring keep the tissue carrier in distance to the bottom of the culture dish. Applying this construction medium provision can take place at the lower and upper side of the tissue carrier.

To prevent curling of the adhesion substrate, the black base ring can be placed on a specific mounting plate (Fig. 74.1). This tool keeps the substrate for cell adhesion in plane position. This plate is not essential but helps in many cases.

Then for example a selected sheet of a nitrocellulose filter with 13 mm in diameter is placed in the black base ring. Finally the white span ring is pressed down to fix mechanically the filter (Fig. 74.2).

Most important are small protrusions on the lower side of the black base ring. They keep later the tissue carrier in distance to the bottom of the culture dish. This specific construction allows medium to reach not only the upper but also the lower side of the tissue carrier. Further the use of a tissue carrier prevents mechanical damage and facilitates the secure handling during transfer from one experimental set-up to the next. Finally, it allows later an exact geometrical placement of the developing cells within any type of container or microreactor during dynamic culture.

**[search: minusheet]**

**Self-study: Get updated about cell culture experiments with a minusheet tissue carrier**

## **16.2. Dismantling a tissue carrier**

The demonstrated Minusheet<sup>®</sup> tissue carrier is constructed not for single but for multiple uses (Fig. 74). In consequence, the tissue carrier has not only be mounted but has also be opened so that the substrate for cell adhesion can be exchanged.

For dismantling a tissue carrier the following procedure is recommended. Fine forceps with sharp thighs are needed. In each case damage of the substrate with adherent cells has to be avoided. To open the tissue carrier, both tips of the thighs are placed at the inner boundary

of the white span ring (Fig. 75.1). Then the white span ring is lifted (Fig. 75.2). Yet the substrate with adherent cells can be taken out of the black base ring. Finally, the base and span rings are transferred to distilled water so that drying of remaining cells and extracellular matrix is prevented before rinsing.

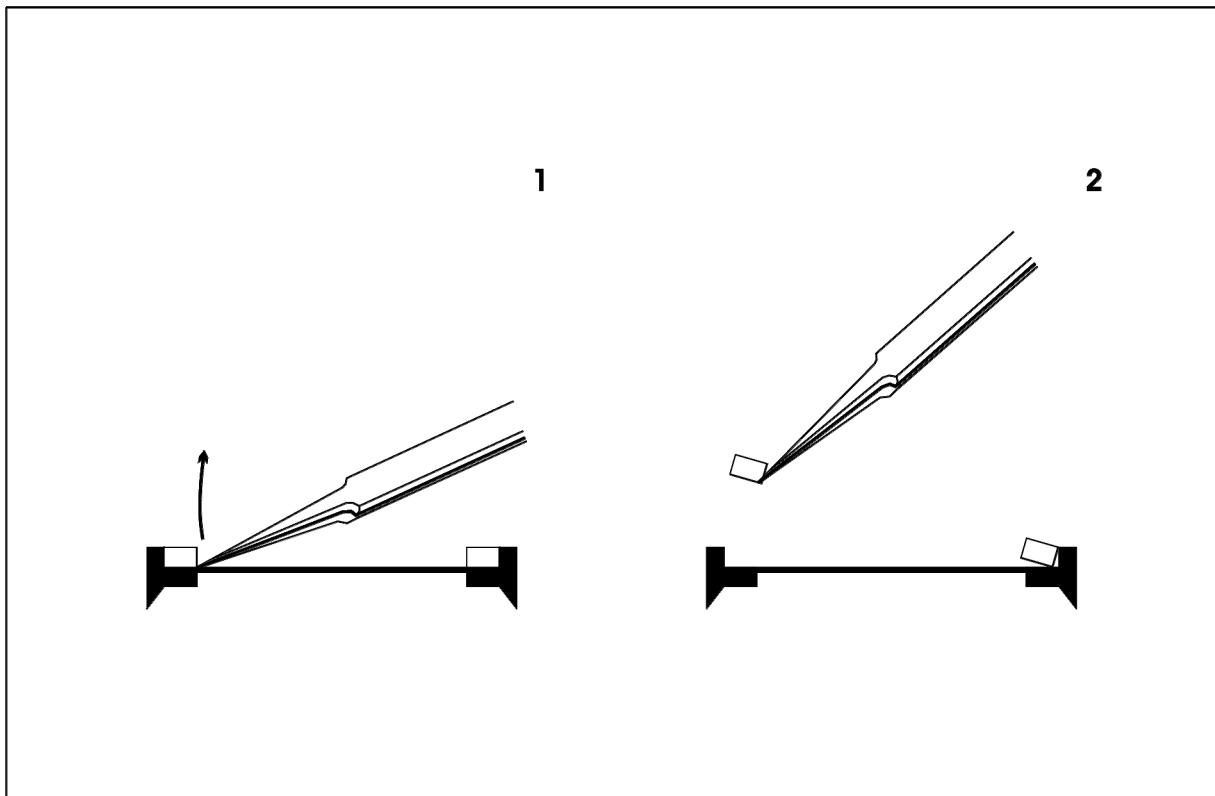


Figure 75: Dismantling a tissue carrier. To open the tissue carrier the thighs of a fine forceps are placed at the inner boundary of the white span ring (1). Then the span ring is lifted (2). Now the substrate with adherent cells can be removed by fine forceps.

### 16.3. Sterilization of a tissue carrier

After mounting a suitable substrate the tissue carrier has to be sterilized. Depending on the cell adhesion substrate it can be sterilized in solutions such as ethanol or formaline for a few minutes. Then the tissue carrier is rinsed several times in phosphate buffered saline (PBS). An alternative method is the sterilization in an autoclave. For this procedure a selection of tissue carriers is placed in a Melafol<sup>®</sup> envelope (Fig. 76.1), a metal box (Fig. 76.2) or a glass

Petri dish (Fig. 76.3). Then autoclaving is performed at an optimum of 105°C and 0.3 bar for 2 x 20 minutes. Due to selected materials the absolute maximum is 135°C and 2 bar for 10 minutes. Finally, an effective sterilization can be also obtained with gas treatment or with commercial irradiation.

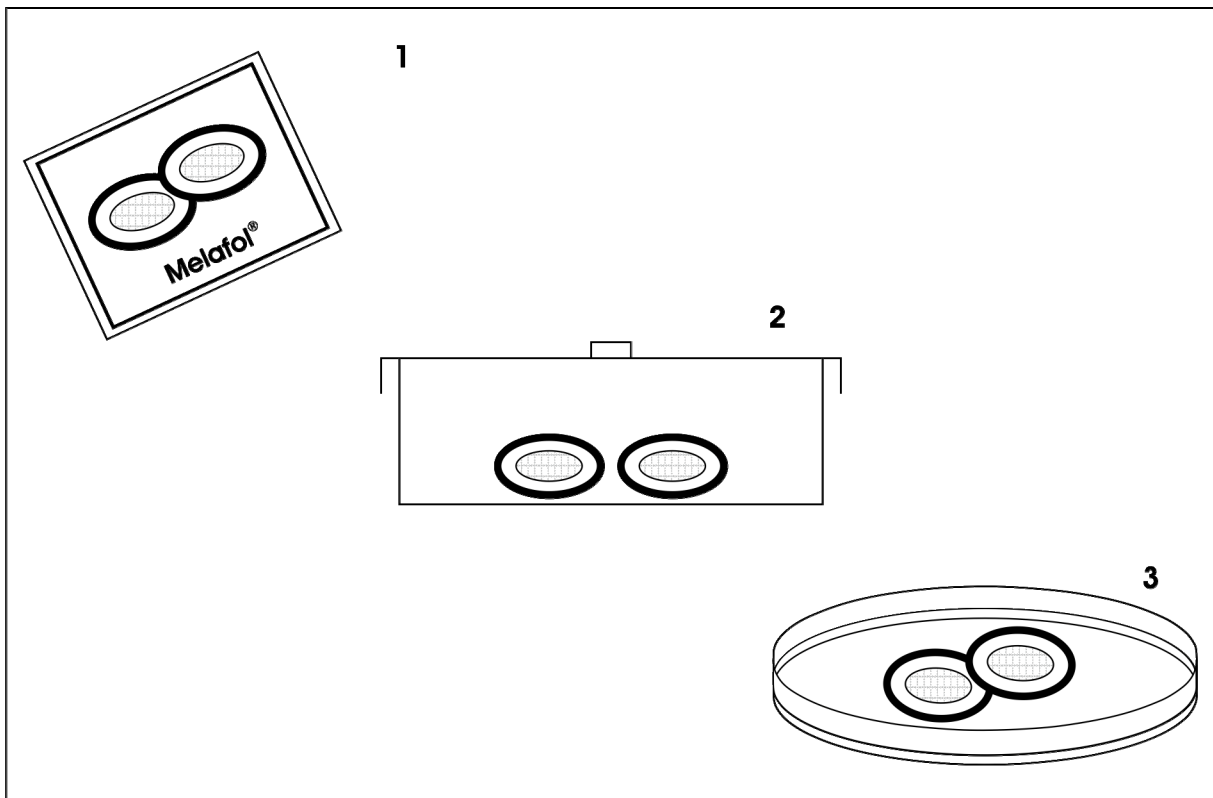


Figure 76: Housing of a tissue carrier during sterilization. For autoclaving a tissue carrier can be placed in a Melafol® plastic envelope (1), a metal box (2) or a glass Petri dish (3).

When the autoclaving process is finished the tissue carrier can be used at once or it can be stored in an envelope for days and weeks in a cooling box.

#### 16.4. Transfer of a tissue carrier to a dish

For seeding of cells first a set of tissue carriers has to be mounted with selected materials for cell adhesion. Then the mounted carriers are sterilized so that the adhesion substrate is not damaged during treatment by vapour, heat, gas exposure, irradiation or chemical treatment.



Figure 77: Tissue carriers mounted with different materials for optimal cell adhesion. The multiple colours illustrate the widespread applications of tissue carriers with selected adhesion substrates.

In the next step the tissue carrier is transferred with a fine forceps to a classical dish. In the case an adhesion substrate is 13 mm in diameter, a 24-well plate can be used for cell seeding and culture.

One has to consider that the adhesion substrate is not damaged during transfer of the tissue carrier. An important hint is therefore that the thighs of the forceps have a grip only on the base and span ring.

It is important to note that the adhesion material in the middle of the tissue carrier must not come in contact with the forceps.





Figure 78: Minusheet® tissue carriers mounted with different adhesion substrates. The tissue carriers are used for cell seeding within a 24-well culture plate.

### 16.5. Seeding of cells on a tissue carrier

After transfer of a tissue carrier into a 24-well plate (Fig. 78), culture medium has to be filled in. It is important to note that only such a little amount of medium is added so that the meniscus of fluid is just moistening the upper surface of the adhesion substrate (Fig. 79).

Frequently it happens during addition of culture medium that gas bubbles accumulate beyond the substrate for cell adhesion. In this specific case the well has to be filled completely with medium. Then the tissue carrier has to be turned by fine forceps so that the gas bubbles can separate. The tissue carrier is then placed back at the bottom of the well.

In the last step culture medium is drawn off so that the meniscus is just moistening the upper surface of the adhesion substrate.

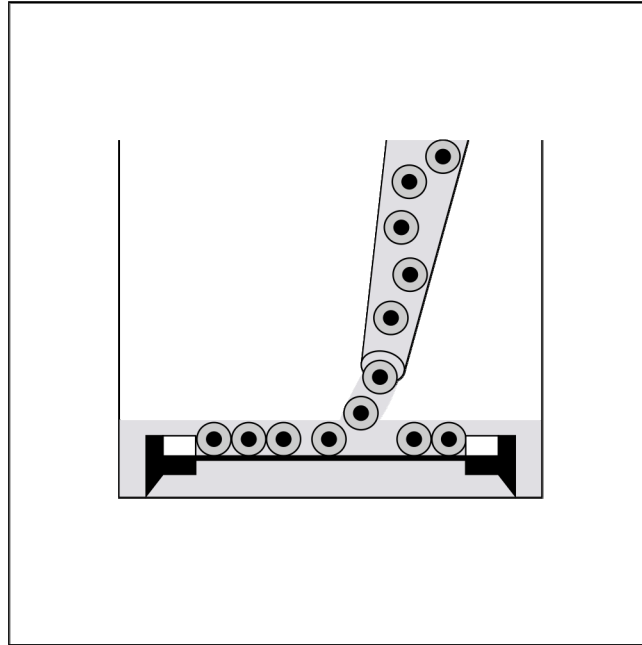


Figure 79: Seeding of cells on a Minusheet® tissue carrier. First the carrier is transferred to a 24-well plate. Then a small amount of medium is added so that the meniscus of the fluid is moistening the upper surface of the adhesion substrate. Finally cells are transferred by a pipette on the substrate.

For seeding an aliquot of a cell suspension is carefully pipetted onto the surface of the adhesion substrate (Fig. 79). When the pipetted volume of culture medium is kept small, the cells will stay at the upper surface of the adhesion substrate. In the case the volume of medium transferred by a pipette is too extended, the cells cannot stay at the upper side of the tissue carrier but will flow together with medium towards the base of the well.

Finally, after seeding of cells culture can start. According the selected buffer system within the medium, cells are incubated either in a CO<sub>2</sub>- incubator or on a warming plate at 37°C under atmospheric air. After adhesion of cells more medium is added so that the fluid meniscus stays a few millimetres over each tissue carrier.

## 16.6. Preparing contacting co-cultures with a tissue carrier

When co-cultures are prepared by the help of a tissue carrier, in the most cases a close contact between both kinds of cells is wished. As shown in the previous experiments first of all a suitable substrate for cell adhesion has to be selected. In the case epithelial cells are cultured on the one side and fibroblast on the other side the selected substrate for adhesion must support proliferation and differentiation of both cell types.

Excellent results were for example obtained with filters made out of nitrocellulose or polycarbonate. One has to consider that the pore size of the filter is not smaller than 0.4  $\mu\text{m}$ .

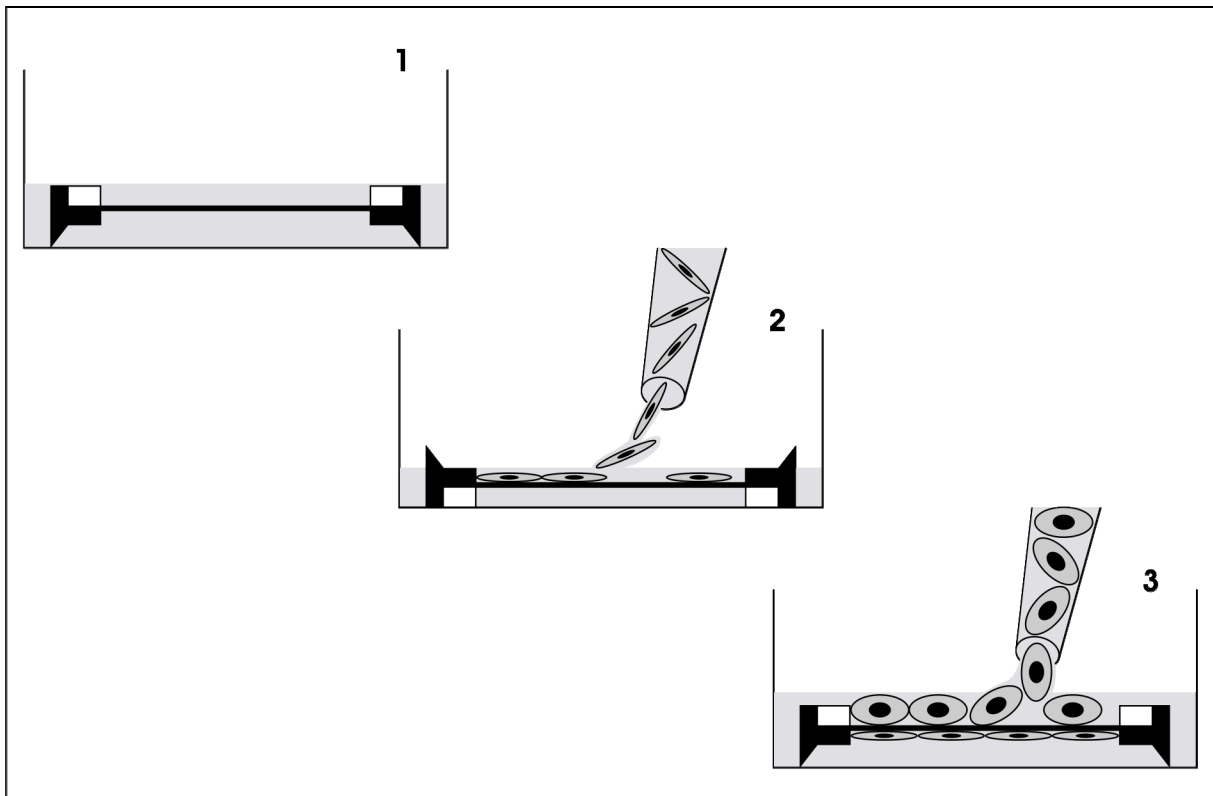


Figure 80: Seeding of cells on a Minusheet® tissue carrier for co-culture experiments. First the carrier is transferred to a 24-well plate (1). Then it is turned so that the white span ring is in contact with the bottom (2). A small amount of medium is added so that the meniscus of the fluid is moistening the upper surface of the adhesion substrate to seed the first type of cells (fibroblast). After adhesion of cells the tissue carrier is turned so that the second type of cells (epithelial cells) can be seeded (3).

In consequence, a nitrocellulose or polycarbonate filter with 13 mm in diameter is placed in a black base ring of a tissue carrier and fixed with a white span ring. After sterilization the

mounted tissue carrier is transferred to a 24-well plate (Fig. 80.1). Then it is turned so that the white span ring is in contact with the bottom of the dish (Fig. 80.2). Medium is filled in until the fluid meniscus is moistening the surface of the filter. In the next step medium with a first cell type such as fibroblasts is transferred by a pipette onto the filter of the tissue carrier. Then culture is started for a couple of hours so that adhesion of fibroblasts can proceed.

After adhesion of cells the tissue carrier is turned so that the black base ring is yet contacting the bottom of the dish (Fig. 80.3). Medium is filled in so that the meniscus of fluid is moistening the surface of the filter. Then an aliquot of the second cell type such as epithelial cells is added. Culture is started so that adhesion of cells can take place. After a couple of hours the tissue carrier is transferred to a new dish, where new medium for nutrition is filled into the well.

Of special interest is the simulation of the blood brain barrier in culture. First endothelial cells from cerebral microvessels can be seeded on one side of a filter. Then the Minusheet® tissue carrier is turned so that astrocytes can be settled on the other side. Such sophisticated cultures are urgently needed, since much research is focused on the development of novel therapeutic agents that permeate the blood brain barrier.

Another example is the culture of the corneal epithelium. In consequence, epithelial cells are seeded on one side of a filter, while on the other side mouse 3T3 fibroblasts are adherent. The fibroblasts previously have to be irradiated or mitomycin C treated so that proliferation and invasion of them is prevented.

Finally, macrovascular or microvascular endothelial cells can be co-cultured with smooth muscle cells to simulate cell interactions within the wall of a vessel.

**[search: minusheet]**

**Self-study: Read about tissue carriers in perfusion culture experiments**

## 17. Bridging static with dynamic culture

The creation of advanced culture conditions should not be an isolated technique but should be based on the task to bridge the gap between the static environment of a classic dish and the dynamic conditions found within multiple microreactors. To realize such a concept, both a cell biological demand and a technical concept have to be combined. On the one hand cells have to be cultured with traditional methods and by the help of classical culture dishes. On the other hand optimal sites for adhesion have to be applied, since the anchorage influences a variety of physiological cell reactions. Finally, due to limited possibilities in static culture a physiological environment is created in perfusion culture and in combination with various types of microreactors (Fig. 81).

<b>Static culture</b>	<b>Perfusion culture</b>
Aging culture medium during time	Provision with always fresh culture medium
Inconstant physiological environment	Constant level of physiological parameters
Decreasing offer in nutrition	Constant level of nutrition
Constant increase of metabolites	Constant level of metabolites
Increasing level of waste molecules	Constant elimination of waste products
Increasing level of paracrine factors	Constant level of paracrine factors
<b>Permanently changing culture conditions</b>	<b>Creation of a physiological environment</b>

Figure 81: List of advantages and disadvantages of environmental parameters, when cells or tissues are kept in static culture and in perfusion culture.

During multiplication of cells in dishes or flasks the substrate does not have a central meaning for the success of the experiments (Fig. 82.1). In contrast, for an optimal

differentiation an effective site of anchorage is needed. For optimal handling the substrate for adhesion must be mounted within a tissue carrier to protect growing cells from mechanical damage. Depending on the experimental design the tissue carriers with adherent cells can be used in static environment of a culture dish (Fig. 82.2) as well as under dynamic conditions within different kinds of microreactors (Fig. 82.3).

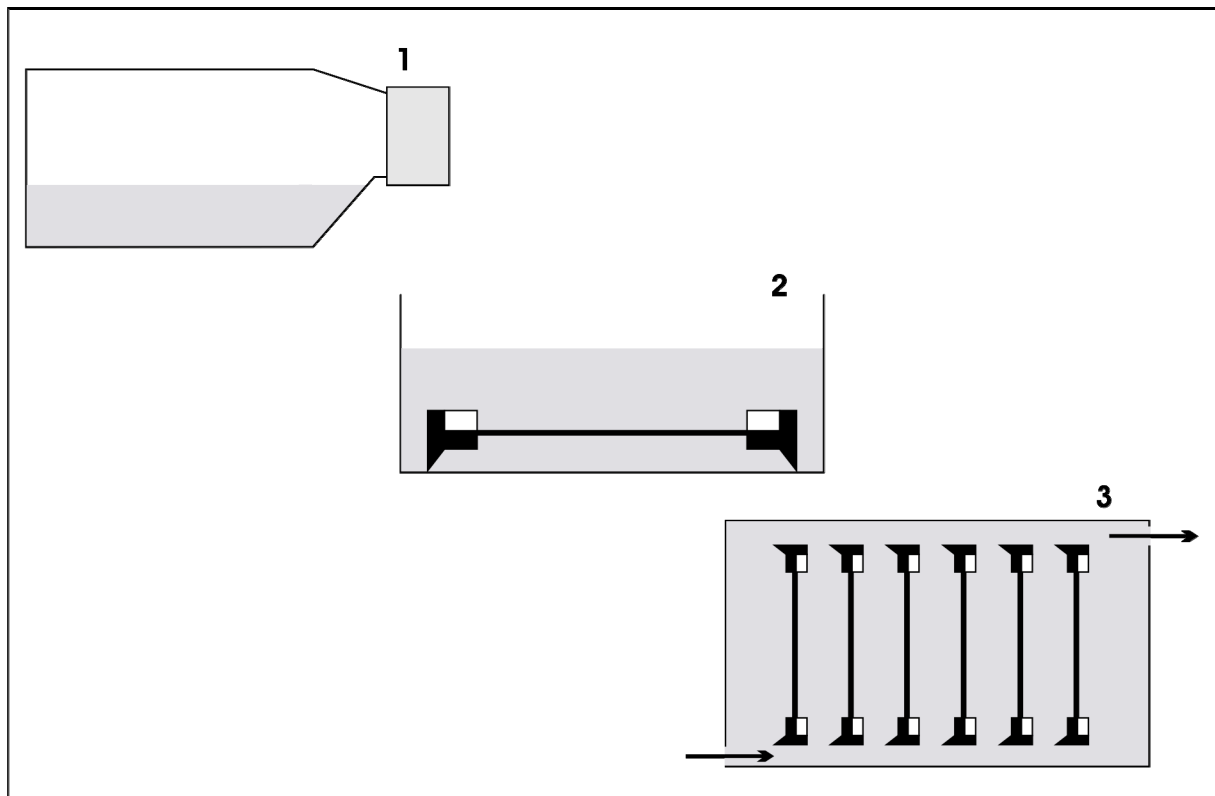


Figure 82: Bridging the technical gap between the static environment of a classical dish, a Minusheet® tissue carrier and dynamic conditions within a microreactor. For multiplication cells are cultured at the bottom of a dish or a flask (1). To improve differentiation, cells are kept on a tissue carrier mounted with a substrate for optimal adhesion (2). Improved environmental conditions are offered in a microreactor by permanent exchange of culture medium (3).

Regarding the different specialized tissue it is obvious that one kind of dish or one kind of microreactor cannot simulate all of the influences stimulating functional development found in the specialized tissues of the organism. In consequence, various aspects such as improved nutrition or rheological stress have to be considered so that differentiation is induced. In turn different kinds of perfusion culture containers with different environmental stimuli have to be



applied. Further it has to be considered that part of the experiments are analyzed after a more or less extended period of culture time, while other experiments need a permanent optical or electrophysiological registration. In so far for each individual experiment a specific microreactor has to be applied.

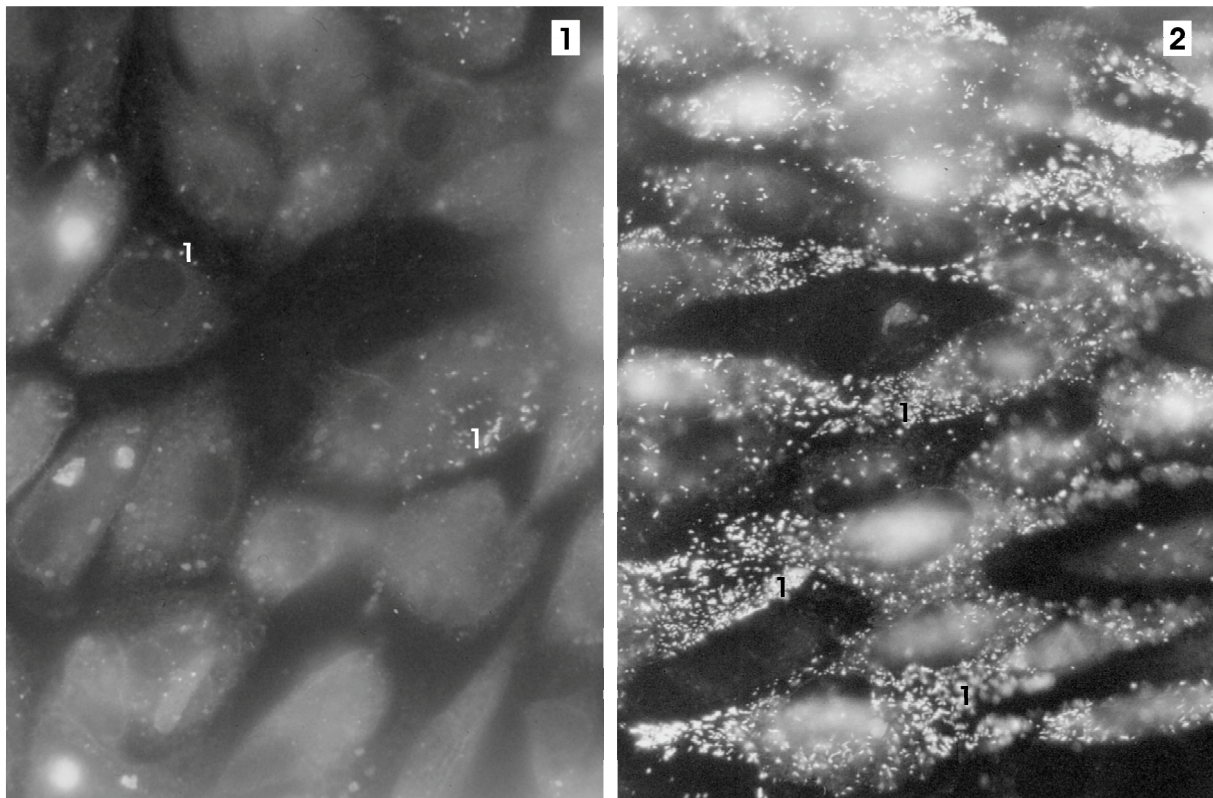


Figure 83: Immunohistochemistry on endothelial cells kept for 13 days under static conditions (1) and under perfusion culture (2). Immunolabel for von Willebrand factor shows, that cells under static culture exhibit only few labeled granula (1.1). In contrast, specimens kept in perfusion culture demonstrate multiple and intense granula (2.1).

Finally, for argumentation an example is given that in static culture not the same results are obtained as seen in perfusion culture (Fig. 83). In parallel endothelial cells were seeded on Thermanox<sup>®</sup> cover slides each inserted in a Minusheet<sup>®</sup> tissue carrier. One group was kept in static environment, while the second group was kept in a perfusion culture container. After a culture period of 13 days the specimens were immunohistochemically labeled for von Willebrand factor. Fluorescence microscopy clearly indicated that under static environment only few and barely visible granula were found (Fig. 83.1). In contrast specimens kept in

perfusion culture demonstrated multiple and intense granula as it is typically found in blood vessels (Fig. 83.2). This simple experiment clearly depicts that cells kept in perfusion culture show histiotypical features, while cells in static environment down-regulate expression of von Willebrand factor.

**[search: perfusion culture]**

**Self-study: Get informed about static and dynamic cell culture experiments**

## **17.1. Versatile containers for perfusion culture**

Looking to many specialized cell types and tissues it is still important to learn from nature. As a consequence the environment inside a microreactor has to be adapted to the specific needs of individual cell types. Numerous investigations have shown f.e. that a perfect functional differentiation does not only depend on an optimal cell adhesion, close communication with the extracellular matrix and the influence of morphogens but is also highly influenced by suitable nutrients, a correct quotient of  $pO_2/pCO_2$ , rheological stress, mechanical load, constant pH, temperature, and finally a favorable electrolyte environment.

The use of a tissue carrier makes it possible to find several solutions for the individual experimental needs. For example, under in-vivo conditions most tissues are constantly supplied with nutrients by numerous capillaries. To simulate this situation a tissue carrier can be placed in a perfusion culture container, where a constant nutrition with always fresh medium is offered (Fig. 84).

Application of a tissue carrier makes it further feasible to hold adherent cells in exact geometrical position within a microreactor (Fig. 84). In turn the constant position of the tissue carrier is yet influencing by its individual form the further distribution of culture medium. The



design of the internal surface within a microreactor in combination with the outer profile of a tissue carrier will steer the flow of culture medium. When both of the parameters are varied, the flow of medium can be adapted to the individual needs of cells and specialized tissues.

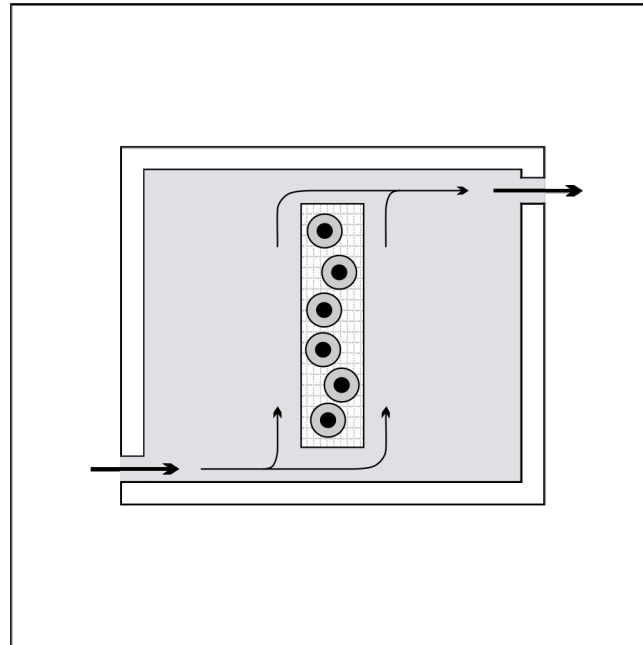


Figure 84: Use of a tissue carrier inside a perfusion culture container. An inlet and an outlet for the culture medium makes provision with always fresh medium possible. Further the use of a tissue carrier makes it feasible to keep adherent cells in always exact position so that medium can reach all of them. When the inlet is placed at the lower and the outlet for medium at the upper side the fluid path facilitates gas bubbles to leave the container.

Using a tissue carrier a microreactor can be designed so that fresh culture medium enters at the basal side, while the metabolized medium is drained at the upper side (Fig. 84). This fluid path facilitates gas bubbles to leave the container. On the passage through the microreactor the medium can flow along one or between a series of tissue carriers.

This way the developing cells are continuously supplied with fresh medium, guaranteeing constant nutrition and preventing an un-physiological accumulation of metabolic products and an overshoot of paracrine factors. In consequence, metabolized medium is not re-perfused in all of the presented experiments.

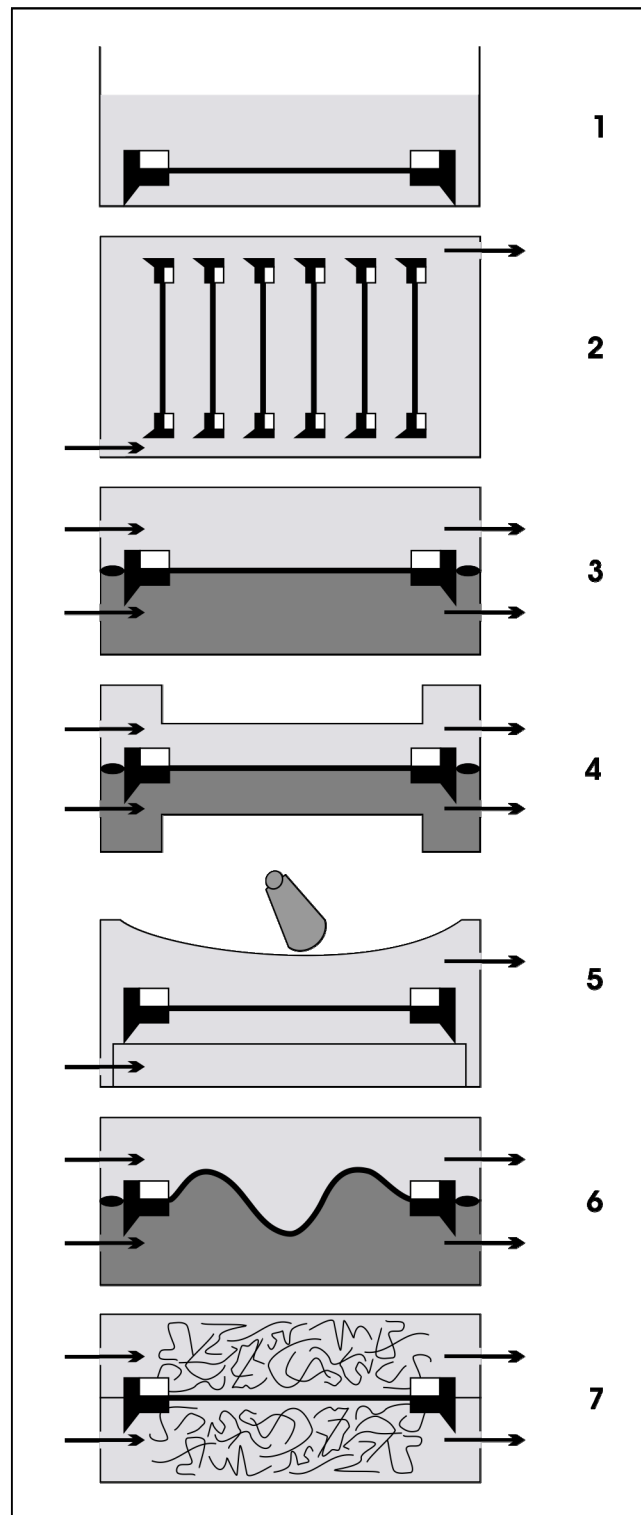


Figure 85: Applications of a tissue carrier inside a culture dish (1) or within different perfusion containers (2-7). For static environment a tissue carrier is used in a dish (1). In one type of container adherent cells are bathed (2). Epithelia cells are kept with different media at the luminal and basal side (3). In a microscope chamber cells are hold between transparent lids (4). A rotating excenter in combination with a flexible wall allows exposure to liquid pressure (5). Tissue constructs can be shaped (6). An artificial interstitium is created by insertion of a fleece (7).

With respect to the numerous factors that influence cell and tissue development in-vivo a variety of microreactors for continuous perfusion was developed on the basis of a Minusheet® tissue carrier that allows to individually control a number of important environmental parameters (Fig. 85).

This technique facilitates adherence of cells on a tissue carrier within a dish (Fig. 85.1), the simple bathing of tissue (Fig. 85.2) under continuous medium perfusion or the exposure of epithelia to a gradient with different fluids at the luminal and basal sides (Fig. 85.3). Another culture container is very flat and is made of transparent material to allow the microscopic observation of the developing tissue (Fig. 85.4). A special container model features a flexible silicon lid (Fig. 85.5). Applying force to this lid by a rotating excenter mimics a mechanical load for developing cartilage or bone tissue.

A broad range of individual cells respectively specifically shaped tissues can be generated in a perfusion tissue engineering container, where holder characteristics and medium supply are adapted to the requirements of an auricle, cartilage or bone construct (Fig. 85.6). Finally, an artificial interstitium within a perfusion culture container can be created by the insertion of a fleece (Fig. 85.7).

All of the introduced perfusion culture containers are constructed by Computer Aided Design (CAD) and machined by Computerized Numerical Control (CNC) out of polycarbonate in a specialized work shop (Fig. 85, 86).

During fabrication the machined surfaces are treated with specific lubricants. This treatment later causes the cells to grow only within the tissue carrier on the adhesion substrate and do not to spread across the inner surface of the perfusion culture container.



Figure 86: Photographic illustration of different types of perfusion culture containers. View to perfusion culture containers for 6 (1) and 24 (2) tissue carriers with 13 mm diameter. A gradient container is available for one (3) or and six tissue carriers (4). Optical glasses are inserted for microscopic observation (5). Tissue engineering container with a 47 mm tissue carrier has specific clamps to keep the construct in position (6).

To show the flexibility in construction various types of perfusion culture containers can be machined (Fig. 86). In a simple version a perfusion culture container can hold 6 (Fig. 86.1) or 24 (Fig. 86.2) tissue carriers with 13 mm diameter. A gradient container is available for one (Fig. 86.3) or and six tissue carriers (Fig. 86.4).

Further optical glasses are inserted in a gradient container for microscopic observation (Fig. 86.5). a tissue engineering container with a 47 mm tissue carrier has specific clamps to keep the construct in position (Fig. 86.6).

**[search: perfusion culture minuth]**

**Self-study: Get informed about perfusion culture containers**

## **17.2. Transport of nutrition and respiratory gas**

Adherent cells are forming often several cell layers in combination with an extended extracellular matrix. Such relatively thick layers of tissue need a continuous provision of nutrition and respiratory gas supporting intact development and maintenance of structures. However, under the static environment within a dish this situation is often leading to unstirred layers of fluid. As a substitute for a missing vessel system transport of culture medium is made within a perfusion culture container maintaining a constant provision with nutrition.

Most favourable are perfusion culture containers, where always fresh medium is transported to the inlet, while the metabolized one is leaving at the outlet to be stored in a waste bottle. By the transport of fresh culture medium always a constant level of nutrition and oxygen can be administered. Further metabolized products are continuously eliminated so that they cannot have a harmful influence on the contained cells. At the same time paracrine cytokines supporting differentiation are kept on an always constant level. In the case biodegradable scaffolds are used, the constant transport of always fresh medium dilutes liberated and harmful molecules such as lactic or glycolic acid. In so far many arguments speak for the constant application of cells in perfusion culture.

In the demonstrated perfusion culture containers fresh culture medium is supplied (Fig. 86). The transport of medium can be made in a continuous flow or in pulses, so that unstirred layers of fluid are avoided. Applying this experimental design the waste medium is collected in separate bottles. In consequence, the medium is not re-circulated. All of these factors taken together are leading finally to controlled culture conditions. It is obvious that such conditions cannot be created under the static environment of a dish.

**[search: perfusion culture nutrition]**

**Self-study: Read about nutrition in perfusion culture**

### **17.3. Dynamic fluid environment for adherent cells**

An essential advantage of perfusion culture within a microreactor is the individual creation of an environment that mimics an adaption to the natural situation for a multitude of cells and specialized tissues. Applying this technique many cell types are developing physiological features, which could not be generated under static culture conditions.

Further it is most advantageous that the modular technique can be used to elaborate on the site of cell adhesion, the action of rheological stress or the exposure to a fluid gradient. The special needs and environmental influences which are required leading to an optimal development of functional tissues under advanced culture conditions.

As a first example the bathing of adherent cells within a perfusion culture container is illustrated (Fig. 87).

When the demonstrated perfusion culture container is working, medium is entering at the basal side to distribute at the bottom of the container (Fig. 87). During further transport the used medium is elevated and flows between the inserted tissue carriers from the base to the top. During this phase medium is contacting the adherent cells mounted on the substrate within the tissue carrier. Yet the exchange of culture medium and respiratory gas takes place.

During flow of medium the individual tissue carriers with adherent cells are constantly exposed to always fresh one. Finally, the medium is leaving the container at the level of the lid. Arising gas bubbles are continuously leaving the container during the transport.

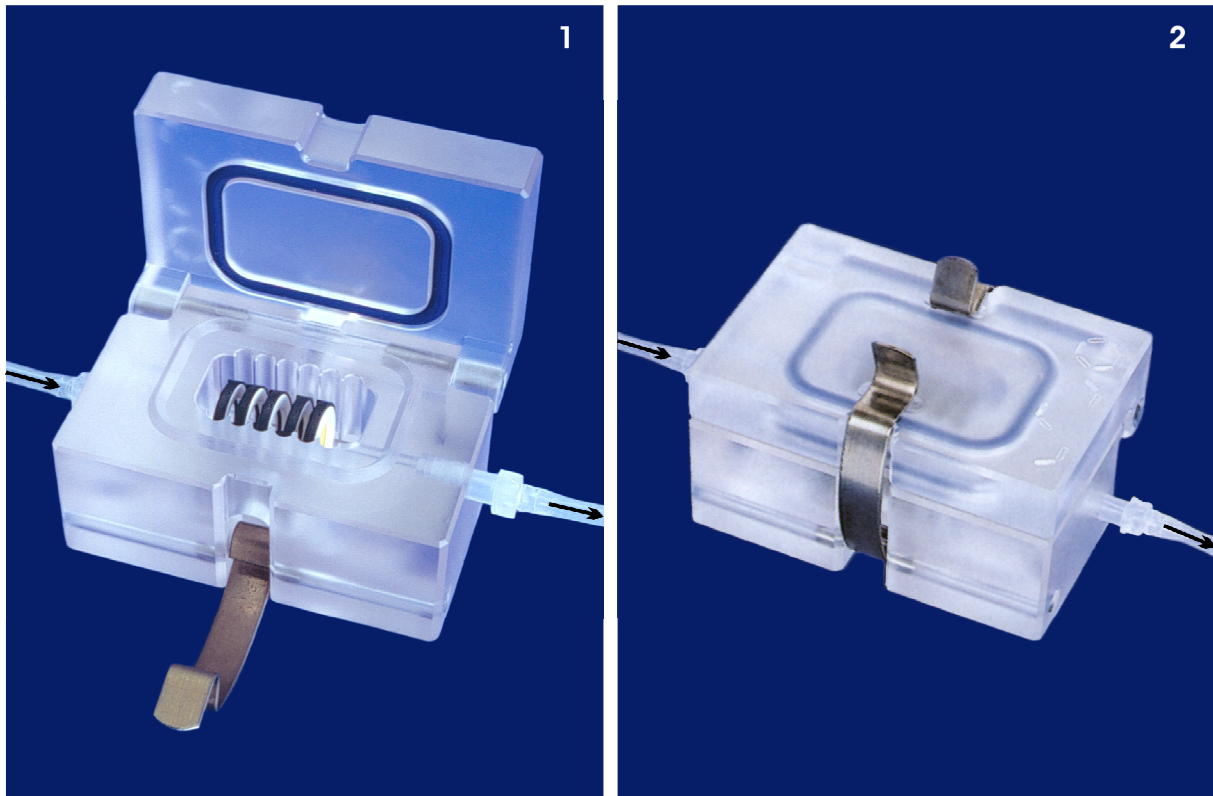


Figure 87: Illustration of a perfusion culture container for the bathing of adherent cells. After opening the lid six tissue carriers are placed in parallel into the base part of the container (1). After closing the lid medium (arrow) enters at the basal side (left), streams through the interior and leaves at the upper side (right) the container (2).

As a second example for a perfusion culture container exposure of epithelia to a fluid gradient is illustrated (Fig. 88).

In the organism epithelial cells are growing as barriers, where they are exposed to different media at the luminal and basal sides. To simulate this unique situation a gradient perfusion culture container was constructed to keep epithelial cells under such tissue-specific conditions (Fig. 88).

First of all epithelial cells have to be seeded on a suitable substrate mounted in a tissue carrier. Then the tissue carrier is transferred to a gradient perfusion culture container consisting of a base and a lid part (Fig. 88.1). When the lid is closed, the tissue carrier separates the gradient container into a luminal and basal side (Fig. 88.2).

In consequence, different media can be transported at the luminal and basal side mimicking a natural environment for epithelia. For example, the typical environment of the stomach, small intestine, gall bladder or kidney can be mimicked by using a hyper- or hypotonic medium on the luminal side, while an interstitial-like fluid is used on the basal side. In a gradient culture set-up the two media are transported at exactly the same speed.

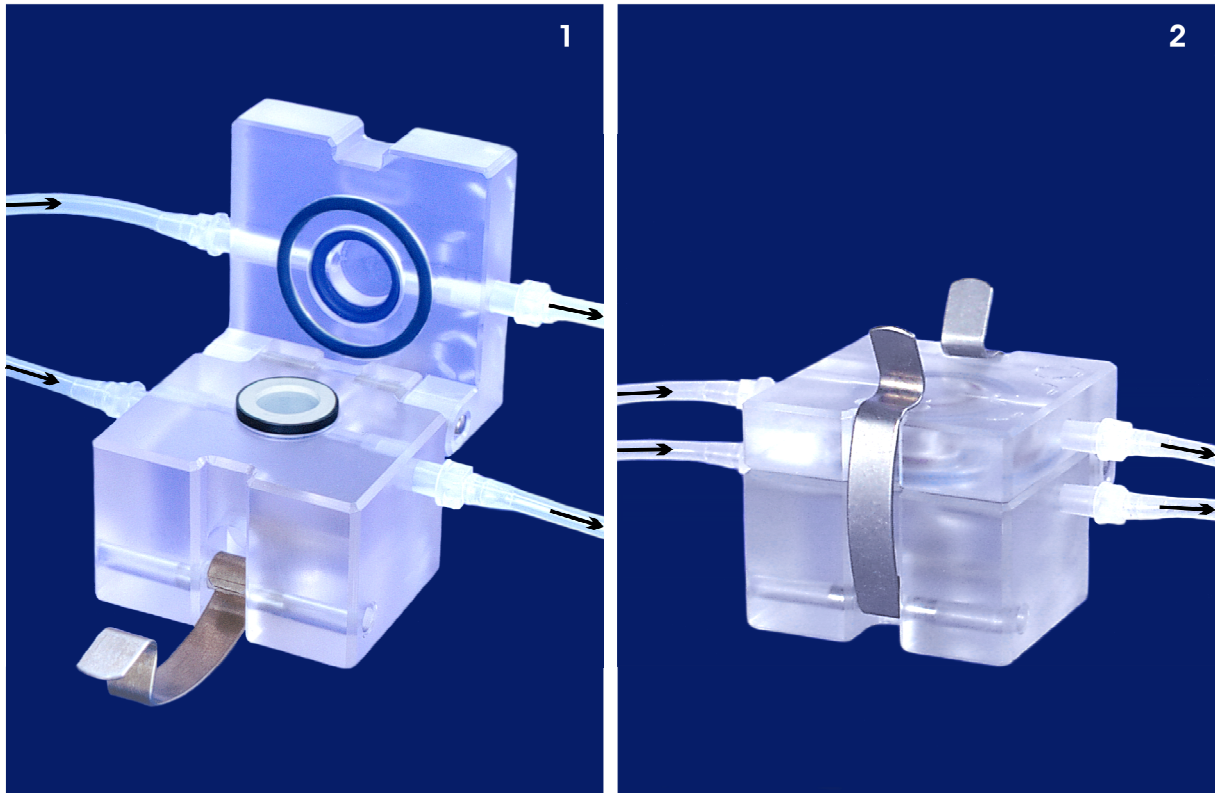


Figure 88: Illustration of a gradient perfusion culture container. After opening the lid a tissue carrier is placed at the base part of the gradient container (1). After closing the lid the tissue carrier with adherent epithelial cells separates the container into a luminal and basal compartment (2). Mimicking a natural situation different media are transported at the luminal and basal sides (arrows).

As a third example perfusion culture under microscopic observation shall be demonstrated (Fig. 89).



In many cases cells have to be analyzed by microscopic methods during perfusion culture. Specifically for such an experimental set-up a perfusion culture container for microscopic analysis was developed (Fig. 89).

It consists of a base and a lid part with inserted glasses for microscopic observation (Fig. 89.1). First a tissue carrier is placed in the basal part of the container. Then the lid is closed by a metal clamp (Fig. 89.2). Yet the tissue carrier is found exactly in the geometrical middle of the perfusion culture container for microscopic analysis. According to this specific design different media can be transported at the luminal and basal side.

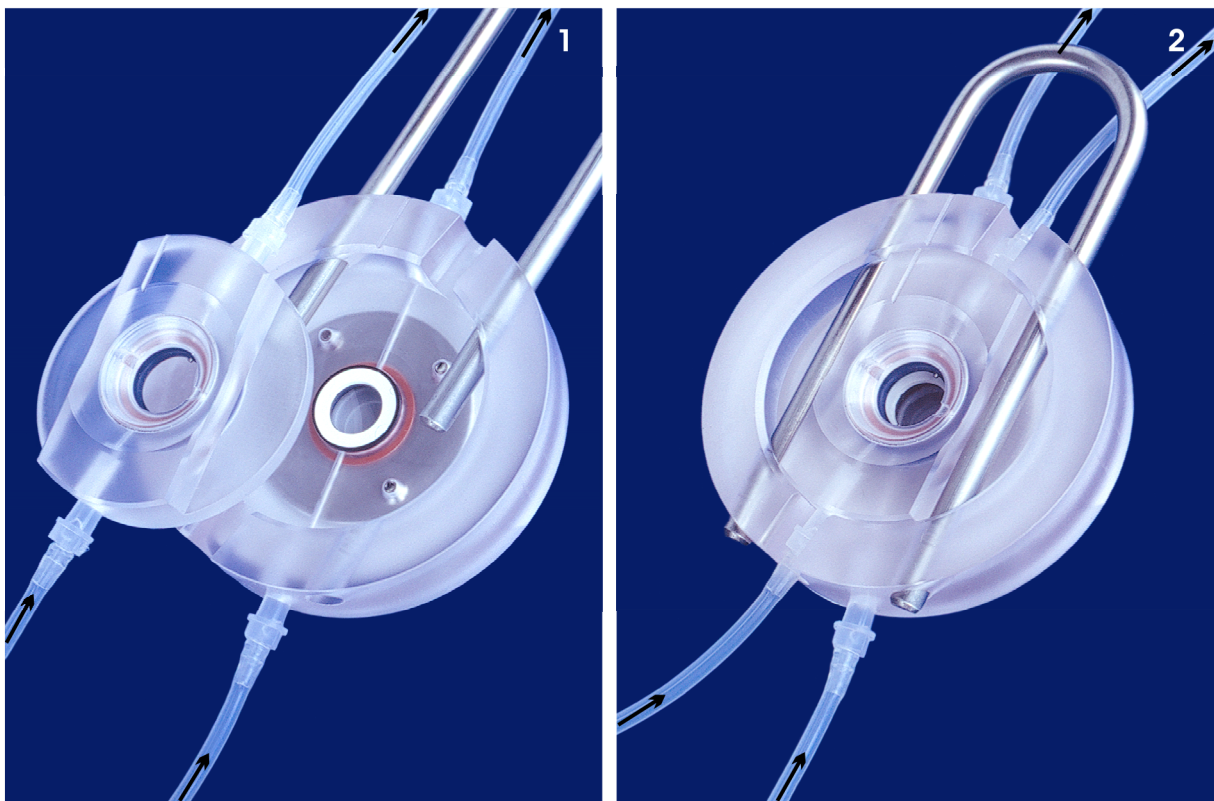


Figure 89: Illustration of a gradient perfusion container for microscopic observation with an inserted 13 mm tissue carrier. The container consists of a lid and a base part (1). After mounting a tissue carrier the lid is closed by a metal clamp (2). Medium can be transported at the luminal and basal side (arrows).

**[search: perfusion culture minuth]**

**Self-study: Inform yourself about rheological stress in perfusion culture experiments**

## 18. Performance of perfusion culture

In presently performed perfusion culture experiments cells are provided with always fresh medium. In these experiments not only the election of a suitable perfusion culture container is important, but also supplementary tools are needed and have to be carefully prepared so that excellent results can be obtained.

First of all specific tubes for the transport of medium and the exchange of respiratory gas have to be selected. Further suitable connectors, fittings, specific screw caps for storage and waste bottles, an optimal peristaltic pump and a secure thermo plate with a cover are necessary. In addition, when medium is transported, contained gas bubbles have to be eliminated by a gas expander module. Finally, in the case the content of oxygen has to be modified, a gas exchange module is needed. When these supplementary tools are connected with a perfusion culture container via tubes with two bottles, one speaks about a perfusion culture working line.

**[search: perfusion culture performance]**

**Self-study: Get informed about long term perfusion culture experiments**

### 18.1. Selecting the suitable tube for medium transport

Many and very different kinds of tubes are commercially available. However, without a really suitable tube professional perfusion culture cannot be performed. For a working line two different kinds of tubes are essential. On the one hand a tube for pumping is needed. It is mounted in the cassette of a slowly rotating peristaltic pump. On the other hand flexible tubes

are needed connecting the storage bottle, the pumping tube, the perfusion culture container and the waste bottle.

Experiments have shown that most suitable for the transport of culture medium are tubes made out of silicone. These tubes exhibit an excellent biocompatibility, do not show evaporation of harmful chemical molecules and last not least they do not show any toxic influence. The silicone material has an enormous flexibility due its Shore grading, it is chemically inert and can be autoclaved. Beside all of these positive properties silicone exhibits a further feature. It reveals an excellent permeability for respiratory gases such as oxygen (O<sub>2</sub>) and carbon dioxide (CO<sub>2</sub>). The permeability coefficient of silicone for oxygen is for example 4715 as compared to 60 in a Tygon® or 200 in a Norprene® tube. For further details see information about specifications of Masterflex® tubes.

Further silicone tubes are frequently used for the production of food and drugs, since the material is biocompatible and does not show any toxic influence. For that reason silicone is accepted by the US Food and Drug Administration (FDA), US Department of Agriculture (USDA), US Pharmacopoeia (USP), US National Sanitation Foundation (NSF) and finally by the Bundesinstitut für Risikobewertung (BfR) für biomedizinische Untersuchungen und Applikationen (Germany).

Silicone tubes fabricated for pumping are calibrated and colour coded. They can be used in a variety of peristaltic pumps and they offer the necessary exactness and reproducibility for the transport of culture medium. The tubes are cut to a defined length.

Onto a pumping tube two stoppers are glued to prevent movement within the cassette. Most suitable for perfusion cultures are tubes with an inner diameter of 1 mm and a wall thickness of 1 mm, when transport of fluid is made between 0.1 and 2 ml/h.

Quality for functional standing of the pumping tube is guaranteed by the supplier for 72 hours. However, numerous experiments revealed that a pumping tube can be normally used for a culture period of 14 days. In extreme cases it was used for 6 weeks in our laboratory. It does not need an explanation, but after a run the tubes are replaced by new ones.

Beside the tubes for pumping further ones are needed connecting storage bottle, perfusion culture container and waste bottle. To reach a high enrichment of oxygen also they are made out of silicone. Most suitable for perfusion cultures are connecting tubes with an inner diameter and a wall thickness each of 1 mm. During perfusion culture medium is transported in the presented experiments with 1 ml/h from the storage to the waste bottle. This relatively small amount of always fresh medium is enriched enough so that cells are provided with always sufficient nutrition and oxygen.

Tubes with a smaller inner diameter than 1 mm do not show recognizable advantages but exhibit disadvantages. When tubes with a smaller diameter are used, it is frequently observed that despite constant pressure in the cassettes the fluid column is not any more continuously transported.

Thus, a constant transport of culture medium cannot be expected in all of the cases during low rotation of the pump. The reason for this phenomenon may be that the fluid pressure in the tube is higher than within the pump. In the extreme case it may happen that the transport of medium stops and is replaced by a constant pulsation so that no medium is exchanged.

Regarding the law of Hagen and Poiseuille

$$R = \frac{8 \cdot l \cdot \eta}{\pi \cdot r^4}$$

the resistance of streaming ( $R$ ) in a tube depends on its length ( $l$ ), on the viscosity ( $\eta$ ) of the fluid and on the fourth potency of the tube radius ( $r^4$ ). In conclusion, a reduction of the inner diameter of a tube of only 16% leads a doubling of the pressure resistance!

Due to this specific reason during perfusion culture tubes and fittings with always the same inner diameter are used in presented experiments. It is obvious that a decrease in the inner diameter of a tube or of a fitting is always leading to a drastic increase in fluid pressure during transport of medium, which has to be avoided.

Further it should be mentioned that the medium within the storage bottle is always kept at the same height of the perfusion culture container and the waste bottle. When an equal level of fluid is not maintained, beside the capillary forces within the tubes also hydrostatic pressure differences have an influence on medium transport rates between storage bottle, perfusion culture container and waste bottle.

**[search: perfusion culture tube]**

**Self-study: Inform yourself about oxygen diffusion in silicone tubes**

## **18.2. Assembling a perfusion culture set up**

To connect the tubes with each other Luer<sup>®</sup> - fittings made out of polypropylene are used. Connections can be made via male and femal parts. Either the fittings are slightly sticked to each other or Luer<sup>®</sup> - lock - fittings are applied. The fittings have a connector nozzle with 1.6 mm in outer diameter so that a silicone tube with 1 mm inner diameter can be shoved on.

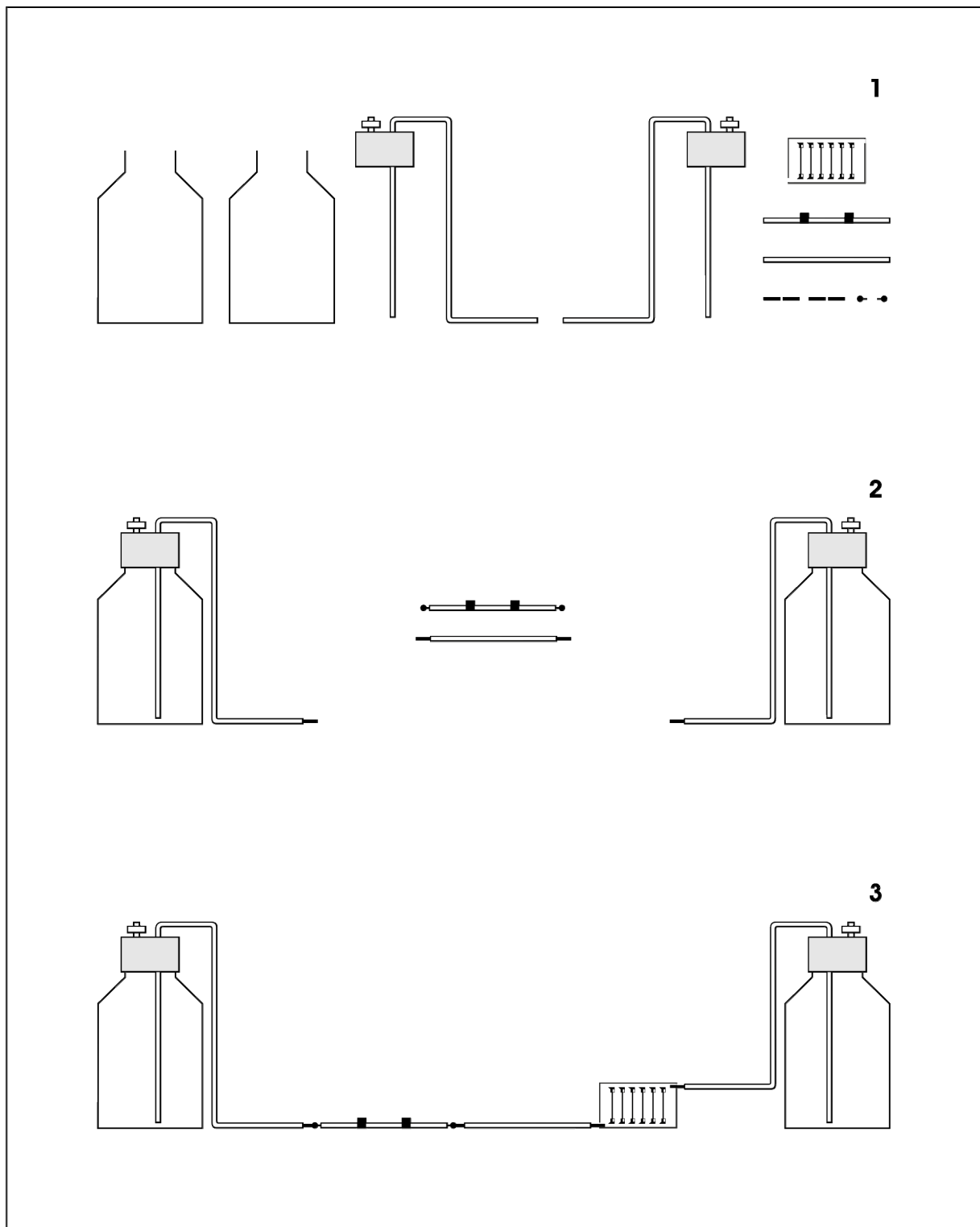


Figure 90: Assembly of a perfusion culture working line. Bottles, screw caps, a pumping tube, a connecting tube, fittings and a perfusion culture container are needed (1). First bottles are closed by screw caps (2). Then connection is made between the storage bottle, the pumping tube, the connecting tube, the perfusion culture container and the waste bottle (3).

To assemble a perfusion culture working line the following parts are needed (Fig. 90.1):

- 2 bottles
- 2 innovative screw caps
- 1 set of tubes and fittings
- 1 selected container

For mounting first the bottles are closed by the screw caps (Fig. 90.2), then the storage and waste bottles are connected with the perfusion culture container by tubes including the pumping tube (Fig. 90.3).

For each set of tubes and fittings a clamp is needed. During transport of a perfusion culture working line from the bench to the peristaltic pump on a laboratory table the clamp is closed so that medium cannot shift in an uncontrolled manner from the storage to the waste bottle.

After the perfusion culture working line is assembled, it is placed in a sterilization bag (Melafo<sup>®</sup>), a metal container or it is enveloped in a thin towel made of pure cotton. Sterilization is made in an autoclave at 105°C and 0.3 bar for 2 x 20 minutes. The absolute maximum is at 135°C and 2 bar for 10 minutes.

To avoid damage, the screw caps on the bottles are not tightly closed and the clamps of the perfusion container remain opened so that the lid is not pressed on the base part. Following this way the vapour can reach all sites of the working line during sterilization. This specific treatment prevents damage of the perfusion culture container so that it can be used for years.

**[search: perfusion culture set up]**

**Self-study: Read about performance of perfusion culture experiments**

### 18.3. Starting perfusion culture

After sterilization the perfusion culture working line is placed under a sterile bench. First of all the clamp on the silicone tube is closed to prevent uncontrolled movement of culture medium. Then culture medium is filled into the perfusion container. In the next step a selection of Minusheet<sup>®</sup> tissue carriers with adherent cells is transferred by fine forceps from a 24-well culture plate into the perfusion container. Then the lid is closed.

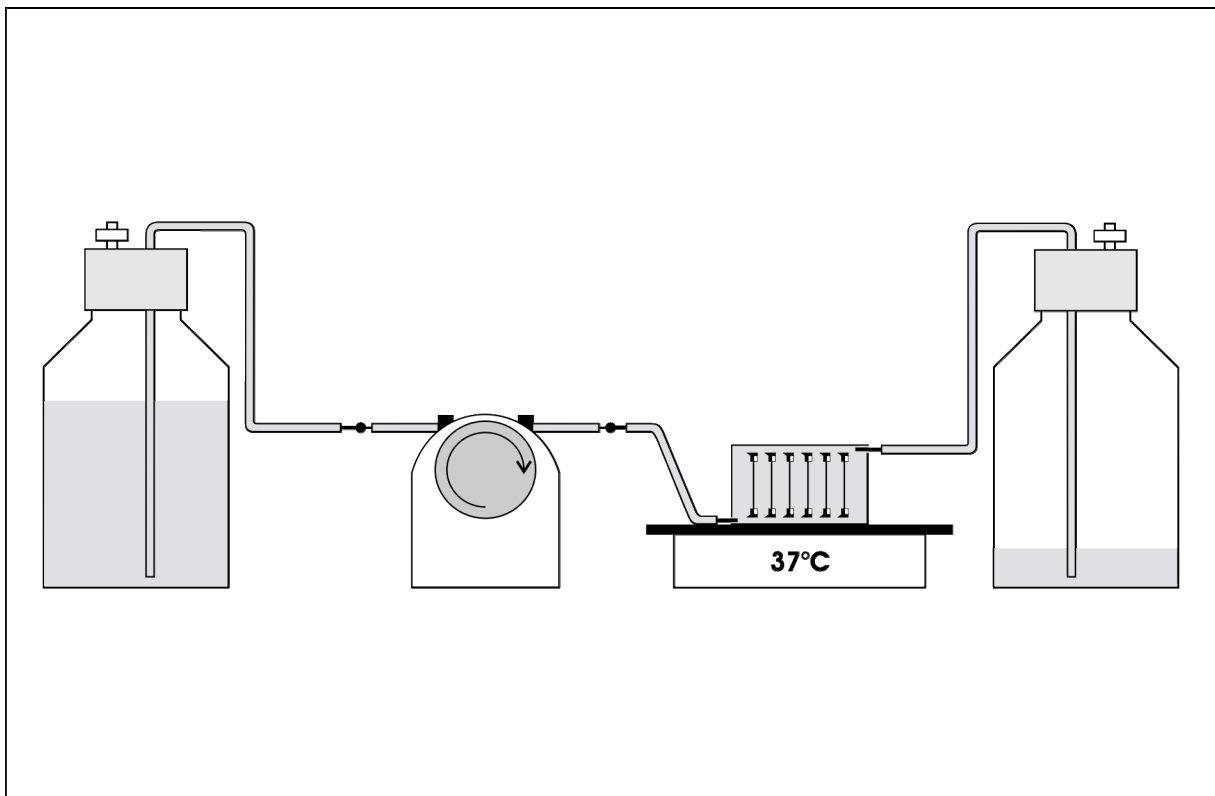


Figure 91: Starting a perfusion culture. Medium is transported by a peristaltic pump from the storage bottle (left) to a perfusion culture container placed on a thermo plate. The used culture medium is collected in a waste bottle (right).

In the next step 500 ml culture medium containing HEPES or BUFFER ALL is filled into the storage bottle. When the transport rate is 1 ml/h, this amount of medium covers the consumption of cells over a period more than 14 days. Finally the screw caps on the bottles are tightly closed. Yet the interior of the perfusion culture line is enclosed in a sterile atmosphere and separated from the unsterile atmospheric environment.



The perfusion culture working line filled with cells and medium is now placed on a tableau to be transferred to the peristaltic pump. Depending on the selected buffer system contained in the medium perfusion culture can be performed either in a CO<sub>2</sub>-incubator or under atmospheric air on a laboratory table.

In the last step the pumping tube is mounted in the cassette of the pump. Then the clamp on the silicone tube is opened. Yet the culture is started either with a continuous or a pulsating flow of always fresh medium (Fig. 91). The exchange of medium prevents unstirred layers of fluid in the interior of the elected culture container. The transport is best accomplished using a slowly rotating peristaltic pump able to deliver adjustable transportation rates of 0.1 to 1 ml per hour. Excellent experiences were made with a multichannel peristaltic pump IPC N8 (Ismatec, Wertheim, Germany). To maintain the correct temperature the perfusion culture container is placed on a thermo plate.

From the cell biological view the transition from the static environment of a dish into perfusion culture is paralleled by drastic environmental changes leading to multiple reactions of the contained cells. Under static environment the cells are kept in the most cases in a full medium containing an overshoot of serum and growth factors so that they proliferate as fast as possible. As a result a multitude of cells can be harvested within shortest time. In contrast, during perfusion culture the content of serum within the medium is reduced. As far as possible serum free or chemically defined medium is yet used. For the development of cells it means that the processes involved in proliferation are down-regulated, instead differentiation and functional development starts. These conditions have to be maintained so that functional features of specialized tissue arise as much as possible.

**[search: perfusion culture assembly]**

**Self-study: Read about tools for perfusion culture experiments**

## 19. Supplementary tools for perfusion culture

During the run of a perfusion culture experiment typically medium is drawn up from the bottom of a storage bottle by a tube to pass through the closure cap before reaching the continuing tube. In this scenario the suction of the pump has to overcome the pressure difference in elevation of fluid within the storage bottle as well as the capillary forces resulting from the inner diameter of the tube. This situation usually does not cause problems at high pump rates. At low pump rates such as 1 ml/h medium is often insufficiently aspirated from the bottle so that the fluid transport is replaced by suction of gas resulting in the formation of gas bubbles within the system. However, arise of harmful gas bubbles is contra-productive in perfusion culture, since they constipate the path of fluid and damage adherent cells and developing tissue.

### 19.1. Innovative closure for media bottles

In earlier times the culture medium was frequently transported through bottle closures that consist of various materials. Typical materials along the medium transport path within the storage bottle were a glass tube and a large diameter silicone tube connected with the interior side of a polysulfone bottle cap. Luer<sup>®</sup> connectors made from polypropylene and a small diameter silicone tube lining to the pump and the perfusion culture container.

Thus, during the start of medium transport four material transitions were given. Further, it has to be considered that the big diameter of a glass and silicone tube and the interior of the bottle closure exhibit an unnecessarily large fluid dead volume. At low transport rates of 0.1 to 1 ml / hour it can take hours for the medium to reach the culture container. Finally the construction was leading to an uncontrolled accumulation of numerous harmful gas bubbles.

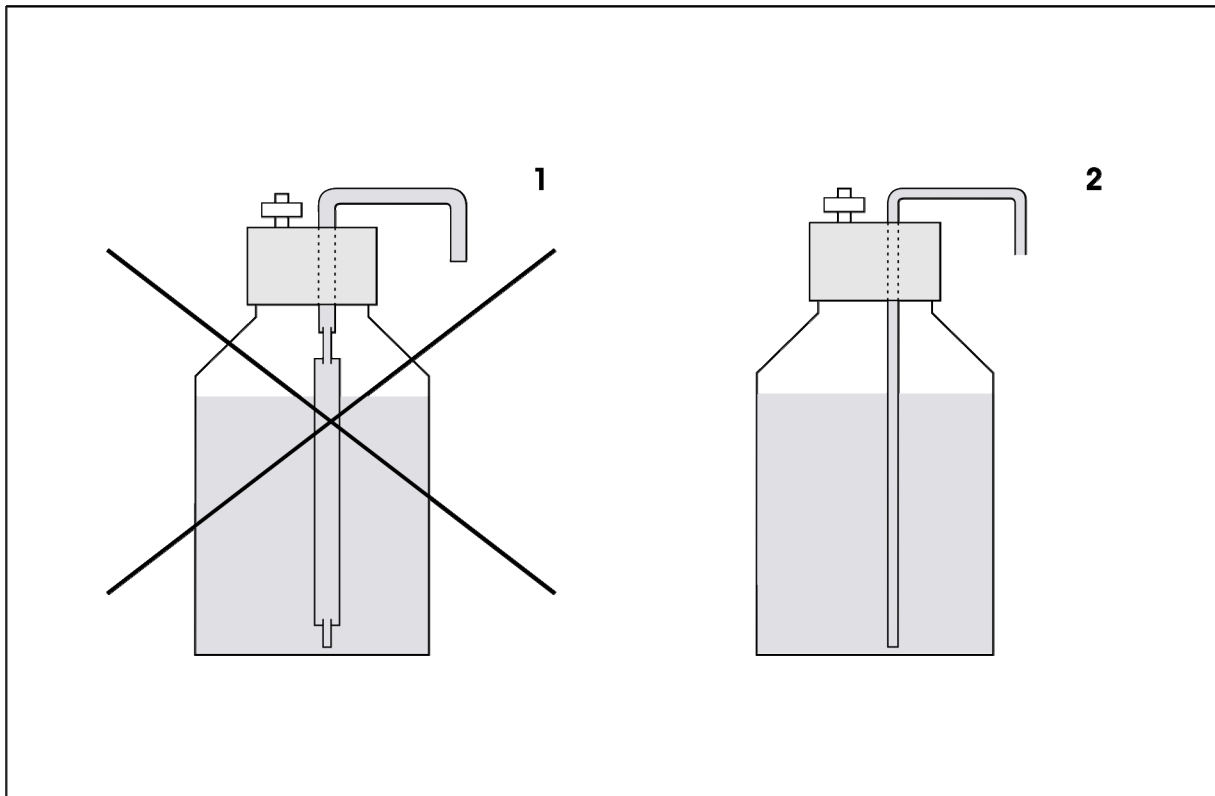


Figure 92: Schematic illustration of unsuitable (1) and suitable (2) screw caps for perfusion culture experiments. For example, typical materials along the medium transport path within the storage bottle were a glass tube and a large diameter silicone tube connected with the interior side of a polysulfone bottle cap (1). A newly developed closure for media bottles minimizes the formation of gas bubbles. A continuous piece of silicone tubing is conducted from the base of the bottle through the closure to allow medium transport without connectors (2).

Further during suction of medium in the storage bottle and during succeeding transport medium is saturated with oxygen along its passage through silicone tube to guarantee optimal supply for the developing tissue. However, the problem in perfusion cultures is that gas bubbles preferentially form at material transitions. These microscopically small gas bubbles are transported along with the culture medium, increase in size and eventually form an embolus that massively impedes the flow.

Due to all these reasons at the start of medium transport a better suction of medium must occur. Further the transport of medium has to be performed as much as possible along minimal material transitions. Thus, closures for media bottles were constructed that facilitate the suction of medium and minimize the formation of gas bubbles. The technical solution is to

use a continuous piece of silicone tube conducted through the bottle closure to allow medium transport without connectors. This construction eliminates further an unnecessarily large fluid dead volume so that formation of gas bubbles is minimized. Thus, in professional closures for media bottles the culture medium has only contact with the lumen of the conducting silicone tube.

**[search: perfusion culture tissue carrier]**

**Self-study: Get informed about transport of fluid through bottle closures**

## **19.2. Transport of culture medium**

After mounting a silicone tube into the cassette of a peristaltic pump medium can be transported from the storage bottle to the perfusion culture container. Depending on the experimental design medium is continuously transported or in pulses. Very comfortable is the use of a peristaltic pump. The advantage is that the pumping head with its rolls has only contact with the external side of the inserted tube. Thus, a direct contact with the transported culture medium is not given. Further the use of a multichannel cassette system allows to mount parallel tubes or to take it out of a single perfusion culture working line without disturbing the function of parallel running lines. Finally the pumping tube can be inserted in the cassette without the risk of infection.

Most important is the reliable transport of culture medium. It is most advantageous, when the peristaltic pump contains several channels so that experiments in parallel can be performed. Very important is that the pump contains a thickset gear box so that small amounts of culture medium in the range of 0.1 to 2 ml/h can be transported. Further the rates of transport must be variable. Successful perfusion culture experiments were performed with rates between 0,1ml and up to 10 ml/h. However, a general recommendation cannot be given. In each case

the physiological parameters within the culture set-up must be controlled by a gas and electrolyte analyzer. As indicated later the samples are measured at the inlet and outlet of a perfusion culture container. In personal experiments in the most cases a transport rate of 1 ml/h was favourable.

For example, in the presented culture set-up medium is transported with a rate of 1 ml/h by a peristaltic pump from a storage bottle to a perfusion culture container loaded with one or more Minusheet<sup>®</sup> tissue carriers including adherent cells (Fig. 93). The metabolized culture medium is collected in a waste bottle. Applying this method the cells are provided with always fresh culture medium containing the same concentration of nutrition and oxygen. In this type of culture set-up the accumulation of harmful metabolites or an overshoot of paracrine factors is avoided.

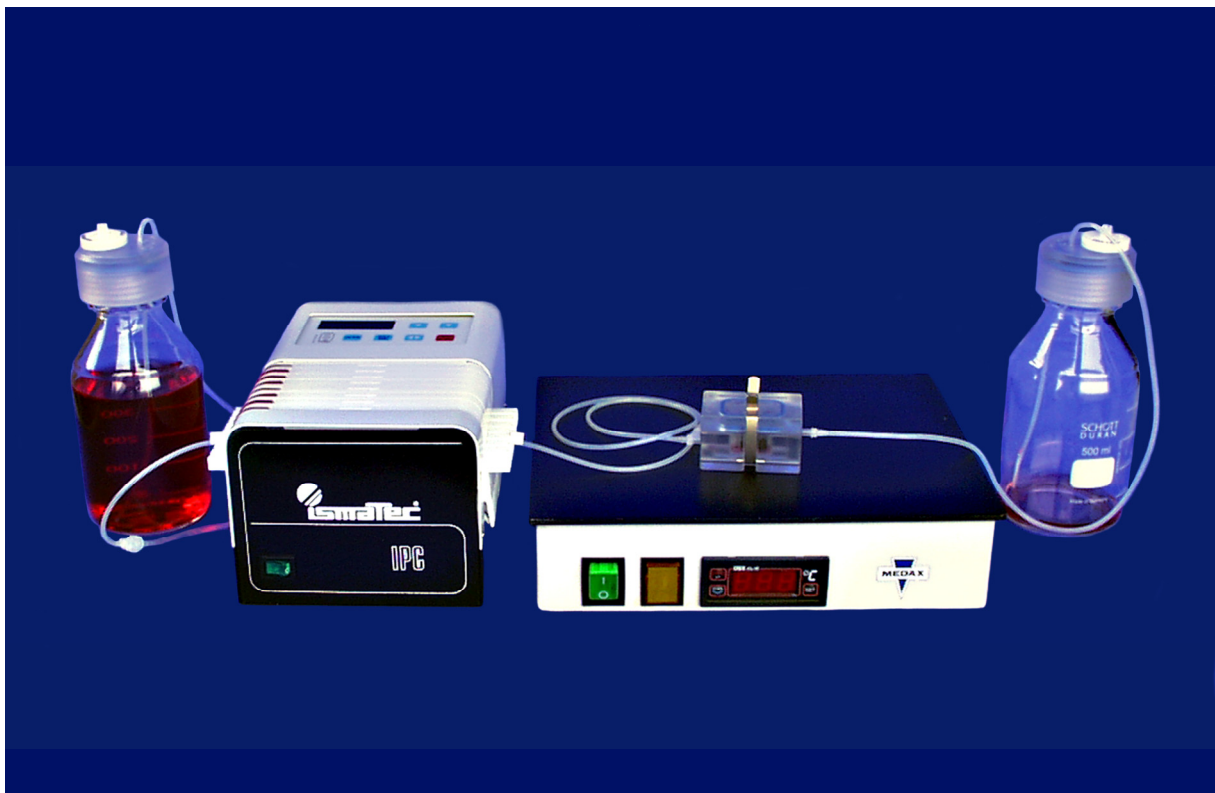


Figure 93: Illustration of perfusion culture set-up working under atmospheric air for weeks. A peristaltic pump transports medium from a storage bottle to a perfusion culture container and then to a waste bottle. A thermo plate maintains the right temperature of 37°C.

The rate of medium transport can be controlled (Fig. 94). Since sterile sensors for such low rates of fluid transport were commercially not available, registration was performed by a digital balance. In consequence, at the start of culture the waste bottle was placed on a balance. After a defined period of time the increase of mass was registered reflecting the amount of transported medium. Applying this simple principle the data exactly indicate rate transported culture medium.

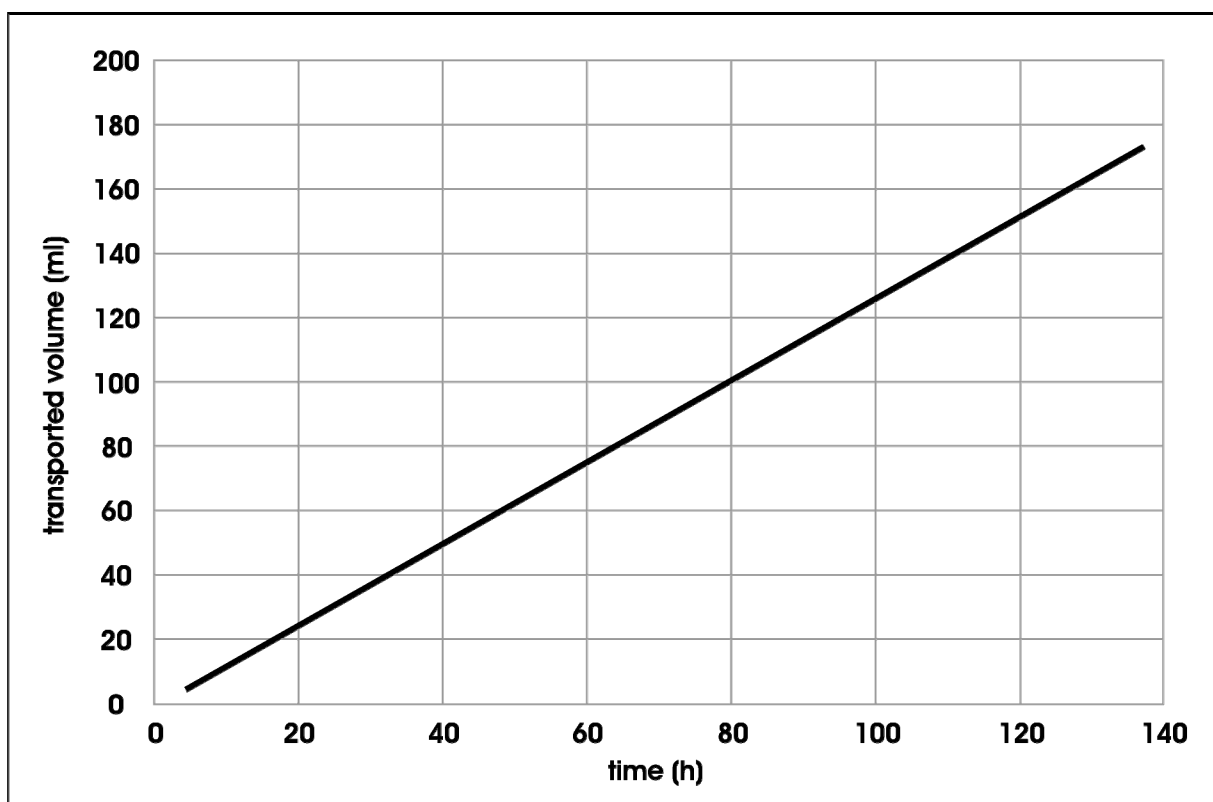


Figure 94: Schematic presentation of culture medium transported during time. A peristaltic pump is transporting medium from a storage bottle to a culture container and then to a waste bottle. The continuous increase in mass during time is registered by a digital balance for 140 hours.

The peristaltic pump should be switchable so that it can transport medium continuously or in pulses. The discontinuous mode is important, when thick layers of cells are contained within a perfusion container or when biodegradable scaffolds are used.

The up and down of fluid motion minimizes the formation of unstirred layers of fluid between cells and the interior of a perfusion culture container. During the pulse medium is transported so that cells are provided with always fresh medium, while in the phases between the pulses diffusion of nutrition and respiratory gas is facilitated.

**[search: perfusion culture medium transport]**

**Self-study: Get informed about pumps for perfusion culture experiments**

### **19.3. Maintenance of the right temperature**

Perfusion cultures have to be performed in the correct temperature environment. One possibility is to place the perfusion culture set-up into a CO<sub>2</sub>-incubator. However, more easily is to perform the experiments under atmospheric air on a laboratory table and by the help of a thermo plate including a covering lid (Fig. 93).

The used thermo plate should have a surface to wash up (Medax-Nagel, Kiel, Germany). Further it must deliver a constant temperature maintaining the cultures at 37°C. Such a thermo plate is often available in laboratories, where paraffin sections for histology have to be stretched (Fig. 95).

Finally a cover made from plexiglass minimizes influences derived from surrounding differences in temperature, sun light or dust. Perfusion cultures can be performed on a thermo plate over weeks. During run of an experiment the temperature data can be controlled.

When experiments have to be performed with specimens kept in an epicritical range of temperature, neither the introduced thermo plate nor a CO<sub>2</sub>-incubator is suitable. Yet the

exchange of temperature between environment and the housing of cells is of essential importance. In this specific case the technical solution is to plunge the perfusion culture container into a bath of warm water as it is used for example for enzyme assays.

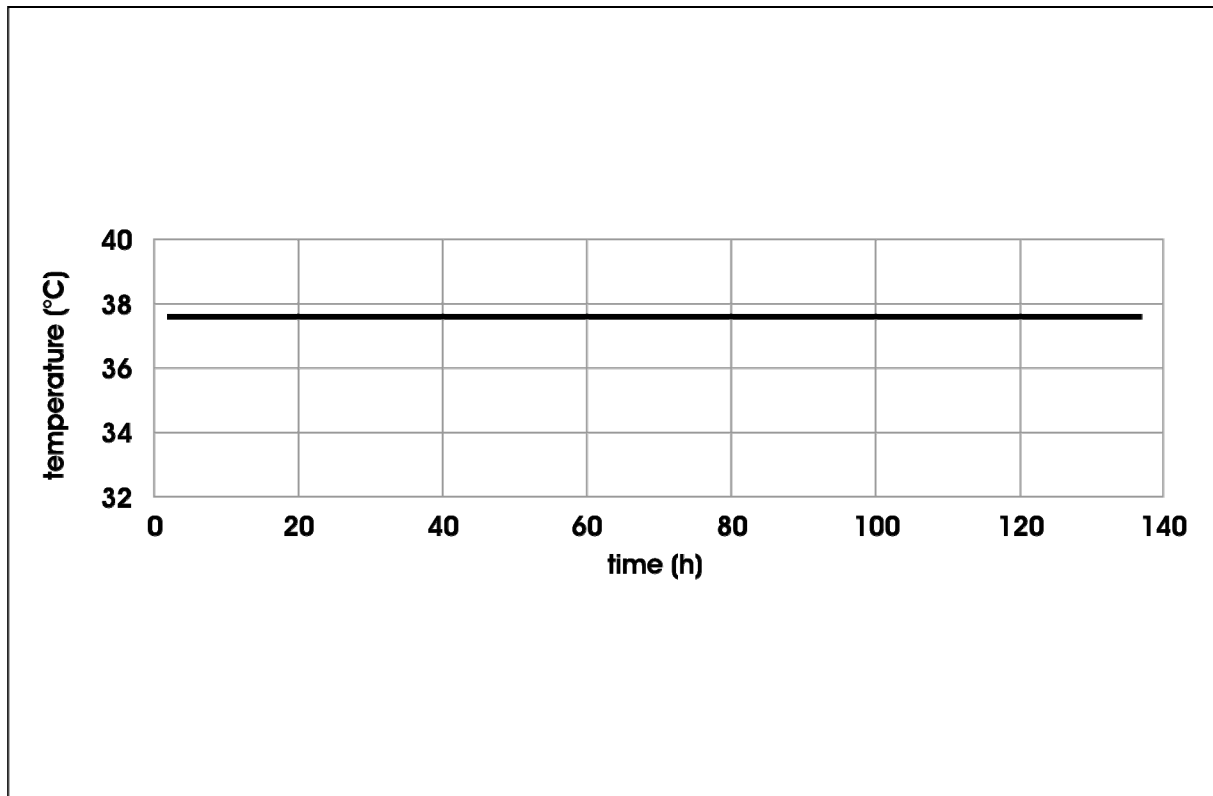


Figure 95: Schematic registration of temperature during perfusion culture. A sensor is placed on the surface of the thermo plate or inside a perfusion culture container. An example shows that the data are registered by a personal computer for 140 hours of culture. During that time a constant temperature is maintained.

**[search: perfusion culture temperature]**

**Self-study: Inform yourself about housings for perfusion culture experiments**

## 19.4. Transfer of respiratory gas

During perfusion culture cells have to be provided with sufficient oxygen to avoid cell death. Two principal techniques exist for the transfer of oxygen into the medium of a perfusion culture set-up. The one possibility is to inject the respiratory gas over a port with electronic regulation. However, during long term culture this method has several disadvantages. On the



one hand the injection of a gas is technically difficult to regulate in a narrow concentration range within a small volume of medium. On the other hand the content of gas has to be constantly measured over an integrated sensor leading frequently to bacterial contamination.

However, one aspect of oxygen injection is extremely contra-productive for perfusion culture set-ups. During injection of a gas not only the oxygen concentration increases but also the formation of numerous gas bubbles is observed. The occurrence of gas bubbles in turn prevents constant provision with culture medium and respiratory gas. Further the path of medium transport becomes obstipated by fusing gas bubbles resulting in varying hydrostatic pressure leading to unequal fluid distribution and damage of growing cells and developing tissues.

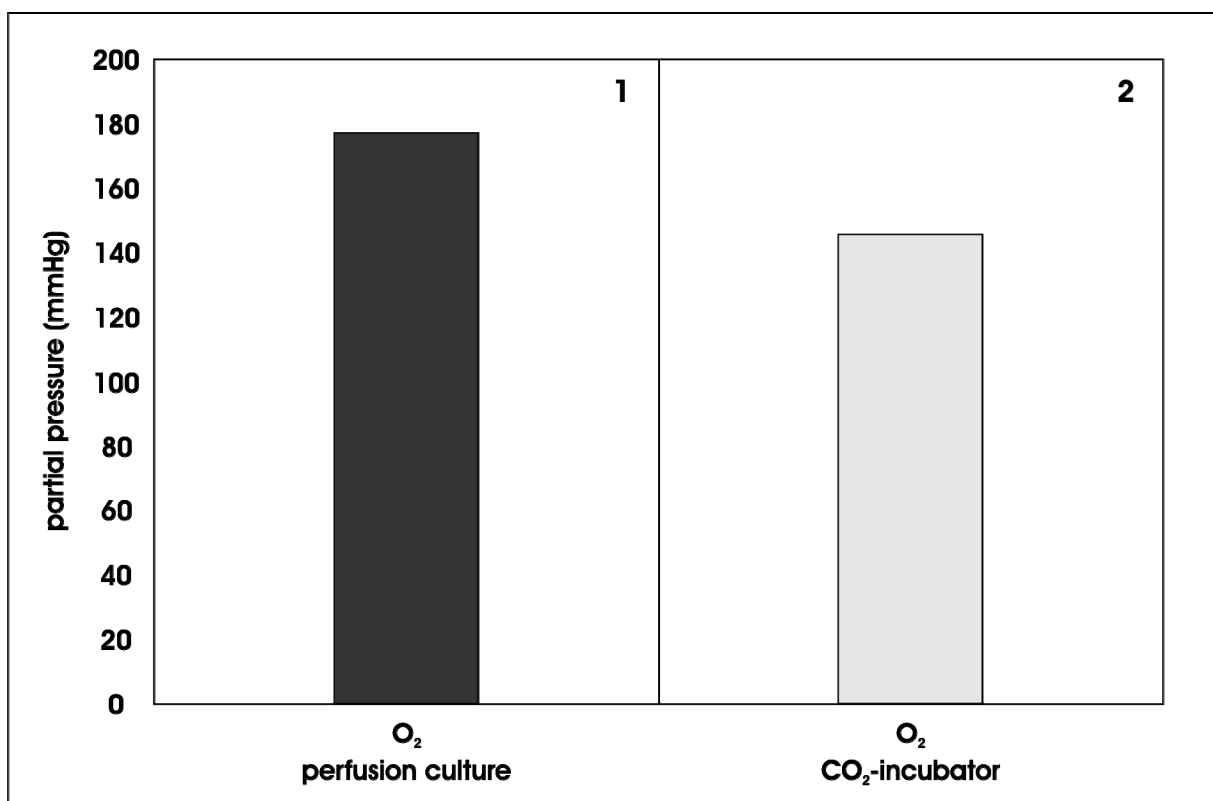


Figure 96: Determination of the oxygen (O<sub>2</sub>) content in IMDM during perfusion culture under atmospheric air (1) and exposed in a CO<sub>2</sub>-incubator (2). In perfusion culture 180 mmHg O<sub>2</sub> are measured, while the sample in the CO<sub>2</sub>-incubator reveals 150 mmHg O<sub>2</sub>.

Beside the injection of oxygen a much better method exists to enrich the culture medium with respiratory gas. As a lung for the perfusion culture set-up the gas-permeable silicone tubes can be used. In consequence, between the storage bottle and the perfusion container the length of a tube for the exchange of respiratory gas must be at least 1 meter. An optimal size for the inner diameter is 1 mm, while the thickness of the wall is also 1 mm. Applying these parameters oxygen can diffuse without limitations from the atmospheric air through the silicone wall towards the culture medium into the lumen of the tube.

For better understanding an example for the content of respiratory gas in a perfusion culture set-up is given (Fig. 96). During run of the experiment a storage bottle for medium is placed in a cooling box at 10°C. The length of the silicone tube between storage bottle and perfusion culture container is 1 meter. The silicone tube has 1 mm inner diameter and a wall thickness of 1 mm. The transport of medium is performed at a rate of 1 ml/h. During transport the medium is warmed up to 37°C before reaching the perfusion culture container.

The determination of respiratory gas in the culture medium can be easily performed. A gas and electrolyte analyzer is found in each hospital. The samples are taken on a T- piece, which is inserted at the inlet of a perfusion culture container. At this port for example a 200 µl aliquot is drawn into a syringe. To prevent a loss of contained gas, the specimen must be transferred within seconds to the needle of a gas respectively electrolyte analyzer. After about two minutes the analyzed data can be read out. Beside the content of O<sub>2</sub> and CO<sub>2</sub> also the pH, the concentration of important electrolytes, glucose and lactate in the medium can be determined.

Using IMDM at a pH of 7.4 in the specimen an astonishingly high value of 180 mmHg O<sub>2</sub> is measured. The example shows that a rather high enrichment of oxygen takes place by equilibration against atmospheric air in transported culture medium between storage bottle

and perfusion culture container (Fig. 96.1). For comparison, in a sample within a CO<sub>2</sub>-incubator only a concentration of 150 mmHg O<sub>2</sub> is detected (Fig. 96.2).

**[search: perfusion culture gas content]**

**Self-study: Read about metabolic parameters in perfusion culture experiments**

## **19.5. Modulation of respiratory gas content**

Optimal oxygen supply is one of the basic parameters for an optimal development of cells and tissues. It is surprising that in a CO<sub>2</sub>- incubator (95% air and 5% CO<sub>2</sub>) an partial pressure of 150 mmHg O<sub>2</sub> is found testing IMDM culture medium. In contrast, when IMDM (3024 mg/l NaHCO<sub>3</sub>, 75 mmol/l HEPES) is equilibrated against atmospheric air in a perfusion culture set-up, partial pressure of 180 mmHg O<sub>2</sub> is detected (Fig. 96). Compared to static cultures in a CO<sub>2</sub>-incubator the oxygen partial pressure in the dynamic culture medium is considerably higher. In so far the used gas-permeable silicone tubes provide an optimal surface for the exchange of gas by diffusion due to the small inner diameter of the tubes (1 mm) and the extended length (1 m).

However, not all of the cells and specialized tissues have the same oxygen requirements. For that reason it is important that the respiratory gas content can be individually adapted in perfusion culture. As previously described a popular method for medium oxygenation is to blow a pressurized gas mixture into the storage bottle. However, the disadvantage of this method is the formation of gas bubbles in the transported medium. Thus, the technical dilemma is to obtain maximum respectively adapted oxygen saturation while avoiding gas bubble formation.

To avoid arise of gas bubbles during transport of medium, a housing for a long thin-walled silicon tube was developed (Fig. 97). The tube is wound up into a spiral inside the gas exchanger module which features a gas inlet and outlet. The tube for the exchange is highly gas-permeable so that it guarantees optimal diffusion of gases between culture medium and surrounding atmosphere. The desired atmosphere within the gas exchanger module is maintained by a constant flow of a specific gas-mixture through the module. This way the content of oxygen, carbon dioxide or any other gas can be modulated in the medium by diffusion. This technique makes it possible to adjust the gas partial pressures within the medium under absolutely sterile conditions and avoiding at the same time arise of gas bubbles. By maintaining a defined carbon dioxide concentration in the medium this method can also be employed to control medium pH via bicarbonate or a further biological buffer system.

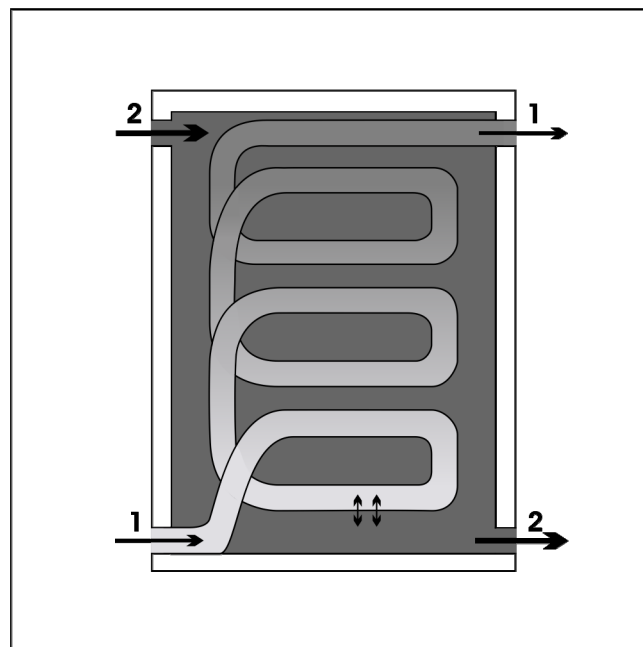


Figure 97: Schematic view of a gas exchanger module to adjust the content of respiratory gases in a medium used in perfusion culture. The module consists of a container including a long thin-walled gas permeable silicone tube for medium to pass through (1). The desired gas atmosphere within the exchanger is maintained by a constant flow of a specific gas mixture (2). Equilibration of the culture medium occurs through the highly gas permeable tube inside the module.

To investigate the influence of different respiratory gas concentrations on cell and tissue development a series of experiments was performed. For example, embryonic renal collecting duct cells were cultured on a collagen support mounted in a tissue carrier. During perfusion culture in a container the developing tissue was exposed to altered oxygen concentrations.

In consequence, during the transport through a gas permeable silicone tube within a gas exchange module the medium was exposed to atmospheric air (controls) or to a gas mixture with decreased oxygen contents. Equilibration of the culture medium with atmospheric air showed a partial pressure of 190 mm Hg O<sub>2</sub>, while reduction led to 155 mm Hg O<sub>2</sub>, and absence of oxygen in the gas mix resulted in a partial pressure of 43 mm Hg O<sub>2</sub> in the medium.

The morphological analysis of the epithelia after a culture period of 14 days showed that independent from oxygen content in all of the cases a perfectly polarized epithelium was developed. Also immunohistochemical analysis revealed that in all series a typical cytokeratin 19 (CK-19) expression was found. However, reduction of oxygen increased cyclooxygenase (COX-2) but decreased Na/K-ATPase expression as a stress response. In control series with high oxygen content COX-2 expression was low, while Na/K-ATPase was high.

The results clearly indicate that oxygen in the demonstrated concentration range has no influence on the morphological development of a polarized CD epithelium. However, the up-regulation of COX-2 and the down-regulation of Na/K-ATPase is due to an influence of a low concentration of available oxygen.

**[search: perfusion culture respiratory gases]**

**Self-study: Get informed about oxygen consumption in perfusion culture experiments**

## 19.6. Elimination of harmful gas bubbles

When culture medium is transported in silicone tubes by a peristaltic pump, it is always observed that gas bubbles accumulate within time on unpredictable sites. First the bubbles arise in a microscopic small dimension then they fuse to become larger. When the gas bubbles are small, the medium can flow without recognizable resistance. However, when they increase in size, a fluid pressure is built up affecting the flow and distribution of medium hampering provision with nutrition. Finally the pressure becomes so strong that it leads to an uncontrolled translocation of the gas bubbles.

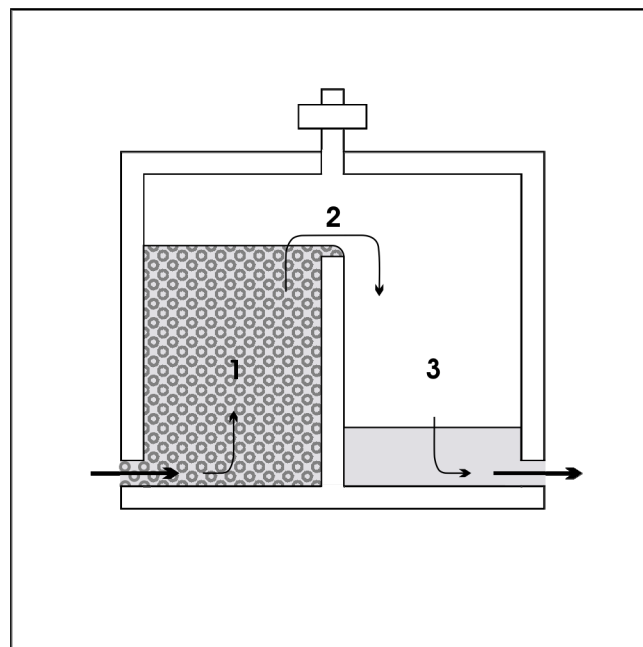


Figure 98: Schematic illustration of a gas expander module removing arising bubbles from the culture medium. Medium is pumped to the inlet of container to reach a small reservoir (1). Then the medium has to cross a barrier (2). Here the medium will expand to separate gas bubbles from the fluid but without losing dissolved oxygen. At the outlet of the container medium is lacking gas bubbles (3).

Since the bubbles do not remain at a specific site but are found after a while at other places, the areas within a perfusion culture set-up are exposed to inconstant fluid flow. Further sites with adherent gas bubbles are not exposed to a constant provision with nutrition and oxygen. Finally, the fusion of them occurs in an explosive manner leading to the damage of

neighbouring cells. It is obvious that due to all these reasons arise of gas bubbles in perfusion culture set-ups has to be avoided.

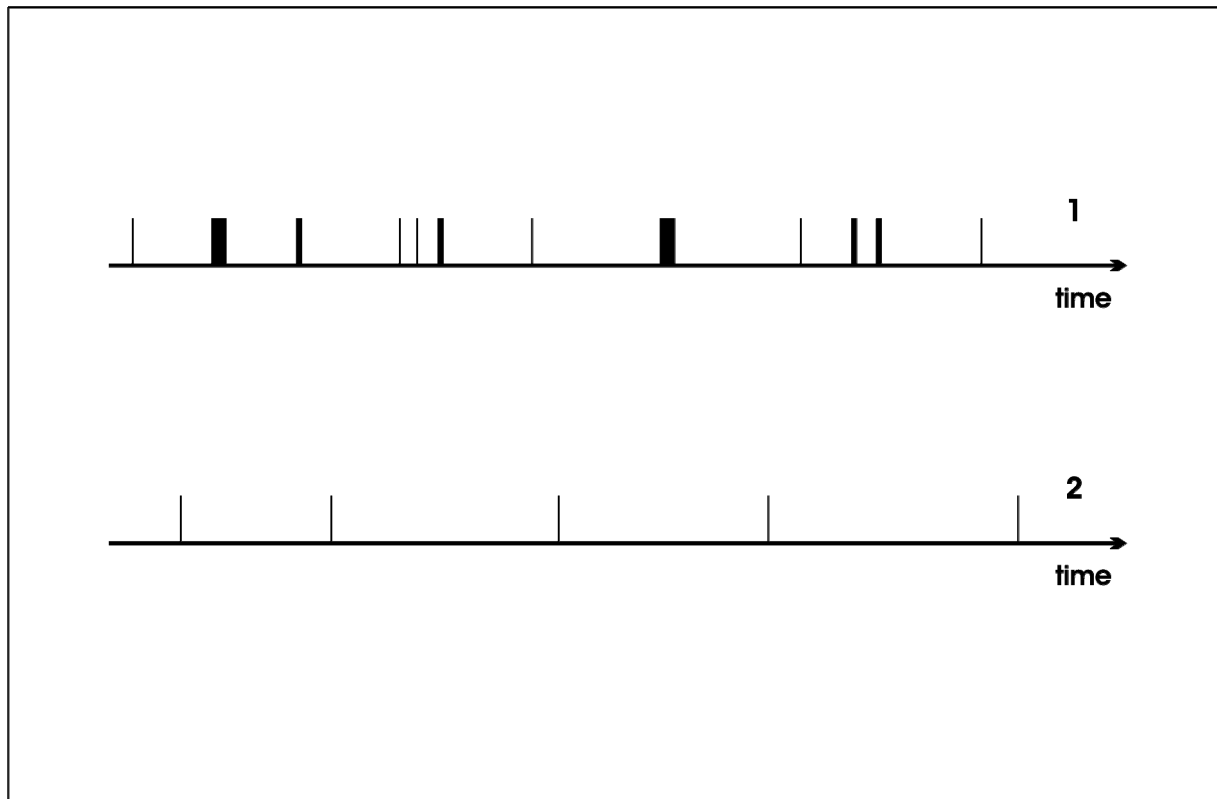


Figure 99: Schematic registration of gas bubbles by a gate control sensor over time. During transport of medium normally numerous gas bubbles are found to be transported with medium (1). Applying a gas expander module the size and amount of gas bubbles is drastically reduced avoiding at the same time the reduction of solved oxygen (2).

Consequently, to transport oxygen-rich culture medium but avoiding the presence of gas bubbles a specific module was developed (Fig. 98). The gas expander module removes bubbles during transport of the culture medium. During operation medium is transported to the inlet of the gas expander module to reach a small reservoir. Then it has to cross a barrier. At this site the medium will expand to separate gas bubbles from the fluid phase but without losing the solved oxygen. After the barrier culture medium is transported to the outlet of the gas expander module. Following this process the medium is oxygen-saturated but free of gas bubbles. The gas expander module itself is ventilated through a port at the top.

Gas bubbles in a perfusion culture set-up can be detected by a sensor, which is placed on a 1 mm inner diameter glass capillary at the inlet port of a container. Floating gas bubbles were registered by an infrared (IR) gate sensor (Conrad Electronics, Wernberg, Germany). Registered impulses were then logged by a personal computer. Performing the transport of medium in combination with a gas expander module demonstrated that arise of gas bubbles could be drastically reduced as compared to the transport without such a module (Fig. 99). Most important is in this regard that reduction of gas bubbles by the described method does not affect the content of oxygen solved in the medium.

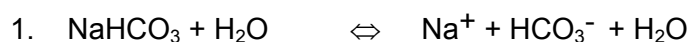
**[search: perfusion culture gas bubbles]**

**Self-study: Read about gas bubbles in perfusion culture set-ups**

## 19.7. Maintenance of a constant pH in the medium

Beside a constant provision with nutrition and oxygen in perfusion culture set-ups a constant pH must be maintained. In the organism the pH is kept to high degree in the range between 7.2 und 7.4 by the solved  $\text{CO}_2$  and the available  $\text{NaHCO}_3$ . Also within a culture medium the concept of buffering is adapted in most of the cases to the naturally occurring sodium-hydrogencarbonate system consisting of  $\text{NaHCO}_3$  and  $\text{CO}_2$ .

In a culture medium  $\text{NaHCO}_3$  is dissociating in two steps:





However, this reaction depends on the CO<sub>2</sub>-partial pressure of the surrounding atmospheric air. At a low CO<sub>2</sub>-partial pressure the culture medium contains more OH<sup>-</sup> ions revealing therefore a more basic pH. To reach a physiological pH of 7.4, in the incubator CO<sub>2</sub> has to be injected so that the desired value is obtained. When the injected gas concentration is lowering, the pH will shift again to a more basic value. In turn, again CO<sub>2</sub> has to be injected. In consequence, this repeated pulsating injection of CO<sub>2</sub>-gas leads to the stabilization of pH within media kept in an incubator.

Thus, when in an incubator a 5% CO<sub>2</sub>-concentration is offered, then for each culture medium an adequate amount of NaHCO<sub>3</sub> must be contained. For example, when only 4% CO<sub>2</sub> are supplied, less NaHCO<sub>3</sub> is added to the medium to maintain a constant pH of 7.4. When 6% CO<sub>2</sub> are offered in the incubator, more NaHCO<sub>3</sub> has to be given to the medium. For that reason the supplier of a culture medium is always indicating the necessary amount of NaHCO<sub>3</sub> and the related CO<sub>2</sub>-concentration within an incubator to reach a constant pH.

When perfusion cultures are running not in a CO<sub>2</sub>-incubator but on a laboratory table under atmospheric air, a constant pH can be easily reached. A necessary presupposition is that for the transport of medium gas-permeable silicone tubes are used. In contrast to a CO<sub>2</sub>-incubator in atmospheric air always a constant concentration of CO<sub>2</sub> is available. While in an incubator for example 5% CO<sub>2</sub> is maintained by injection, in atmospheric air only 0.3% CO<sub>2</sub> is contained.

The experimental consequence is easily to recognize. When a culture medium normally used in a CO<sub>2</sub>-incubator is standing for a while under the bench, it can be observed that the phenolred indicator alters to lilac colour indicating a shift in pH into the alkaline area. This reaction is due to the high content of NaHCO<sub>3</sub> within the medium and minor concentration of solved CO<sub>2</sub> (0.3%) in the medium and atmospheric air.

In consequence, to maintain in perfusion culture under atmospheric air a constant pH between 7.2 and 7.4, the concentration of  $\text{NaHCO}_3$  in the medium has to be decreased.

However, alone by a decreased concentration a constant pH over a long period of time cannot be obtained. Therefore for the stabilization of the pH an additional  $\text{CO}_2$ -independent buffer has to be added. Excellent experiences were made by the use of biological buffers such as HEPES or Buffer All (Fig. 100).



Figure 100: Correction of the pH of a medium used in perfusion culture under atmospheric air. In each well 1 ml culture medium of a 24-well plate is pipetted. Then aliquots with increasing concentrations (0.5 - 2.5%) of Buffer All are added. Then the samples were equilibrated under atmospheric air at 37° C over night. The result shows that colour of the indicator does not correspond to the correct pH. The correct pH has to be determined by measurement with an electrode. According to the result 1.25% HEPES has to be added so that a correct pH of 7.4 is maintained under atmospheric air over prolonged periods of time.

In any case the maintenance of a correct pH during perfusion culture under atmospheric air is essential for the outcome of a successful experiment (Fig. 100). For each administered

medium the correction of the pH has to be made in the described way. To avoid the addition of extensive concentrations of biological buffer, medium with the lowest concentration of  $\text{NaHCO}_3$  has to be ordered, when perfusion culture experiments under atmospheric air are performed.

It is very easy to adapt the buffer system of a medium kept under atmospheric air. First of all a culture medium with low  $\text{NaHCO}_3$  content is ordered. Then a titration with the medium is made by pipetting 1 ml in each well of a 24-well culture plate. After this step to each of the aliquots an increasing amount of biological buffer such as HEPES or Buffer All is added.

Then the plate is incubated over night at  $37^\circ \text{C}$  on a thermo plate and under atmospheric air. The next morning the pH in each well is measured by an electrode.

The results will demonstrate that phenol red is in the range between pH 7.2 and 7.4 an inaccurate indicator. For this reason the pH of the medium must not be estimated according the colour tone but has to be determined by an electrode to avoid misinterpretations.

In consequence, the measured pH in an individual well of the culture plate clearly informs about the concentration of biological buffer, which has to be added to medium for perfusion culture. Adding the correct amount of biological buffer, a pH in the range between 7.2 and 7.4 can be maintained for perfusion cultures under atmospheric air over prolonged periods of time. In the presented equilibration 1.25% HEPES has to be added (Fig.100).

**[search: perfusion culture control pH medium]**

**Self-study: Inform yourself about correction of pH in perfusion culture experiments**

## 19.8. On-line monitoring of perfusion cultures

Principally perfusion cultures can be performed without registration of physiological parameters (Fig. 101.1). However, in many cases it is of interest, in which physiological environment cells and tissue are contained during perfusion culture.

To obtain information about these parameters, invasive and non-invasive measurements with sensors can be performed. One possibility is to insert sensors into the tube system or into the perfusion culture container so that continuous registration of parameters via a personal computer becomes possible (Fig. 101.2).

A further possibility for the registration of metabolic parameters is the insight via T-pieces. Such pieces can be inserted before the inlet or at the outlet of a perfusion culture container. On the one hand the T-pieces can be used to harvest an aliquot of medium by a syringe for the measurement in a gas and electrolyte analyzer. On the other hand T-pieces can be used to infuse for a short period of time a growth factor or a drug. During that time the provision with culture medium is not interrupted (Fig. 101.3).

The use of T-pieces in the tube system exhibits advantages in comparison with integrated sensors. Personal experiments revealed that integrated sensors or electrodes undergo in most of the cases corrosion, biofouling or an overgrowth with fibroblasts during extended periods of culture time.

In contrast, inserted T-pieces made it possible to take at any time under sterile conditions and without direct contact to cells a sample for the measurement of gas, electrolyte or other physiological parameters.

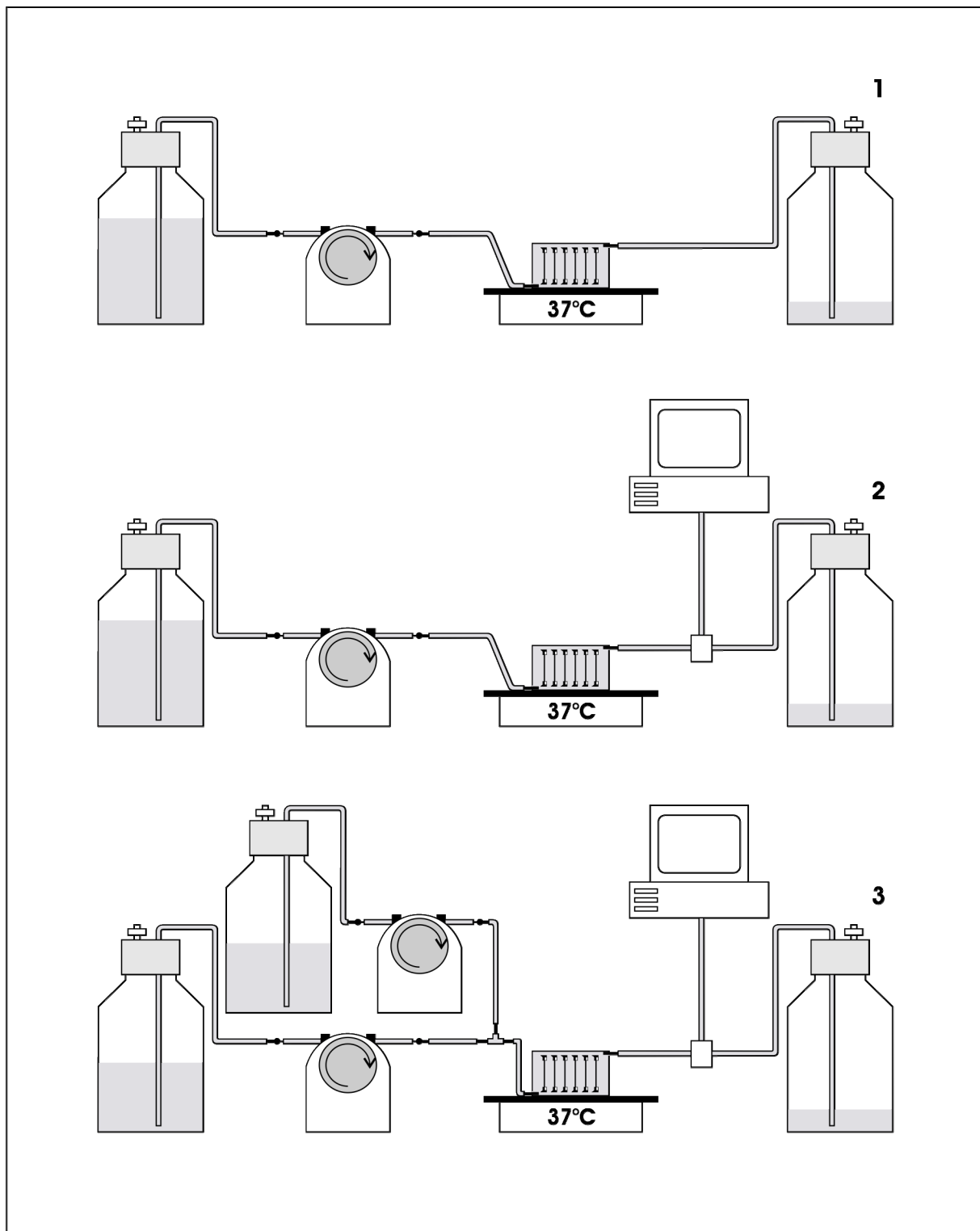


Figure 101: Registration of physiological parameters during run of perfusion culture. Principally perfusion culture can be performed without registration of physiological parameters (1). However, by insertion of a sensor into the tube system measurements via a personal computer can be performed (2). During run of perfusion culture the mounting of a T-piece makes it possible to infuse growth factors, hormones or drugs over a short period of time (3).

With a 100 µl sample a multitude of different measurements can be simultaneously performed with a gas and electrolyte analyzer. For example, measurements show that the content of glucose is high (1760 mg/l) at the inlet, but low in the vicinity of growing tissue (1100 mg/l) and increased (1550 mg/l) at the outlet of the container.

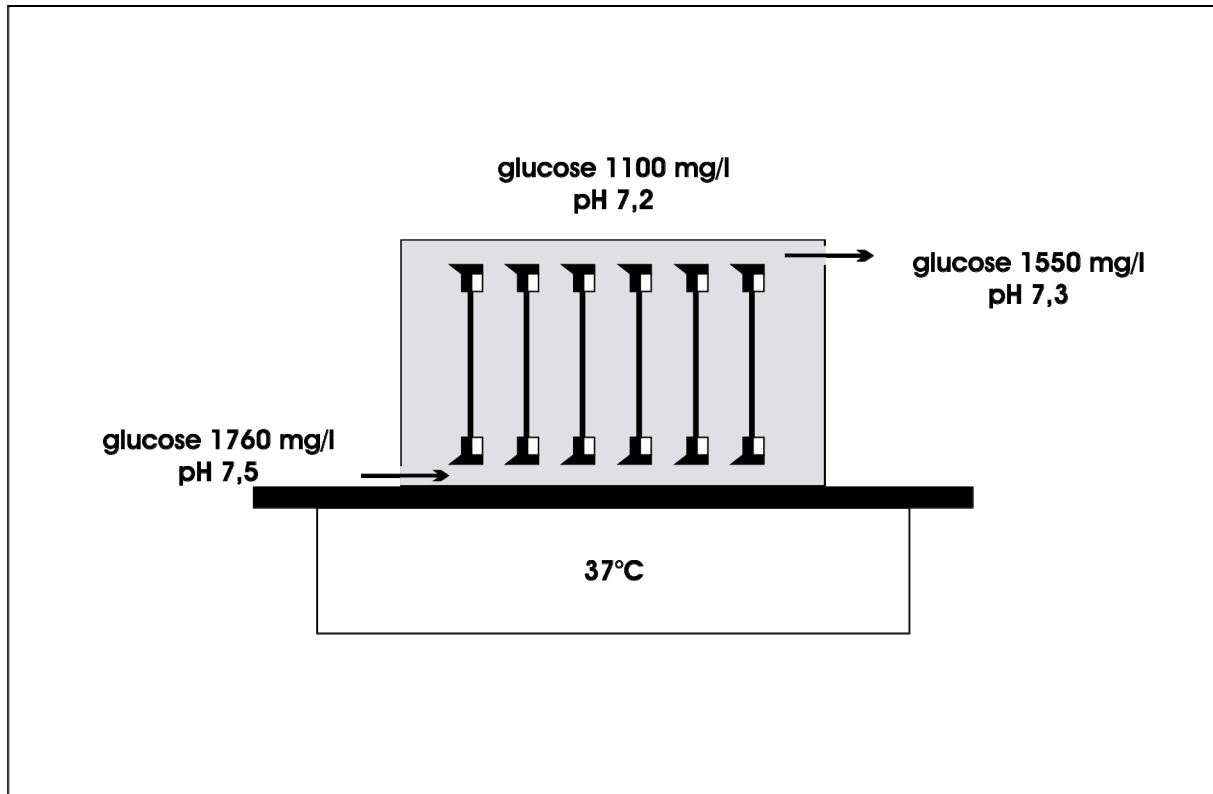


Figure 102: Determination of metabolic factors during run of perfusion culture. Either aliquots of medium can be taken or sensors can be placed at the inlet, in the interior of a perfusion culture container or at the outlet. For example, measurements show that the content of glucose is high (1760 mg/l) at the inlet, low in the vicinity of growing tissue (1100 mg/l) and again increased at the outlet (1550 mg/l).

The low pH at the site of tissue may be caused by the degradation of a scaffold containing poly-lactide. When the low pH at the site of tissue will further decrease in culture due to increasing concentrations of liberated lactate or due to cellular metabolism, the rate of medium transport has to be carefully observed. To adapt the rate of medium transport has to be slightly increased. In each case further measures of the actual physiological environment have to be performed.

**[search: perfusion culture on line monitoring]**

**Self-study: Get informed about nutrition in perfusion culture experiments**

## 20. Selection of a suitable culture medium

Beside tissue carriers and microreactors for the performance of perfusion culture experiments a suitable culture medium is needed.

Since decades culture media are commercially offered by a variety of companies. At best the selected medium for perfusion culture is ordered in a bottle containing 500 ml fluid. When the transport is performed with 1 ml/h during run of the experiment, this amount of medium provides cultures with nutrition over more than 14 days.

During delivery and storage the culture media must be kept in a bottle fridge. However, when they are kept at a too low temperature, precipitation of chemicals will take place. To avoid decomposition of contained molecules by natural or UV-light, bottles filled with medium are stored in the dark.

An ordered culture medium is filled either in a glass bottle or in a bottle made out of polycarbonate. The closure consists out of a perfectly sealing screw cap. A seal guarantees sterility, originality and quality. In this form the media can be used at once. Performing culture with adherent cells in a CO<sub>2</sub>- incubator (5 – 7.5 % CO<sub>2</sub>), an adequate concentration of NaHCO<sub>3</sub> must be contained. However, when the cultures are running under atmospheric air on a laboratory table, the buffer system has to contain only a minimal amount of NaHCO<sub>3</sub> due to the low content of CO<sub>2</sub> (0.3 %) in the air. Most important, in this specific case a suitable biological buffer, further glutamine and other additives such as hormones or growth factors have to be added to the culture medium before the start of the experiment.

Often media are offered in a tenfold (10x) concentration. Before the media can be used, an adequate amount of distilled water and a suitable buffer have to be added. In a very simple way media can be prepared from the pre-packed powder. First the powder is transferred to a measuring cylinder, then sterile water is filled in so that the meniscus does not reach the 1l

mark. After solving the powder by stirring the pH of the solution is measured and if necessary a correction is made. Finally water is filled up to the 1 l mark. Applying concentrated media and powder media, the quality of distilled water must always be perfect.

**[search: cell culture medium]**

**Self-study: Inform yourself about the selection of a suitable culture medium**

## **20.1. View to the variety of culture media**

Many of the presently used media were developed in the years between 1950 and 1970. In these earlier times the aim for culture experiments was to apply media so that the cells can proliferate as fast as possible. Following this concept it was unimportant, which differentiated features were expressed in the cultures. Development of tissue specific characteristics or functional development of organs was not so much in the focus of research at that time.

Main ingredients for a culture medium are inorganic salts, nucleic acids, amino acids, vitamins and further other more or less important substances. Due to the contained chemicals the variety of commercially available culture media becomes visible, when a catalogue of a supplier is regarded. The offer comprises on the one hand many classical culture media invented decades ago. On the other hand numerous media are offered, which were specifically developed for the culture of hybridoma cells and other producer cell lines. New media were tailored with formulations needed for the successful culture of stem/progenitor cells and specialized tissues.

A small selection of commercially available culture media is given here. It is obvious that the list of mentioned media cannot be complete.



1. As **classical media** are listed:

**Basal Medium Eagle (BME)** is used for the primary culture of mammalian cells.

**BGJb Medium** is needed for the growth of foetal bone.

**Brinster's BMOC-3 Medium** is taken for the culture of mice embryos.

**CMRL Medium** is suitable for many parenchyma cells contained in specialized organs.

**Dulbecco's Modified Eagle Medium (DMEM)** is standard for many types of cells.

**Glasgow Minimum Essential Medium (G-MEM)** is used for cells in connective tissue.

**Iscove's Modified Dulbecco's Medium (IMDM)** can be applied for many cell types.

**Medium 199** is a specific medium for fibroblasts.

**Minimal Essential Medium (MEM)** can be used for a variety of mammalian cells.

**Williams' Medium E** was developed for the culture of hepatocytes.

2. As **actual media** are listed:

**Dulbecco's Modified Eagle Medium / Nutrient Mixture F-12 (DMEM/F-12)** is suitable for a variety of mammalian cells.

**Keratinocyte SFM** is developed for the culture of skin epidermis cells.

**Knockout DMEM** is promoting the growth of many cell types of mice.

**StemPro Medium** is used for the culture of stem/progenitor cells.

**Neurobasal Medium** can be applied for the culture of many types of neurons.

**Hibernate Medium** is suitable for the maintenance of many neural cells.

**Endothelial SFM** is adapted for the culture of endothelial cells.

**Humanes Endothelial SFM** is used for the culture of human endothelial cells.

3. As **innovative media** are listed:

**UltraCULTURE Medium** is suitable for many types of cells.

**PC-1 Medium** is serum free and serves for the multiplication of adherent cells.

**Nephros LP Medium** is serum free to support culture of renal cells.

**ProVero 1 Medium** is protein free and serves for the culture of many epithelial cells.

**ProPer 1 Medium** is chemically defined and can be used for embryonic cells.

**IVF Culture Medium** is frequently applied for the culture of embryonic cells.

4. As **challenging media** for stem/progenitor cells are listed:

**StemPro hESC SFM - Human Embryonic Stem Cell Culture Medium** is a fully defined, serum and feeder free medium for the growth of human Embryonic Stem Cells (hESC).

**StemXVivo serum-free NSC Base medium** is a formulation for the expansion and differentiation of neural stem and progenitor cells. All of the components have been selected for human, mouse and rat cells.

**StemPro NSC SFM - Serum Free Human Neural Stem Cell Culture Medium** is specifically formulated for serum free growth and expansion of human Neural Stem Cells (hNSC).

**StemPro MSC SFM – Human Mesenchymal Stem Cell Culture Medium** is specifically designed for the expansion of human Mesenchymal Stem Cells (hMSC).

**StemPro Chondrogenesis Differentiation Kit** is used for the generation of cartilage on the basis of stem/progenitor cells.

**StemPro Osteogenesis Differentiation Kit** is taken for the generation of bone on the basis of stem/progenitor cells.

**StemPro Adipogenesis Differentiation Kit** is used for generation of adipose tissue on the basis of stem/progenitor cells.

5. As **tissue-specific medium** for cancer research is listed:

**PrEGM** - Prostate Epithelial Cell Growth Medium is a commonly used medium to culture human prostate epithelium.

[search: cell culture medium proliferation differentiation]

**Self-study: Get informed about cell and tissue specific culture media**

## 20.2. Selection of a medium for perfusion culture

For perfusion culture with adherent cells principally all of the listed culture media can be used. When the culture is not performed in a CO<sub>2</sub>- incubator but under atmospheric air, the media must contain a low concentration of NaHCO<sub>3</sub>. For that reason media with the lowest content of NaHCO<sub>3</sub> are ordered, when perfusion culture is performed under atmospheric air. In this case biological buffer such as HEPES or Buffer All has to be added as described before.

In addition, the following media can be used:

**Leibovitz's L-15 Medium** is based on a phosphate buffer system and is therefore suitable to be used in an environment, which is not incubated with CO<sub>2</sub>.

**CO<sub>2</sub>- independent Medium** contains a special mixture of biological buffer so that under atmospheric air can be worked over prolonged periods of time.

**Heparmed Vito 141** is frequently used for perfusion culture including application of hepatocytes in bioreactors.

The surface view to the different culture media cannot be complete. In so far it has to be seen as a selection of examples. From each of them further modifications exists. It gives insights in the choice of multiple media to culture adherent cells. Regarding a catalogue it can be further seen that the spectrum of culture media applications has been changed over the last years. While in earlier times a medium was used for many different kinds of cells, many modern ones are formulated for a single cell type and a very specific application.

**[search: cell culture medium perfusion]**

**Self-study: Read about molecules contained in culture media**

### **20.3. Basic compounds of a culture medium**

Since decades culture media were supplemented with various concentrations of serum. Sometimes undefined additives such as boullion or extracts from different endocrine glands or specialized immune tissues were added. However, during the last years more and more media are offered consisting of clearly defined ingredients. In many cases such chemically defined media are not tailored for many different cells but only for an individual cell type. In some cases one part of the available culture medium kit consists of clearly defined compounds, while some additives are not chemically defined. Regarding these kinds of media one has to reflect in how far it makes sense to work with such a black box.

As a base the culture media consist of very different chemical compounds solved in buffered saline solutions such as PBS (Phosphate Buffered Saline), EBSS (Earle's Buffered Saline Solution), GBSS (Gey's Balanced Salt Solution), HBSS (Hanks' Buffered Saline Solution) or

Puck's salt solution. Which kind of saline solution is most advantageous, is dependent on the individually used cell type.

During culture the cells have to synthesize proteins. For that reason a culture medium contains amino acids and related molecules such as L-alanine, L-arginine-HCl, L-asparagine x H<sub>2</sub>O, L-asparagine acid, L-cysteine / HCl, L-glutamine acid, L-glutamine, glycine, L-histidine HCl x H<sub>2</sub>O, L-isoleucine, L-lysine / HCl, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophane, L-tyrosine and L-valine.

It is obvious that in the most cases the L- and not the D-isoform of the related amino acids are contained. The reason for it is that in animal and human cells only the L-form of an amino acid is used for the synthesis of proteins. However, epithelial cells are special, since they are able to metabolize a D-amino acid into the related L-amino acid. Fibroblast cannot do it. In consequence, a medium for the elimination of fibroblasts and the selection of epithelial cells will contain D-valine instead of L-valine.

Intact function of a cellular metabolism is dependent on the presence of vitamins. Due to that reason culture media contain for example biotin, choline chloride, D-Ca<sup>++</sup>-pantothenate, folic acid, D,L-6,8d-lipon acid, nicotinamide, pyridoxine HCl, riboflavine, i-inositol, thiamine HCl and vitamin B<sub>12</sub>.

In many kinds of culture experiments it is aimed that cells divide. For that reason molecules for DNA and RNA synthesis and for the energy metabolism are needed. To these substances belong adenine, thymidine and glucose, further linolic acid, putrescin-2 HCl and sodium pyruvate. Further metal ions are contained supporting catalytic processes in the growing cells.

The buffer system in a culture medium is selected according to the environment. In the case the experiments are performed within a CO<sub>2</sub>- incubator, a NaHCO<sub>3</sub> buffer system is applied. When perfusion culture is performed under atmospheric air, only a low NaHCO<sub>3</sub> concentration in combination with a biological buffer such as HEPES or Buffer All is used. The pH of the culture medium must stay constant between 7.2 and 7.4.

For a control of the presently existing pH, phenol red is contained in most of the culture media as indicator. However, one should be cautious, since phenol red does not indicate recognizable pH changes in the range between 7.2 and 7.4. Further addition of phenol red must be omitted, when cells in culture are expressing estrogen receptors. Phenol red has a certain affinity to this kind of receptor so that it may interfere with binding of the hormone. Due to that reason culture media are offered lacking addition of phenol red.

Finally, culture media contain a certain concentration of detergents such as Tween 80 to prevent precipitating of barely soluble chemicals. Performing toxicological test with cultured cells this fact has to be considered.

**[search: cell culture medium composition]**

**Self-study: Get informed about tissue specific culture media**

## **20.4. Driving forces within a culture medium**

In the organism cells and specialized tissues are provided with nutrition and respiratory gas by the interstitial fluid. Under culture conditions the interstitial fluid is mimicked by the culture medium. One could believe that the electrolyte content of the interstitial fluid and of a culture medium is more or less the same.

Most interesting is to investigate the composition of electrolytes within a culture medium. When media such as IMDM, BME, William's medium, McCoys 5A medium and DMEM are measured in an electrolyte analyzer and compared with plasma as a mirror for the interstitial milieu, it surprisingly appears that the values do not match. In contrast, differences of up to 20 % in the composition are found. The reason for this discrepancy is that during development of culture media in earlier times the exact simulation of the interstitial fluid was not required. Instead one was interested to find by trying and error an electrolyte environment stimulating as much as possible proliferation and not differentiation.

	Serum (mmol/l)		
	Rabbit	Human	IMDM (mmol/l)
<b>Na<sup>+</sup></b>	136	140	112
<b>Cl<sup>-</sup></b>	99	103	85
<b>K<sup>+</sup></b>	6	4	4
<b>Ca<sup>++</sup></b>	1.7	2.5	1.1

Figure 103: Comparison of the electrolyte composition of serum and IMDM (Iscoe's Modified Dulbecco's Medium). Except the K<sup>+</sup>- concentration the content of electrolytes in IMDM is much lower as compared to serum. The low concentration of electrolytes in IMDM stimulates cells to proliferate.

It was shown in many experiments that the continuous decrease in the concentration of electrolytes is surprisingly stimulating the permanent proliferation. Following this strategy many cell lines show high rates of mitosis, while at the same time the period of interphase is drastically shortened. This technique made it possible to harvest within shortest time a maximum of cells. All of these culture experiments could be performed without the administration of serum or other growth factors.

However, when functional features in cultured cells are needed, the medium supporting proliferation has to be replaced by one that stimulates differentiation. Keeping these



arguments in mind, for each culture experiment a plan has to be made considering on the one hand a period of time for proliferation and on the other hand a long phase for the differentiation. Thus, only in this succeeding interphase period the up-regulation of functional features can be expected.

When culture media are selected according a catalogue, one has to decide if medium with or without addition of glutamine is ordered. The amino acid L-glutamine is an essential molecule for the metabolism and must be contained in sufficient amount in all of the culture media. However, it was shown that L-glutamine is rather in-stabile when kept at a temperature beyond -10°C.

When media are under storage over longer periods of time, it is difficult to estimate how much intact L-glutamine is still contained. For that reason L-glutamine should be added during preparation of the culture medium. Some media are commercially available containing stabilized glutamine so that they can be kept under storage for months.

For a culture medium in many cases hormones and growth factors are needed. Experiences demonstrate that these substances reveal very different chemical characteristics. Many of them such as steroid hormones are for example barely soluble, so that a special procedure must bring them in solution to prevent precipitation. When for example steroid hormones have to be applied in a culture medium, they can be brought in solution within ethanol. Then the alcoholic solution is added under permanent slow stirring in droplets to the pre-warmed medium. However, one has to consider that the medium is now containing a certain amount of ethanol. For that reason the added amount of ethanol has to be kept as small as possible.

Further it has to be mentioned that many other additives such as peptide hormones have in the culture medium only a short bioavailability. It means that such substances are degraded within shortest time by proteinases and other causes so that they are inactivated. For that

reason such biodegradable substances have to be added as short as possible before the start of the culture experiment.

Finally, during run of culture experiments in both classical dishes and in a perfusion culture set-up molecules can be absorbed at inner material surfaces such as the surface of a glass bottle, within a pipette, on the bottom of a dish, in the lumen of a tube or at the adhesion site of cells. This phenomenon is leading to a strongly reduced bioavailability. Although the concentration of the administered hormone was originally high enough, only a reduced amount of the substance is coming in contact with the cells. However, the decreased concentration in solution does not evoke a reaction of the cell.

When absorption onto a material surface is assumed, the available concentration of the chemical substance in solution has to be determined by the help of a professional laboratory. Following this strategy exact information about the concentration of biologically active and available substance becomes known. A special meaning has such a test, when the toxic influence of a hormone must be tested in a chemically defined culture medium.

**[search: cell culture medium review]**

**Self-study: Inform yourself about exact electrolyte composition of culture media**

## 21. Applying the best culture medium

Adherent cells need during the phase of proliferation and the succeeding period of differentiation a suitable culture medium. In previous times it was normal to add serum or organ extracts to a basal medium supporting both proliferation and differentiation. However, this classical strategy is questionable.

Beside the well known basal media, yet new complete and specialized ones are offered to culture cells without the addition of undefined molecules supporting either proliferation or differentiation. In each case media applied for special purposes have to be tailored to the individual personal needs.

Looking to a catalogue a variety of culture media is commercially available. In most of the cases the offered ones are not ready for use but have to be tuned by addition of serum, growth factors and other stimulating factors. However, in principle four different categories of media exist exhibiting varying capabilities and tasks.

1. **Serum containing media.** This group of media contains animal or human serum in concentrations between 1% and 25%. Depending on the price of Foetal Bovine Serum (FBS) or even called Foetal Calf Serum (FCS) the specimens are obtained by a needle puncture within the uterus, by a venous puncture of newborn or shortly born animals. Beside bovine specimen serum is also available from other animal species or human donors. In the biomedical area of research or practice serum can be obtained from a young or an adult donor person.

**[search: cell culture serum containing medium]**

**Self-study: Read about serum content in different culture media**

2. **Serum free media.** In this category of media addition of complete serum is not performed. However, proteins derived from serum of animal species or humans are present. Typical additives are albumin or protein hydrolysates from serum.

**[search: cell culture serum free medium]**

**Self-study: Get informed about the composition of protein lysates in culture media**

3. **Protein free media.** In this kind of media cells are cultured without addition of animal or human proteins or protein hydrolysates. However, these protein free media can contain additives consisting of proteins, peptides or hydrolysates of vegetable origin.

**[search: cell culture protein free medium]**

**Self-study: Read about the selection of a protein free culture medium**

4. **Chemically defined media.** In this group of media proteins of unknown animal or vegetable origin are not contained. Instead all of the substances or additives are clearly declared and used in described concentration.

**[search: cell culture chemically defined medium]**

**Self-study: Inform yourself about the exact composition of a chemically defined media**

## 22. Use of serum as a culture additive

To improve surviving and to promote the proliferation of cells in culture, addition of serum is frequently necessary. However, applying serum one has to consider that a possible risk of infection by viruses, bacteriae, fungi and prions can exist. On the one hand the risk of infection should not be overestimated, since enzymes such as trypsin or accutase normally used for cell isolation during subculture may be also contaminated. On the other hand it is known from the last decade that more than 50% of offered serum charges were positively tested for viruses.

Due to risks of infection international guidelines were published. They regulate that commercially available charges of serum have to comply certain criteria of security. International directions are found for example in the US-FDA Code of Federal Regulations 9CFR113.53,1997, the EU Balai Product Directive CD 92/118/EEC, the Directive CD 96/405/EEG, the WHO Guidelines for the use of serum in cell culture and the Annex to the Monograph on Oral Polio Vaccine, WHO, 1990.

Foetal Calf Serum (FCS) was and is used as a standard additive mostly at 5 to 10 % within complete media. Usually, each laboratory selects the best batch of FCS for their culture purposes by testing. For example, parallel cultures with bone marrow derive stem cells were prepared with the same endotoxin free basic medium supplemented with the same amount of aliquots derived from selected batches.

For example, the analysis at day 10 of a culture experiment revealed that in the presence of one batch of FCS matured cells were found. Another one demonstrated minor differentiation, while a further batch revealed not differentiation of cells. These differences cannot simply be attributed to varying endotoxin levels in the serum. No correlation was found for other tested

contained factors such as corticoids and glucose. Thus, optimization and further standardization of serum additive is needed for research as well for clinical purposes.

**[search: cell culture foetal calf serum]**

**Self-study: Get informed about molecular compounds in serum**

## **22.1. Nutritional influences of serum**

One cannot deny that a barely manageable big number and a variety of experiments have shown a positive influence after serum addition on the results of adherent cells in culture. However, all of these findings are attributed to the fact that not a single but various stimulating factors improved the content of serum.

In part these factors such as serum spreading factor have a stimulating influence on cell proliferation. The presence of numerous growth factors such as Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF), Nerve Growth Factor (NGF), Endothelial Cell Growth Factor (NCGF), Platelet derived Growth Factor (PDGF), Insulin like Growth Factor (IGF) interleukins, interferons and Transforming Growth Factors (TGF) further explains that not a single cell type, but a variety of cells react after administration of serum. In addition, attachment molecules such as fibronectin or laminin allow the cells to find first contact sites with the surface of an adhesion substrate.

The buffer system contained in serum stabilizes the pH through phosphates and proteins. A lot of molecules are able to neutralize for example the toxic influence of accumulating lactate or other metabolites. Important trace elements and minerals such as mangan, copper, cobald, zinc and selenium or sulfate improve the fluid environment so that cells can better metabolise. In serum important molecules such as proteins, amino acids, nucleosides,

choline and pyruvate are contained. Also fatty acids, cholesterol and special lipids are available. In consequence, addition of serum is leading to an optimization of the nutritional offer.

Finally, the extracellular environment of commercially available culture media for adherent cells is stabilized by added proteins such as immune globulins, albumin and transferrin improving principally the quality of osmolarity respectively osmolality.

It is obvious that serum is a complex fluid mixture containing proteins, hormones, growth factors, electrolytes and other not clearly defined molecules. In total more than 5000 different molecules should be contained. However, depending on the delivered charge of serum the composition of contained molecules can vary. Since by the administration of serum many different and also unknown molecules are added, the amount of biologically active factors varies. For that reason one cannot speak about the creation of a clearly defined culture environment.

**[search: cell culture serum nutrition]**

**Self-study: Read about supplementation with serum in culture experiments**

## **22.2. Origin and plurality of serum**

In the most cases of culture experiments serum derives from animal species. It is obtained from bovine, horse, monkey, pig, sheep, rabbit, chicken, rat and mouse species is commercially available and is normally used in a concentration range between 1 and 25 % within the culture medium. Beside known substances serum contains also still unknown molecules. In addition, the concentration of substances varies in delivered charges and species. For that reason it is difficult to produce always reproducible results with media

containing serum. In consequence, for extended research projects always a big amount of a special serum charge has to be ordered so that reproducible experiments can be performed over prolonged periods of time. When the stock is exhausted, new charges have to be tested so that the series of experiments can be brought to a successful and reproducible end.

The addition of serum to a basic culture medium is made due to several reasons. First of all, it will stimulate cell proliferation and adhesion. Then the fluid surrounding is improved for the contained cells by stabilizing pH and oncotic pressure. Barely soluble molecules such as steroid hormones can be administered in culture by the presence of serum. In this case the hormones are bound to proteins. In many instances addition of serum is completing nutrition as compared to the basic culture medium lacking serum.

In each case one has to accept that serum is a natural product. Accepted companies collect it according industrial standards in different countries round the world. Serum from horses for example is obtained from a group of animals kept under permanent supervision of a veterinarian. Foetal Bovine Serum (FBS) respectively Foetal Calf Serum (FCS) is isolated in a slaughter house. The isolation of such individual charges of serum is rather difficult, since in many cases the heart has to be punctured. After coagulation the serum is separated by centrifugation from blood cells and fibrin. Finally charges derived from different animals are combined.

The obtained charges of serum are then filtered in several steps using always smaller pore sizes down to 0.2 and 0.1  $\mu\text{m}$ . After this step the necessary sterility is given. A sterilization process in an autoclave is not possible, since serum cannot stand high temperature. A treatment by heat would result in molecular destruction. The contained proteins would precipitate and the available growth factors will loose biological activity. Finally, the filling of serum in bottles takes place in sterile rooms under a bench so that conditions free of



particles are fulfilled for example according to British Standard BS5295; 1989 and US Fed Standard – 209E.

**[search: cell culture serum origin]**

**Self-study: Get informed about the origin of serum for culture experiments**

## 23. Risks of serum application

Within the last years in many cases it was shown that serum is added to the culture medium although this would not be necessary.

Further numerous sets of culture experiments have shown that it does not play a role if serum derived from calf, monkey or horse is applied. In contrast, when cultures are used in the biomedical area or for therapeutic applications only serum from humans are applied to avoid a possible risk of infection.

Before human serum is used it must be guaranteed that there is no risk of infection. Further in culture medium containing bovine serum there is a danger that a contamination with prions exists leading possibly to a BSE infection (Bovine Spongiforme Encephalopathy). Arguments that the origin of serum is outside of Europe do not count, since diseases analogous to BSE such as Mad Cow Disease exist. The same risk is present, when for example an extract of the bovine pituitary gland is administered.

Despite several steps of filtration serum involves the risk of an infection by viruses and mycoplasmas. Although most of mycoplasmas are eliminated after filtration through a 0.1 µm filter, a certain risk of infection remains. In some cases a treatment with γ-irradiation will help to inactivate viruses. If this method is also suitable for serum contaminated with prions remains to be elucidated.

Not to underestimate is a general bacterial contamination. One of these pathogens in delivered serum might be MAP (Mycobacterium Avium Paratuberculosis). In each case one would make sure that medium containing serum is always carefully filtered.

Serum is delivered in a frozen state so that it can be put on storage for years. The thawing of it should slowly happen within a cooling box. In the thawed state serum must not be shaken, but gently moved back and forwards. This treatment prevents a denaturation of proteins and mixes different phase of fluid. Finally, for a running experiment the necessary amount of serum can be taken. The rest of serum is transferred by a pipette in same amounts of aliquots that are frozen. A measured aliquot has the advantage that for example for 100 ml basic culture medium the necessary serum aliquot can be simply added without the transfer of a pipette. This way saves time, reduces steps of further transfer with pipettes and avoids several cycles of freezing and thawing. It must be finally mentioned that several cycles of freezing and thawing are damaging the biological activity of serum.

Finally, quantitative and qualitative fluctuations of the biologically active molecules in charges of serum exist. For that reason culture with medium lacking serum should be principally considered before administration of it is performed.

**[search: cell culture serum bse donor]**

**Self-study: Read about risks of serum application in culture experiments**

## 24. Replacement of serum in medium

Regarding the risks of serum in a culture medium quite a natural idea is to work with an alternative. However, the experiences with various adherent cells show that the conversion from a serum containing to a serum free medium is not easily to perform and needs a lot work and time.

When the concentration of serum is reduced in steps, in parallel it can be observed that the cells show a decreased rate in proliferation and a loss of adherence to the bottom of the dish so that they grow in a more or less semi-adherent state. In many cases cells do not show further adhesion to the bottom of the dish but round up to grow in suspension. Regarding these severe alterations one will recognize the enormous influence of serum not only on the proliferation but also on the adhesion of cultured cells.

**[search: cell culture serum protein free]**

**Self-study: Get informed about replacement of serum in culture experiments**

### 24.1. From serum to serum free conditions

In consequence, a switch from serum containing medium to one with low serum content or to one lacking serum has to be made in steps. It takes a long time over many days or probably weeks, until the cells are trained to tolerate a serum free culture environment.

Cultured cells like it, when a changeover from a serum containing medium to a serum free medium is not abruptly made but occurs within an adaption phase. The successive adaption is not made with a complete change of the culture medium. Instead, when cells are fed with new medium, only part of medium is replaced by new medium containing yet a lowered

concentration of serum. The next change of the culture medium is made in the same way, until cells are fed only with serum free medium.

A further motivation for a changeover from a medium containing serum to a medium lacking serum might be the costs. When numerous experimental series are performed, media containing serum become expensive. Yet one has to reflect if the changeover to a serum free medium might be the optimal option.

The application of a Serum Free Medium (SFM) allows the performance of culture experiments under better defined conditions as it is possible by the use of one containing serum. However, one has also to consider that in serum free media relatively big amounts of serum proteins such as albumin and transferrin are added.

Further the protein free media do not contain by definition proteins, but protein lysates such as peptides are present. Regarding these criteria a serum free or a protein free medium is not clearly defined.

In the meantime additives such as SerEX exists, which exhibit stimulating features for the proliferation of cells as it is described for serum. Further Express Media are on the market supporting serum free culture with CHO (Chinese Hamster Ovary), MDCK (Madin-Darby Canine Kidney), Vero (African Green Monkey Kidney), HEK (Human Embryonic Kidney), BHK (Baby Hamster Kidney), L-M (connective tissue) and L929S (fat tissue) cell lines.

**[search: cell culture serum containing serum free]**

**Self-study: Read about culture media containing and lacking serum**

## 24.2. Additives in serum free media

Advantages for the application of a serum free culture medium are in each case the reduced risk of infection and the low costs. Further these media contain better defined additives and a less varying composition of administered molecules. A single disadvantage is that it might take a long period of time until the cells are trained to grow in a serum free culture environment. For example, neuron cells derived from hippocampus and other types of neurons can be cultured without any difficulties in serum free medium. When for example Neurobasal Medium is used, supplements such as B-27, N-2, bFGF and EGF have to be added. Further one has to decide if the medium is ordered with or without phenol red. When experiments dealing with hormone receptors have to be performed, phenol red might interact with the binding of a hormone.

When a reduction of serum in the culture medium is made, alternative supplements have to be added so that the cells proliferate. An important mitogen contained in serum is the Platelet Derived Growth Factor (PDGF). This factor is a dimeric glycoprotein consisting of an A- and B-chain. For example, fibroblasts, glia cells and smooth muscle cells are spreading very well, when PDGF is added as a single substance to the culture medium.

Working with serum free or protein free culture media a classical cocktail for a stimulating environment is ITS consisting of Insulin (5 mg/l), Transferrin (5 mg/l) and Selenium (5 µg/l). Beside ITS also hydrocortisone, testosterone, progesterone, estradiol-17 $\beta$ , ECGS, EGF, triiodothyronine and phosphoryl ethanolamine can be administered.

In contrast, chemically defined media do not contain serum, proteins, hydrolysates or other molecules of unknown composition. In each case all of the contained molecules are declared and must be commercially available. It is obvious that in chemically defined media also hormones, growth factors or cytokines have to be added. When such substances are

administered, they must be chemically pure and have to be added in the announced concentration.

**[search: serum free culture conditions growth factors media]**

**Self-study: Inform yourself about additives in culture media**

### **24.3. Preventing risks of infection**

At the start of a culture experiment it must be guaranteed that the adherent cells are exposed to a sterile milieu. However, despite any care it may happen that bacterial or mycotic infections are present that affect adherent cells. In static culture this is more problematic than in perfusion culture, since the cells are provided with always fresh medium diluting the infection particles. In some cases application of perfusion culture can help to prevent an infection and a loss of the experiment by the provision with always fresh medium. In other cases application of antibiotics and antifungal drugs has to be made.

For the application in a culture medium substances such as penicillin G or streptomycin can be made. Also one can use commercially available cocktails consisting of an antibiotic and antifungal solution. However, in each case one has to consider that antibiotic and antifungal substances may exhibit a toxic influence of individual cell types. Such an action is for example typical for the antibiotic Tylosin and the antifungal drug Fungizone. Due to possible toxic influences the quality standard of the own work should be that during performance of the experiments an application of antibiotics or antifungal substances is not necessary. In contrast, performing primary cultures with numerous preparations and manipulations under a semi-sterile atmosphere in many cases it cannot be done without the application of antibiotics or antifungal substances.

Cell cultures provide an ideal environment for contaminating mycoplasmas. It was reported that between 30% and 90% of mammalian cultures are contaminated with them. Most interestingly, only in few cases a real intense infection is observed leading to a loss of the culture. In most of the cases the infection is silent. However, mycoplasmas are strongly influencing the growth and metabolism of adherent cells. To be sure that such a silent infection is not present, the specimens have to be analyzed in a laboratory specialized in the detection of mycoplasmas.

A further possibility is to label the cultures with fluorescent DNA markers and then to analyze it under the microscope. DAPI- or bisbenzimidazole-staining is used to detect infection with mycoplasmas. When beside the nucleus other areas within the cytoplasm reveal a fiber like label, most probably stained DNA from mycoplasmas is detected. In this case the culture appears to be infected. In consequence, the infected culture has to be treated several times with a Mycoplasma Removal Agent (MRA). From the European Collection of Animal Cell Cultures (Porton Down, U.K.) a concentration of 0.5 µg/ml in medium is recommended.

**[search: cell culture medium infection]**

**Self-study: Read about risks of serum application in culture experiments**



## 25. Exposure of cells to always fresh medium

Little knowledge is available about the mechanisms leading from embryonic to adult and from homogenously to heterogeneously composed epithelia found in various organs including the kidney. For example, the renal collecting duct epithelium derives from stem/progenitor cells found in the collecting duct ampulla of the earlier ureter bud. Each of the collecting duct ampullae can be detected in the stem/progenitor cell niche located in the outer cortex of the neonatal kidney. Here and further downwards in the ampulla neck frequent cell divisions are found causing elongation of the differentiating collecting duct tubule. In the mid cortical region and beyond the ampulla neck the matured and heterogeneously composed epithelium is developing consisting of Principal cells (P) and different kinds of InterCalated (IC) cells.

Up to date it is unknown, which factors are influencing the development from an embryonic to a specialized renal collecting duct epithelium. The development may depend on the one hand on growth factors, morphogenic substances such as vitamin A derivatives or steroid hormones such as aldosterone. On the other hand the development depends on specific extracellular matrix proteins distributed in the interstitium. Further it was assumed that changes in the pH or osmolarity, but also variations in the surrounding electrolyte composition might play an essential role in the development of those specialized cell characteristics.

In previous experiments it was shown that embryonic renal collecting duct cells develop in perfusion culture after administration of aldosterone features found within the matured collecting duct epithelium of the adult kidney. Beside a typical morphological appearance in P cells an amiloride sensitive sodium transport in combination with physiological tightly sealing characteristics were developed during 13 days of perfusion culture in a serum free medium. Interestingly, administration of aldosterone was influencing not only the development of P

cells, but also features of  $\beta$ -type IC cells were found to be up-regulated as demonstrated by binding of peanut agglutinin (PNA).

Further development of typical P and IC cell features was obtained simply by increasing the NaCl concentration in the culture medium up to physiological values. In this coherence one has to consider that the individual concentration of electrolytes in most culture media is significantly lower as compared to serum reflecting the fluid of the interstitium. Regarding the individual cell reactions during performance of the experiments, we did not realize before how sensitive the embryonic collecting duct epithelia reacted to changes in electrolyte composition of media.

### **25.1. Offering the same cells different culture media**

First of all we were interested in the amount of electrolytes found within culture media frequently used with renal cells. However, comparing the lists of electrolytes within a catalogue we were not able to recognize the final sum of each electrolyte within a medium. Please have a look in a catalogue, then you will understand.

To surmount the situation six different media were then selected for planned experiments. For the presented culture experiments all of the media were purchased from Gibco® Cell Culture/Invitrogen (Darmstadt, Germany).

1. Iscove's Modified Dulbecco's Medium (IMDM, Nr. 21980)
2. Medium 199 (M 199, Nr. 31153)
3. Basal Medium Eagle (BME, Nr. 41010)
4. Williams' Medium E (WME, Nr. 32551)
5. McCoys 5 A Medium (MCM, Nr. 22330)
6. Dulbecco's Modified Eagle Medium (DMEM, Nr. 21063)

To obtain information about physiological parameters in the different media such as contained electrolytes  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$  and  $\text{Ca}^{++}$ , glucose and osmolarity were determined in an undiluted 200  $\mu\text{l}$  sample by a STAT Profile 9 Plus analyzer (Nova Biomedical, Rödermark, Germany) (Fig. 104).

	<b><math>\text{Na}^+</math></b> mmol/l	<b><math>\text{Cl}^-</math></b> mmol/l	<b><math>\text{K}^+</math></b> mmol/l	<b><math>\text{Ca}^{++}</math></b> mmol/l	<b>Glucose</b> mg/dl	<b>Osmolarity</b> mOsm
IMDM	117	81	3.9	1.1	418	250
M 199	138	125	5.1	1.5	99	270
BME	146	111	4.8	1.4	94	286
WME	144	117	4.8	1.4	186	288
MCM	142	106	4.8	0.5	270	289
DMEM	157	116	4.8	1.3	382	323

Figure 104: Parameters of electrolytes, glucose and osmolarity in different culture media.

In the demonstrated table the culture media were placed in order due to their low (IMDM), medium (M 199, BME, WME, MCM) and high (DMEM)  $\text{Na}^+$  content (Fig. 104). It is obvious that the media differ not only in their electrolyte composition but also in their amino acid content and nutritional offer. These data are not shown.

In a series of further experiments the effect of different culture media on the differentiation of embryonic renal collecting duct epithelia was tested. The aim was to find out if adherent epithelial cells survive in all of them over prolonged periods of time.

Finally it was important to elaborate if generated features of differentiation in epithelia were the same or were different, when various culture media are used.

To establish the renal collecting duct epithelium for an initial period of 24 hours the culture medium contained 10% Foetal Calf Serum (FCS). During the experimental run in perfusion culture for 13 days none of the culture media contained any serum or growth factors. Only aldosterone ( $1 \times 10^{-7}$  M) was added. Depending on the sodium bicarbonate concentration HEPES was used in individual culture media to maintain a constant pH during experimental run on a laboratory table under atmospheric air (0.3% CO<sub>2</sub>). Transport of medium was performed with 1ml/h.

After a period of 13 days in perfusion culture the generated epithelia were analyzed. It was a surprising observation that independently from the medium in all of the experimental series a perfectly polarized collecting duct epithelium was found. Regarding the epithelia under pure morphological criteria differences in quality could not be observed (Fig. 105). All of them were well preserved and demonstrated a clear orientation. At the luminal side they were exposed to the different culture media, while their basal side was in contact with the basal lamina. Since after the use of IMDM, M 199, BME, WME, MCM and DMEM an excellently polarized epithelium was harvested, all of the tested media appear suitable for future culture protocols.

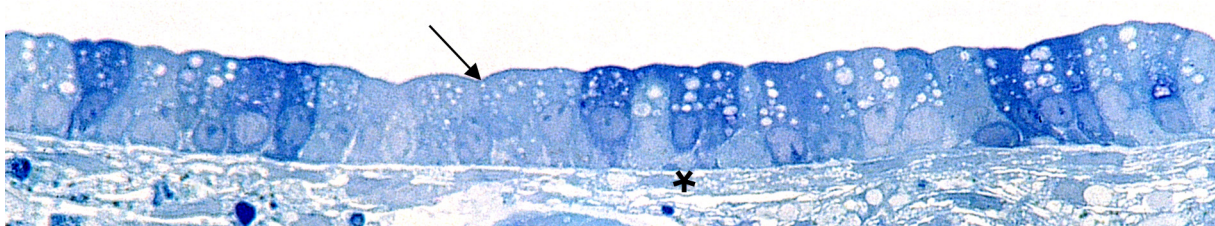


Figure 105: Morphological view to a collecting duct epithelium generated within 13 days in perfusion culture. Arrow illustrates lumen, while asterisk shows basal lamina.

While the morphological features of cultured collecting duct epithelia did not differ in the experimental series, the immunohistochemical profile might be influenced by the different administered culture media. In this case one has to mention that protein expression is influenced by a facultative and a constitutive mechanism.

Thus, constitutively expressed molecules in the generated collecting duct epithelium are for example cytokeratin 19, renal collecting duct specific P<sub>CD</sub> 9, Na/K ATPase and laminin. All of these proteins were present in collecting duct epithelia cultured with above mentioned media.

<b>Binding on cells (%)</b>	<b>Mab 703</b>	<b>Mab 503</b>	<b>PNA</b>
IMDM	5 - 10	5 - 10	80 - 90
M 199	30 - 40	5 - 10	5 - 10
BME	90 - 100	5 - 10	80 - 90
WME	90 - 100	40 - 60	10 - 20
MCM	90 - 100	50 - 70	5 - 10
DMEM	90 - 100	70 - 90	70 - 80

Figure 106: Illustration of facultative antigen expression in collecting duct epithelium generated in perfusion culture with six different culture media. Number of positive cells was determined after label with collecting duct specific antibodies such as mab 703 and mab 503. Further binding of PNA lectin was tested.

In contrast, regarding facultative expression some proteins are found to be up-regulated not in all but only by some of the presently used culture media (Fig. 106). This group of facultatively expressed molecules comprises renal collecting duct specific antigens detected with monoclonal antibodies (mab) and lectins. For example between 90 and 100 % of cells were labelled by mab 703 after culture in BME, WME, MCM and DMEM. Most interestingly, only 10% positive cells were detected in epithelia cultured in IMDM. A similar low rate of labelled cells was detected after mab 503 staining. In contrast, an intense rate of PNA-binding was found in epithelia cultured in IMDM, BME and DMEM, but not after culture in M 199, WME and MCM.

It appears likely that the variation in osmolarity of tested culture media might be responsible for the change in cell differentiation of generated collecting duct epithelia. However, it was found that culture of the epithelia in media with nearly the same osmolarity such as BME, WME and MCM did not show the same profile of differentiation as expected (Fig. 106). In contrast, a quite different and rather individual pattern was observed. This important observation demonstrated that environmental factors other than osmolarity or electrolytes influence the pattern of differentiation. In so far it would be interesting to find out if other culture media might be much more successful to generate a high degree of differentiation than commonly used media.

Finally, the presented culture experiments can now be performed by online measurements with microelectrodes produced for example by Shelfscience<sup>®</sup>. Micro flow electrodes are available for pH, pO<sub>2</sub>, pCO<sub>2</sub>, Na<sup>+</sup>, Cl<sup>-</sup>, K<sup>+</sup> and NH<sub>3</sub>. The various flow cells can be utilized in combination with silicone tubes. Most important, autoclavable electrodes and flow cells are also available.

**[search: collecting duct epithelium perfusion culture minuth schumacher]**

**Self-study: Read about electrolytes and osmolarity of culture media**

## **26. Offering an improved fluid environment**

In biomaterial testing as well as in tissue engineering innovative materials and artificial matrices are brought in contact with living cells to investigate cell and surface interactions. Beside the optimization of polymer or metal implants, a challenging task in modern biomedicine is the production of living tissue constructs such as cartilage, bone, tendon or skin for the implantation in severely injured patients. Further to bridge the temporal loss of important vital functions, an intense demand is present for implantable pads containing stem/progenitor cells for the regeneration of diseased parenchyma of liver, pancreas or kidney. Beside these pure clinical applications a multitude of tissue specific cultures is urgently needed for toxicological tests in pharmaceutical research and for consumer protection.

### **26.1. Providing external and internal surfaces with nutrition**

For most of the cultures in pharmaceutical and biomaterial research cells in suspension are transferred by a pipette to the bottom of a dish, a film, a porous filter mounted in an insert, a newly structured biomaterial or a three-dimensional fleece matrix. Here the cells have to find an optimal anchorage site so that typical functional features of specialized tissues can develop.

Due to pores in foams or spaces between fleece fibers some of the offered materials exhibit not only external but also internal surfaces for the anchorage of cells. In consequence, provision with nutrition and respiratory gas must take place not only on the outer surface but also on barely accessible anchorage sites in the interior of a relatively thick scaffold or other kind of matrix. To assure survival of cells in each case the formation of unstirred layers of fluid has to be prevented.

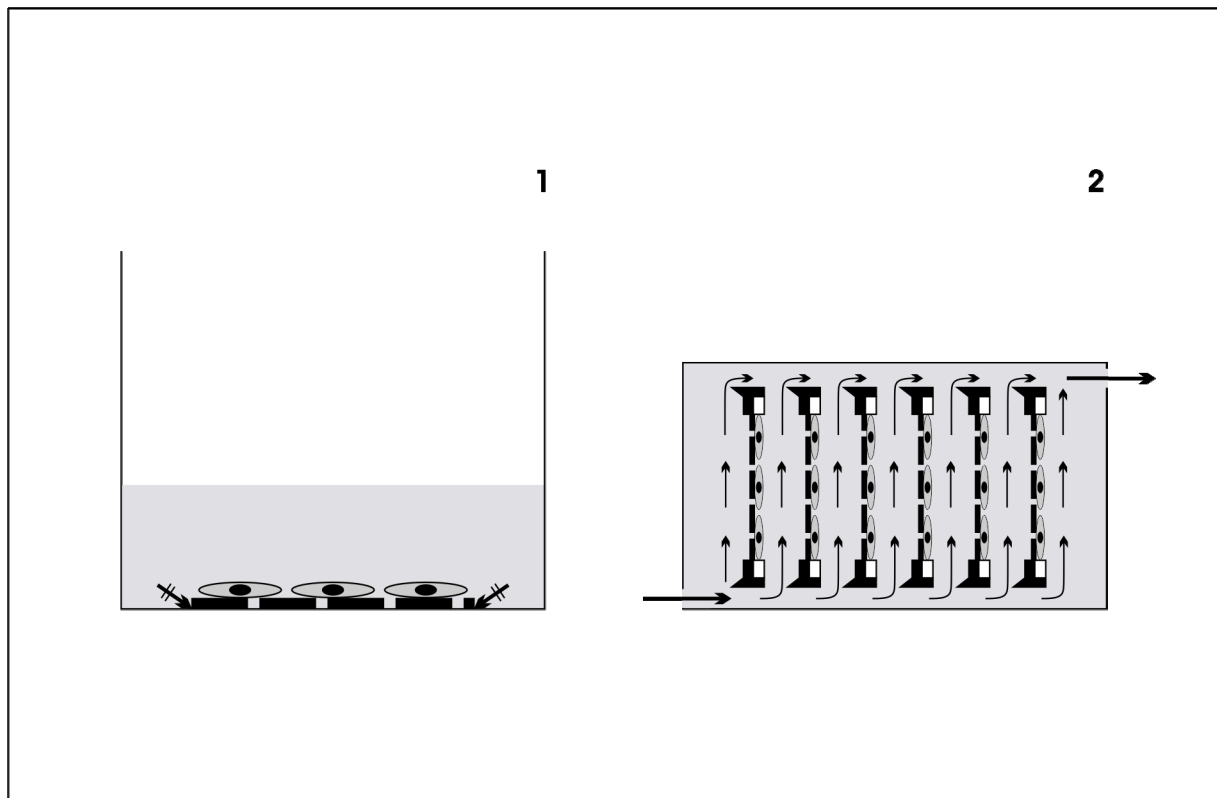


Figure 107: Illustration of cells on the bottom of a dish and within a perfusion culture container. When a porous substrate with adherent cells is placed on the bottom of a dish, provision with medium is restricted at the base site (1). In contrast, when substrates are placed within a tissue carrier inside a perfusion culture container, distribution of fluid takes place on both sides of substrates and cells (2).

It is obvious that these requirements cannot be fulfilled in the static environment of a culture dish, since the interface between the bottom of the dish and the inserted substrate is not accessible for culture medium to provide cells with sufficient nutrition and respiratory gas (Fig. 107, 108).

A technical solution is to mount the selected biomaterial for cell anchorage in a tissue carrier. Then the tissue carrier can be placed in a perfusion culture container. Thus, mounting of a selected biomaterial including the adherent cells within a tissue carrier is leading first to an exact geometrical placement within a perfusion container preventing in turn damage of adherent cells.



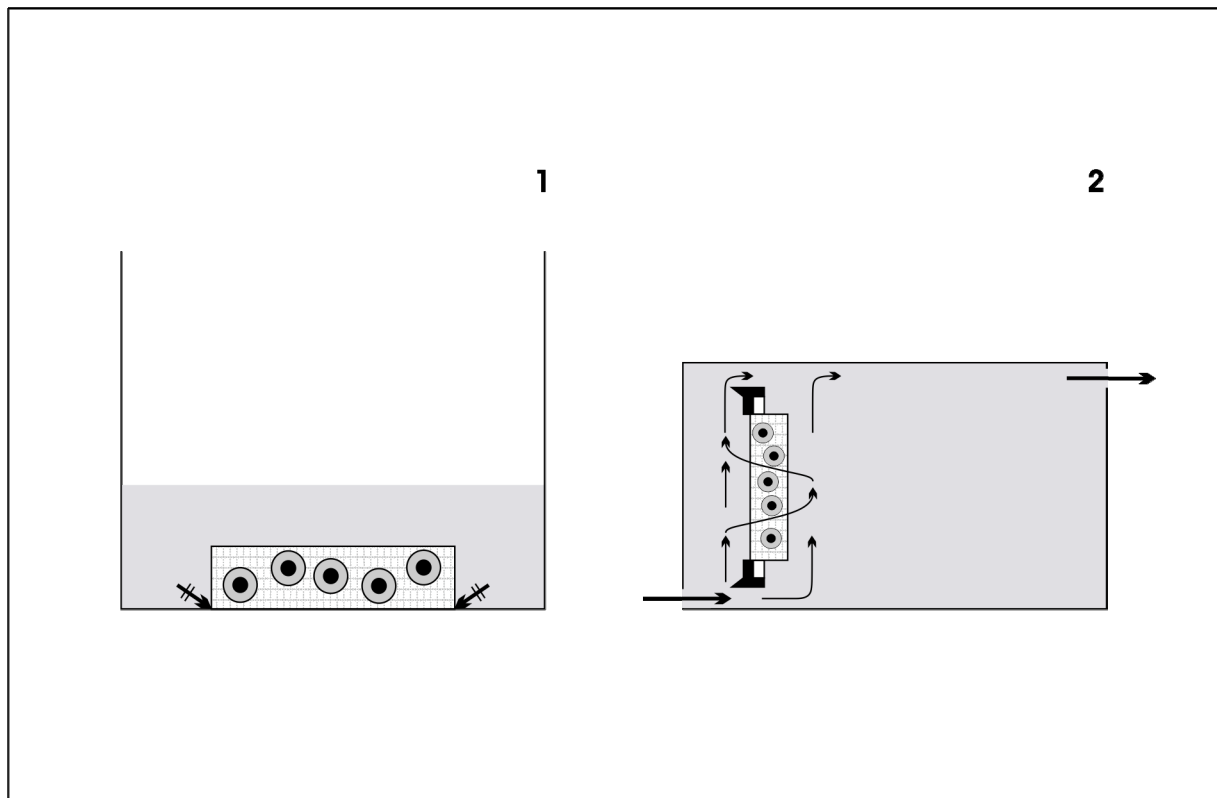


Figure 108: Illustration of restricted medium provision at the interface of a substrate containing chondroblasts and the bottom of a dish (1). In contrast, when a permeable scaffold is mounted in a tissue carrier always intense provision with fresh medium is given on both sides and in the interior of the scaffold (2).

Further on, the exact placement in turn is causing always an exact distribution of transported culture medium within the container. This geometrical interaction between the placement of a tissue carrier and the resulting space for fluid distribution within a culture container is the base for an optimal transport of medium resulting in a constant provision of cells with nutrition and respiratory gas (Fig. 107, 108).

In this scenario both sides and the interior of the applied substrate respectively porous scaffold material are yet exposed to a permanent exchange of culture medium. The streaming of the fluid is providing both the external and internal surface containing adherent cells with sufficient nutrition and respiratory gas. Further unstirred layers of fluid are avoided, since the metabolized medium is continuously eliminated.

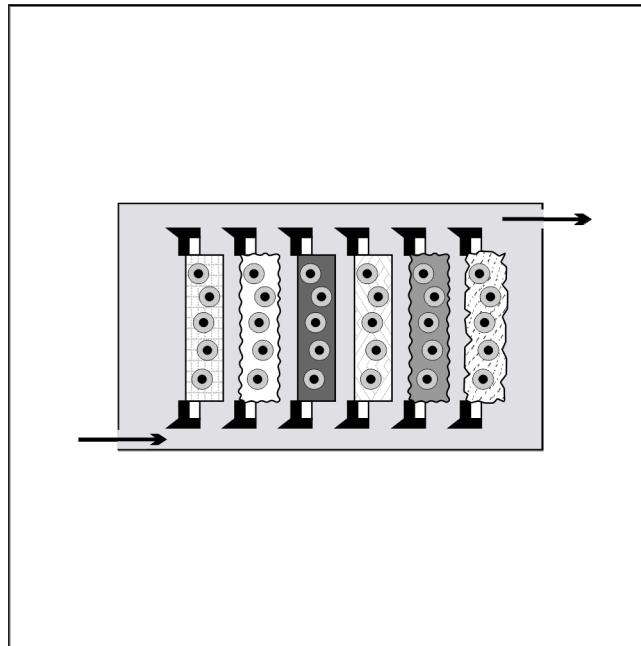


Figure 109: Illustration of different kinds of scaffolds mounted in a tissue carrier within a perfusion culture container. The surface and the porosity of the substrate respectively scaffold material will determine the flow of culture medium on the surface and in the interior of the selected materials. As a result quite different pattern of adherent cells can arise.

Depending on the thickness and the porosity of the applied substrates respectively scaffold materials one has further to decide if a continuous transport of medium has to be performed or if a transport in form of pulses is made (Fig. 109). This special mode decreases the formation of unstirred layers of fluid, when cells are cultured not on flat films or filters but on thick scaffolds made out of biodegradable polymers or collagen.

**[search: cell scaffold perfusion culture]**

**Self-study: Get informed about microreactors for perfusion culture experiments**

## 26.2. Distribution of fluid within a perfusion culture container

In order to visualize the distribution and exchange of medium within a perfusion culture container several possibilities exist. One possibility is to use a special computer program to

feed it with geometrical measures of the inner surface of the individually used perfusion culture container, the outer surface of inserted tissue carriers and their placement inside the container. Then the program calculates the flow of medium and offers in turn solutions to optimize it. As a result nice illustrations are generated. However, all of this information is based on assumptions without considering the actual roughness and porosity of inserted scaffold material, the adhesion of cells or the possible occurrence of unstirred layers of fluid and individual narrow slits within the perfusion culture container influencing drastically the exchange of culture medium.

Considering these arguments and looking for a solution of an unsolved problem an alternative way was selected. In order to visualize the exchange of culture fluid during an experimental run, phenol red contained in medium was used as a marker.

Thus, for the presented experiments either acidified yellow medium or alkalized purple medium was transported in pulses through a culture container. The surface of the container was polished so that registration of fluid flow could be performed. During run of the pump the transition from yellow colour to purple colour was registered by a camera from various perspectives (Fig. 110).

Using a container with six inserted tissue carriers it was observed that during a transport rate of 1ml/h after 15 seconds medium is entering at the inlet of the container (Fig. 110.1). Surprisingly, it completely distributes within 20 minutes at the basal part of the container (Fig. 110.2). In the side view it can be seen that between 30 minutes (Fig. 110.3), 1 hour (Fig. 110.4), 1.5 hours (Fig. 110.5), 2 hours (Fig. 110.6) and 2.5 hours (Fig. 110.7) the level of the be-coloured medium is continuously elevated between the inserted tissue carriers. After 3.5 hours it has reached the highest point to leave the container through the outlet near the lid (Fig. 110.8).

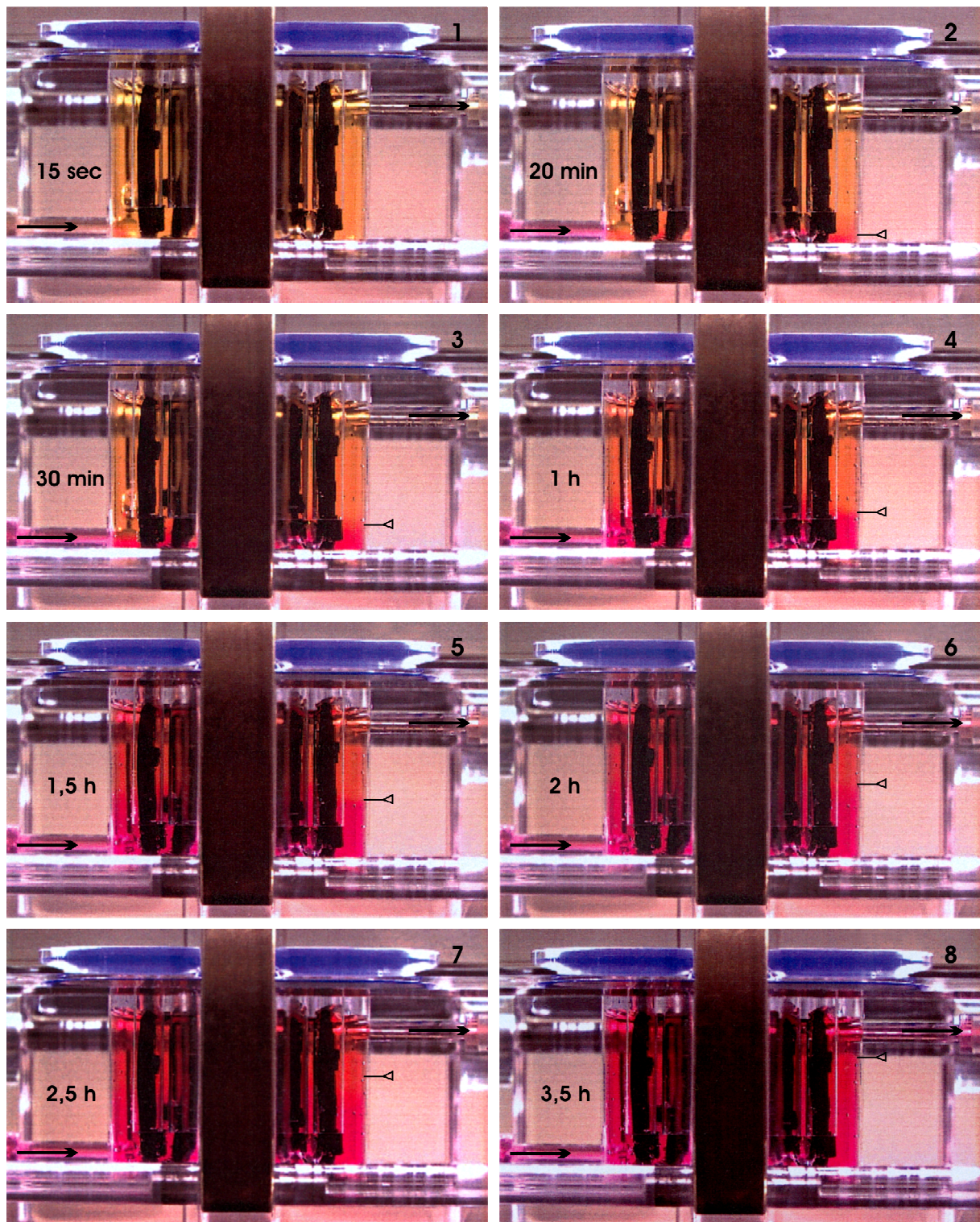


Figure 110: Distribution of culture medium within a perfusion culture container. Medium is transported with 1 ml/h after 15 seconds to the inlet of the container (1). Then it distributes within 20 minutes at the basal part of the container (2). It can be seen that between 30 minutes (3), 1 (4), 1.5 (5), 2 (6) and 2.5 hours (7) the level of the coloured medium continuously is elevated between the inserted tissue carriers. After 3.5 hours the medium has completely left the container through the outlet at the lid (8). Arrow head depicts level and distribution of medium.

It is obvious that the applied technique is not yet perfect to show the spatial distribution of fluid inside a perfusion culture container. However, in future fluorescent cell markers are applied in the culture medium so that they can be detected later by immunohistochemistry on generated tissue. This technique will make it possible to register the distribution of fluid inside a thick layer of cells or within an extended scaffold over prolonged periods of time. All of these experiments have to be performed by the help of a perfusion container and with living cells during a running experiment. As learned by experiences, the distribution of fluid within a perfusion culture container cannot be solely done with a more or less suitable computer program.

**[search: microreactor fluid flow]**

**Self-study: Inform yourself about fluid flow in microreactors**

### **26.3. Parameters of medium inside a perfusion container**

As a model in presented culture experiments Iscove's Modified Dulbecco's Medium (IMDM) was transported by a rate of 1 ml/h through silicone tubes. Since the perfusion culture system works on a laboratory table under atmospheric air (0.3% CO<sub>2</sub>), the pH must be adjusted.

IMDM contains for example 3024 mg NaHCO<sub>3</sub>. In consequence, to maintain a constant pH of 7.4 under atmospheric air, a titration of medium has to be performed. The result shows that up to 50 mmol/l HEPES has to be added.

To register physiological data of the culture medium, a T-piece is inserted in the silicone tube at the inlet of the perfusion container. Then a 200 µl sample can be soaked up by a syringe to analyze it in a Stat Profile gas respectively electrolyte analyzer. The data show for



example that in the running experiment 174 mmHg O<sub>2</sub> is contained in the medium (Fig. 111). This amount is much more oxygen than contained in serum (99 mmHg O<sub>2</sub>). In each case this present amount of 174 mmHg O<sub>2</sub> is sufficient to provide developing cells and tissue with enough oxygen within a perfusion culture container.

<b>pH 7.4</b>	<b>pO<sub>2</sub></b> mmHg	<b>pCO<sub>2</sub></b> mmHg	<b>Na<sup>+</sup></b> mmol/l	<b>K<sup>+</sup></b> mmol/l	<b>Cl<sup>-</sup></b> mmol/l	<b>Ca<sup>++</sup></b> mmol/l	<b>Glucose</b> mg/dl	<b>Osmolarity</b> mOsm
<b>perfusion</b>	174	9	120	4	86	1.2	393	255
<b>serum</b>	99	40	142	4	103	2.5	100	290

Figure 111: Physiological parameters found in IMDM during perfusion culture under atmospheric air. An individual 200 µl sample was analyzed in a Stat Profile analyzer.

Although IMDM medium is often used in cell culture experiments, its measured osmolarity of 255 mOsm is surprisingly low as compared to 290 mOsm found in serum specimens (Fig. 111). In contrast, rather high is the content of 393 mg/dl glucose in medium, while in serum only 100 mg/dl is found. Specimens of serum revealed for Na<sup>+</sup> (142 mmol/l), Cl<sup>-</sup> (103 mmol/l) and Ca<sup>++</sup> (2.5 mmol/l) quite normal concentrations. However, as compared to serum the content of Na<sup>+</sup> (120 mmol/l), Cl<sup>-</sup> (86 mmol/l) and Ca<sup>++</sup> ( 1.2 mmol/l) in culture medium is surprisingly low. Only the concentration of K<sup>+</sup> (4 mmol/l) is the same. It is obvious that a rather high discrepancy exists between the content of electrolytes in a culture medium and serum seen as a mirror for the fluid environment within the interstitium.

**[search: cell perfusion culture medium composition]**

**Self-study: Get informed about physiological parameters in perfusion culture**

## 26.4. Examples to generate specialized connective tissues

A special issue in regenerative medicine is the treatment of cartilage and bone defects by artificial tissue constructs containing various scaffold materials. In numerous cases perfusion culture can definitively improve the quality of generated tissues.

To generate cartilage constructs for implantation bioresorbable scaffolds were in earlier times frequently applied in combination with chondroblasts or chondrocytes within a culture dish. However, in the static environment an increasing concentration of biodegraded molecules such as lactate is liberated resulting in a damage of the growing tissue during time. In consequence, to eliminate continuously biodegraded molecules Minusheet® perfusion culture was applied successfully for the generation of cartilage. Applying this method it became possible to elaborate realistic data concerning kinetics of the degradation process from different scaffold materials. In addition, by perfusion culture the cell biological quality of generated tissue could be improved by stepwise modifications of the scaffold material so that the risk for implantation could be minimized. Surprisingly, it was shown that the application of natural extracellular matrix such as a conventional collagen sponge does not improve the quality of generated cartilage. In contrast, scaffold materials with modified polyethylene coating or gelatine-based Spongostan® focused in excellent results. Further on, the generation of intact cartilage constructs by perfusion culture revealed as an ideal model to investigate factors affecting destructive joint diseases.

In bone tissue engineering described perfusion culture technique was applied to investigate the development of osteoblasts on ceramic materials, decellularized spongy bone, collagen membranes, mineralized collagen, hydroxyapatite scaffold, PLGA sheets, laminin-coated polycarbonate membranes and textile chitosan scaffolds. Most important for clinical applications are experiments related to effects influencing tissue development after sterilization of scaffold materials consisting of poly-D,L-lactic-co-glycolic acid.

A further problem is the occurrence of unstirred layers of fluid within a tissue engineered construct, since it develops during perfusion culture a permanently increasing thickness. For that reason a constant provision with nutrition and oxygen plays an essential role during generation of the construct. Last but not least, learning from bone in perfusion culture may lead to an effective strategy for the regeneration of dentin.

**[search: perfusion culture scaffold cartilage / bone]**

**Self-study: Read about the generation of specialized tissue in tissue engineering**



## 27. Simulation of an environment for epithelia

In the organism epithelia belong to the fourth group of basic tissues beside the nervous tissue, the muscular tissue and the connective tissue. Without exception all of the epithelia exhibit important barrier functions. Regarding this aspect the epithelia are exposed in an adult organism to a different environment at the luminal and basal sides.

As seen from a morphological point of view the epithelia are homogeneously or heterogeneously composed. They can occur as simple, pseudostratified or stratified epithelia. The geometrical shape varies from squamous to cuboidal and up to columnar cells.

The physiological tasks of epithelia are manifold. To protect underlying tissues is the role of epithelium covering the external surfaces and orifices, while transport of mucus and particles is performed by ciliated epithelia found in secretory, respiratory and genital ducts. The epithelia of the intestine, liver and kidney are involved in absorption, secretion and filtering of molecules from the lumen to the interstitium or in the vice versa direction. In the follicles of the thyroid gland or the ovary related epithelia are covering the free surface of the hormone reservoir respectively the surface of the egg cell. Taste buds and olfactory mucosa epithelia are involved in sensory reception.

It is typical for all of the epithelia that the contained cells are resting on a layer of a very specific extracellular matrix known as basement membrane or basal lamina. It is heterogeneously composed and consists in the typical case of a lamina rara, lamina densa and lamina fibroreticularis.

**[search: cell culture epithelium]**

**Self-study: Read about environment of epithelia**

## **27.1. Necessity of epithelial models in biomedicine**

Epithelia act in the organism as a physiological barrier both separating and controlling the exchange of molecules between the luminal and basal sides. To investigate the development of these vector functions, to find improvements for the adhesion of cells on new biomaterials or to analyze the metabolism of newly developed drugs cultures with epithelial cells are of great importance in biomedical research. For example, to elaborate new physiological features or to find out toxic influences of newly developed drugs cultures were prepared from hepatocytes, pneumocytes, skin and endothelium. Cultures from tracheal, esophageal and urothelial epithelium were found to be important models to test the integration of new scaffold materials or to control the profile of differentiation in tissue engineering.

Although epithelia were cultured for decades, in many cases the necessary degree of functionality is not developed under described in vitro conditions. Reasons for the suboptimal differentiation are the very sensitive reactions of isolated epithelial cells to changes of the environmental fluid and the offered substrates. For example, it was shown that the differentiation of cultured epithelial cells is highly dependent on several influences such as the surface of offered biomaterials, molecular adhesion sites, intercellular communication and the environmental fluid situation during culture. Frequently it is forgotten that all of these factors have to interact in culture so that optimal differentiation can take place and that in parallel the development of atypical features by dedifferentiation is prevented.

Thus, a presupposition to reach a high degree of cellular differentiation in epithelia is that the culture experiments are performed under conditions adapted to the individual needs. In turn the environment will stimulate the development of a correct functionality urgently needed in biomaterial testing, tissue engineering and pharmaceutical research.

However, problematic is that the culture environment for the single epithelia must be special.

Adult epithelia from skin, lung and cornea are exposed to air at the luminal and fluid at the basal side. All the other ones act as a functional barrier between two very differently composed fluids at their luminal and basal sides. In contrast, only during the phase of foetal development epithelial cells are exposed to an environment exhibiting the same fluid composition at the luminal and basal side (Fig. 112.1).

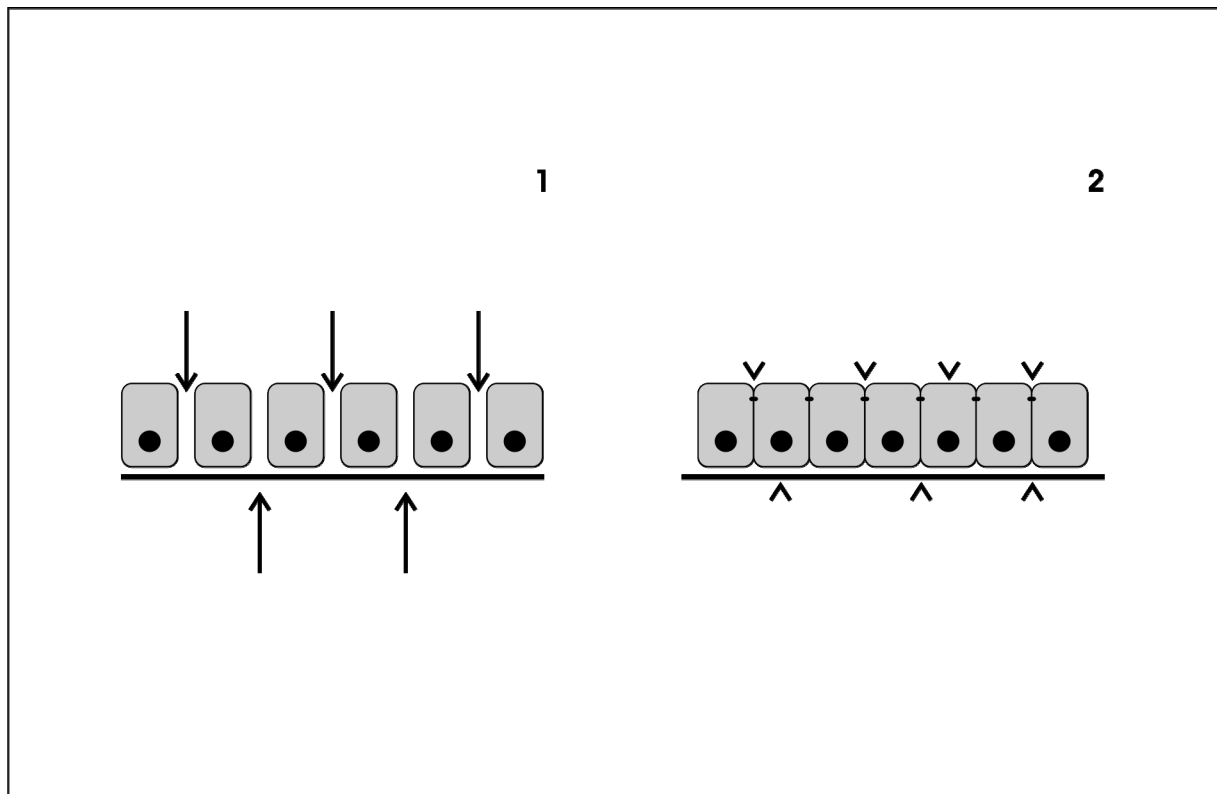


Figure 112: Control of luminal and basal fluid environment on immature (1) and mature (2) epithelial cells resting on a basal lamina. An immature epithelium does not exhibit a barrier function (1). The polarization including the cellular junctions is not developed. In consequence, an uncontrolled passage of molecules is possible via the paracellular shunt (arrows). In contrast, during maturation the epithelium develops polar differentiation (2). The tight junctions are sealing so that the paracellular shunt is closed. Yet the epithelial barrier is intact so that the cells control selective passage of molecules through the epithelium (arrow head).

The leaky characteristics of foetal epithelia are changing during proceeding development. When neighbouring cells are developing intercellular junctions, in parallel the luminal and basal sides of the epithelium become polarized so that a tight functional barrier can establish (Fig. 112.2). When this process is finished, the cells are exposed to different fluid

environments at the luminal and basal sides. Such mature epithelia exhibit an individual but continuous barrier function throughout their life span. They are yet able to control the transport of molecules from one side to the other.

**[search: cell culture epithelium model]**

**Self-study: Get informed about epithelia in bicameral culture systems**

## **27.2. Selecting a suitable strategy for the culture of epithelial cells**

Several possibilities exist to keep epithelial cells in culture. To investigate the proliferation, epithelial cells can be cultured in classical culture dishes. When a serum containing medium is added, the cells will adhere to the polystyrene surface to proliferate until a more or less confluent monolayer is established (Fig. 113.1). However, in most of the cases it is observed that after spreading cells do not develop the required degree of polarization and functional differentiation when kept in this environment. This limiting result is most probably caused due to the unequal distribution of nutrients and oxygen supply. One side of the epithelial cells is resting on the bottom of the dish, where a significantly reduced supply of nutrients and oxygen is found. Only the upper side of cells is fully exposed to the culture medium. Comparing the adherence of epithelial cells on a natural basal lamina, the bottom of a culture dish does not reflect a physiological situation for the developing cells but prevents the development of functional polarization.

For improvement of the culture environment for epithelia filter inserts can be used (Fig. 113.2/3). In this culture set up the cells grow on a filter, which is mounted on a hanging plastic cylinder. Offering a culture medium the epithelial cells adherent to the filter are now exposed to an equal distribution of nutrients and oxygen supply at the luminal and basal sides.

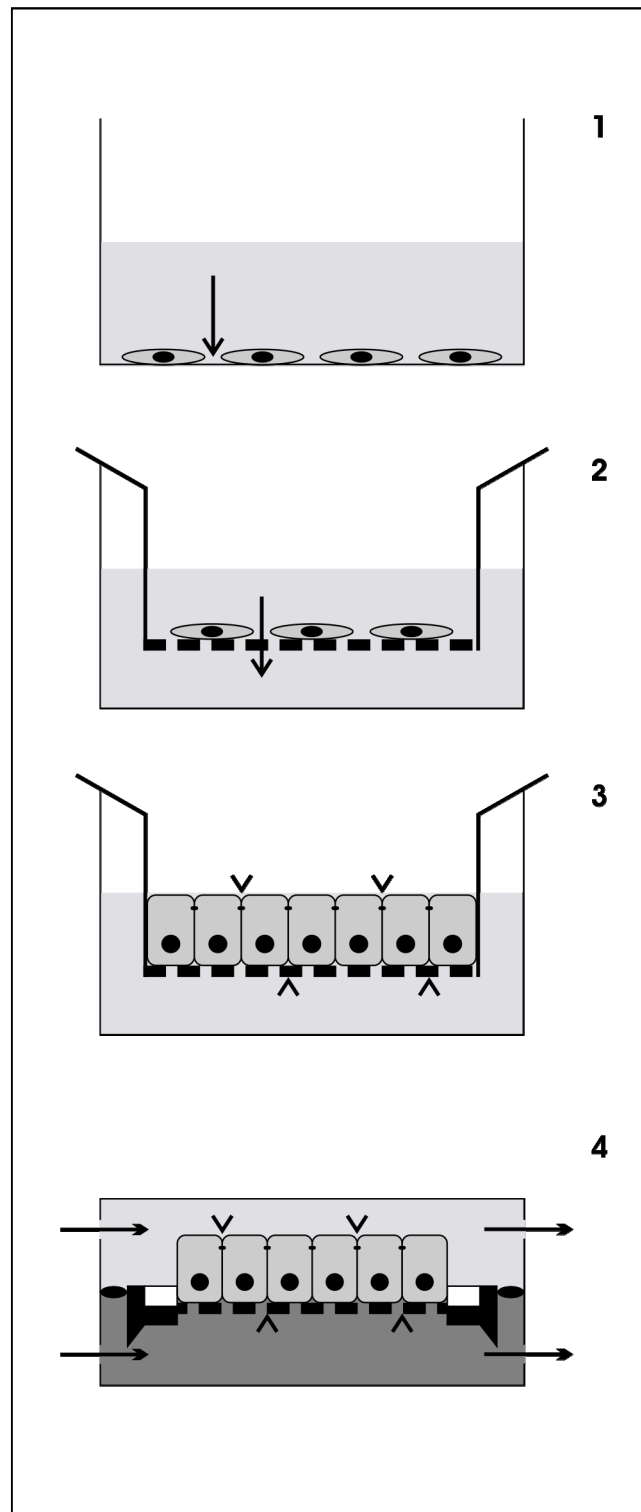


Figure 113: Strategies for the culture of epithelial cells. Conventional culture is performed in a dish (1). To offer a basal lamina substitute cells are cultured on a filter (2). Uncontrolled exchange of fluid occurs between non-confluent cells (arrow). Confluent cells show polarization and intercellular junctions so that exchange of molecules is controlled (3). A typical environment is created, when cells are cultured within a gradient perfusion container (4). Different media are transported at the luminal and basal sides. Intact barrier is indicated by arrow heads.

As long the cells are non-confluent a free exchange of molecules between the luminal and basal sides of the epithelium can take place (Fig. 113.2). However, a mixing of media between the luminal and basal side of the epithelial cell layer represents a non-physiological situation resulting in an atypical biological short circuit current.

When finally a confluent monolayer of epithelial cells is established within the filter insert, yet media of different electrolyte composition can be offered at the luminal and basal sides (Fig. 113.3). At this stage full polarization is developed so that transport features are found to be up-regulated. In a physiological set up with electrodes the activity of an established epithelium can be recognized by measuring the transepithelial potential difference respectively Trans Epithelial Electric Resistance (TEER). However, the maintenance of epithelia kept in a filter insert over long periods of time is problematic. Only a small volume of medium is present in the luminal compartment, while a relatively big volume of medium provides the cells with nutrition in the basal compartment. Due to the static environment a continuous fluid gradient between the luminal und basal sides of the epithelium cannot be maintained over prolonged periods of time.

To provide epithelia by a continuous transport of always fresh medium at the luminal and basal sides, gradient perfusion culture has to be applied (Fig. 113.4). Following that strategy confluent epithelia cells are anchoring on a porous substrate mounted on a tissue carrier within a gradient perfusion culture container. Over prolonged periods of time developing or developed epithelia can be exposed to tissue specific fluid environment. This offer meets individual needs for epithelia resulting in a high degree of cellular differentiation and long term stability over weeks.

**[search: cell culture epithelium TEER]**

**Self-study: Inform yourself about transepithelial transport**

## 28. Technical prerequisites for gradient culture

During gradient perfusion culture an epithelium is maintained as a living barrier, in a permanent fluid gradient and over prolonged periods of time. Applying this technique one can learn about adherence of epithelial cells on selected biomaterials used as basal lamina substitute. Further during gradient perfusion culture epithelia have to tolerate fluid stress, while medium of different composition is transported at the luminal and basal sides over prolonged periods of time. In parallel features of differentiation are up-regulated in the epithelia so that the barrier function can permanently be maintained.

In consequence, specific environmental conditions have to be applied for different types of epithelia. The aim is that they develop in gradient perfusion culture cell biological features closely resembling their functional counterparts found within the organism. However, all these attempts can only be achieved by solving technical needs in gradient perfusion culture in combination with cell biological requirements of applied cells.

By the first view performance of gradient perfusion culture appears quite simple. However, closer examination reveals that gradient perfusion culture is a sophisticated technique, which requires exact knowledge, experience and extensive practical work in order to generate intact epithelia in long term culture experiments.

In the organism epithelia are continuously supplied with always fresh nutrition and oxygen, while the metabolic products are eliminated. To offer such a physiological environment under in vitro conditions sophisticated technical equipment is necessary so that development of a sealing epithelium within the gradient perfusion culture container can take place. The sealing in turn depends on an optimal adherence and confluent growth of cells on a selected substrate. All this occurs at the interface, where an epithelium, a suitable adhesion substrate and a tissue carrier mounted inside a gradient perfusion culture container come in contact.

Only an optimal interaction of the epithelium with the technical equipment such as a tissue carrier mounted within a gradient container will finally result in the arise of an intact physiological barrier. In conclusion, generation of functional epithelia within a gradient perfusion culture container is a surprisingly close interactive process between the culture equipment and the contained cells.

**[search: gradient perfusion culture epithelium]**

**Self-study: Read about gradient perfusion culture experiments**

## **28.1. Adherence of cells on an individually selected substrate**

In a gradient perfusion container epithelia are exposed on the one hand to fluid stress and on the other hand media of different composition are transported at the luminal and basal sides. A prerequisite for an optimal epithelial development under these harmfully appearing conditions is therefore the positive interaction of cells with a substrate selected as a basal lamina substitute. To obtain a perfect confluent growth of cells the substrate has to exhibit an excellent biocompatibility exhibiting numerous adhesion sites.

The spectrum of prospective materials is broad. More rigid support materials such as cellulose, nitrocellulose, polycarbonate, silk or polyester are available in form of membranes, foils, meshes or fleeces. All of these materials are applied to improve the mechanical stability as well as to promote differentiation of adherent epithelial cells. Rigid materials such as nitrocellulose or polycarbonate can best withstand deformation during fluid transport and hydrostatic pressure differences in the gradient perfusion container.

Comparable to the basal lamina the selected substrate has to be permeable and must have enough porosity promoting free exchange of molecules. In consequence pores of filters must



not be smaller than 0.2  $\mu\text{m}$  so that they do not represent a hindering barrier for the exchange of essential molecules.

Filters with pore sizes between 0.4 and 1.0  $\mu\text{m}$  in diameter are used for co-culture as well as transport experiments, secretion analysis and diffusion studies of small molecules. In contrast, filter with larger pores sizes ranging between 3.0 and up to 8.0  $\mu\text{m}$  in diameter are taken for migration, metastasation or chemotactic assays. Transparent and translucent filters are suitable to investigate the growth of cells by light and electron microscopy. Especially translucent filters are also most suitable to register Trans Epithelial Electric Resistance (TEER).

Many of described materials are commercially available as discs measuring 13 mm in diameter. A series of companies is offering membranes in the described size consisting of Polyethersulfone (PES), Mixed Cellulosester (MCE), Nylon (N), Polytetrafluorethylen (PTFE), Polyethylene Tetraphthalate (PET), Polypropylen (PP), Cellulose Acetate (CA), Polypropylen (PP) mesh, Nylon (N) mesh, Polycarbonate track etched (PC) or Regenerated Cellulose (RC). Other materials have to be excised by a punching tool so that they fit into the introduced tissue carrier.

Adherent cells can be cultured on filters for a wide spectrum of applications. It comprises migration, metastasation and chemotaxis. In contrast, epithelial cells can be retained by pores sizes of 0.4 or 1.0  $\mu\text{m}$  in diameter so that they built up a barrier function. Most suitable are these kind of filters also for co-culture experiments. A wide spectrum of applications is dealing with transport, diffusion and secretion of all kinds of molecules. The exchange in mass transport such as electrolyte pumping can be easily analyzed by electric and other methods. Finally the rigid filter support facilitates analysis in light and electron microscopy and immunohistochemistry.

Depending on the experimental design and the type of used cells, membranes can be coated with serum proteins or proteins of the extracellular matrix to improve adhesion of cells. In other cases a sheet of prepared collagen or pieces of the renal capsule are used for cell anchorage. Finally, before cell seeding the selected substrates have to withstand an individual sterilization procedure without losing its perfect properties for cell adherence.

**[search: cell culture epithelium filter]**

**Self-study: Inform yourself about culture of epithelial cells on filters**

## **28.2. Protection of cells within a tissue carrier**

In order to prevent damage of adhering epithelial cells the selected substrates are mounted in a Minusheet®. To provide a certain stability the tissue carrier is injection molded with Procan® (Fig. 114).

Mounting for culture is performed by placing the selected substrate into the base part of a tissue carrier (Fig. 114.1). Then a white tension ring is pressed in (Fig. 114.2). The fixation of the selected substrate between the base part and the tension ring, which leads to an exact orientation, stabilizes the developing epithelium, prevents mechanical damage and facilitates handling during transport from the dish to the gradient container.

However, it is important to point out that the mounting of a substrate within a tissue carrier does not lead to a sealing between the upper and lower part of the tissue carrier! In contrast, the biological sealing is exclusively made by the growth of cells on the substrate and the adhesion to the material of the tissue carrier.



Figure 114: Mounting a tissue carrier (1) with a substrate (2) and transfer to a 24-well plate (3). For mounting a selected substrate such as a filter with 13 mm diameter is placed in a base part (1, black) and fixed by a tension ring (2, white). After sterilization the mounted tissue carrier is placed in a 24-well culture plate for cell seeding (3).

After sterilization the tissue carrier is transferred by forceps to a 24-well culture plate (Fig. 114.3). Yet medium is filled until the meniscus is just wetting the substrate. Then cells are

transferred by a pipette within a small aliquot of medium. Since the tissue carrier is resting on small protrusions, medium supply is given at its upper and lower side. It depends on the experimental design if only a single cells type is adherent on the substrate. For example, a second cell type can be seeded, when the tissue carrier is turned up side down. Performing this way numerous thinkable kinds of co-culture experiments become possible. Examples are the blood-urine barrier within the renal glomerulus or the blood-brain barrier

**[search: cell culture epithelium tissue carrier]**

**Self-study: Read about epithelial cells cultured on tissue carriers in perfusion culture**

### **28.3. Epithelia within a gradient perfusion container**

To expose the seeded epithelial cells to different fluids at the luminal and basal sides, the tissue carrier has to be placed in a gradient perfusion culture container Fig. (115.1,3,5). The flat form of a tissue carrier guarantees an exact geometrical placement of the growing epithelium within a gradient container after closing the lid (Fig. 115.2,4,6). Further this specific construction facilitates later the uniform supply of medium to the luminal and basal sides of seeded cells.

A gradient container typically can hold one (Fig. 115.1,2,5,6) or six tissue carriers (Fig. 115.3,4). Gradient culture experiments under visual control can be performed using a special microscope container (Fig. 115.5,6). In this case the tissue carrier mounted with a transparent 13 mm diameter substrate is placed between a base part and a lid containing glass covers.

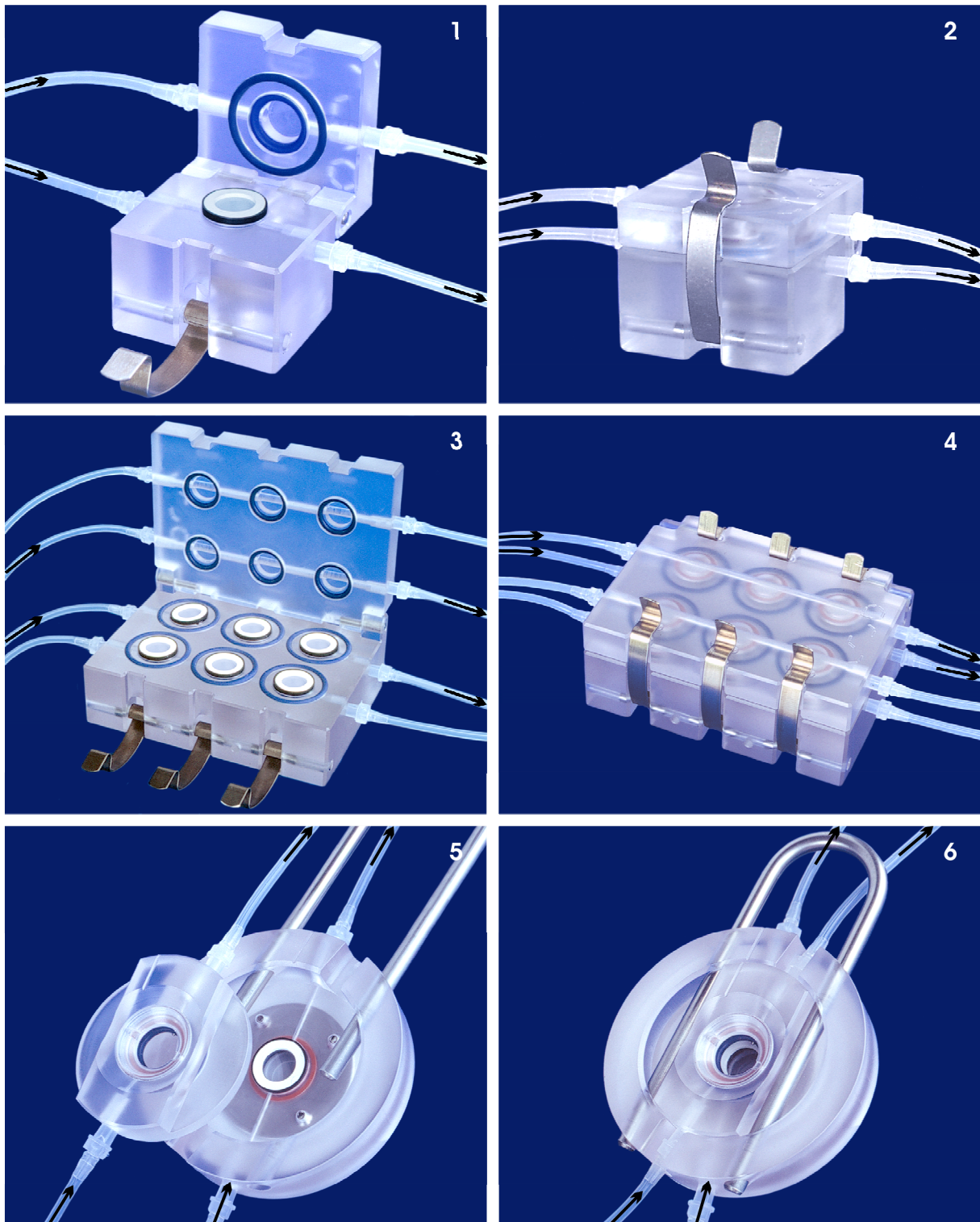


Figure 115: Examples for gradient perfusion culture containers to hold Minusheet® tissue carriers. One (1) or six (3) tissue carriers are placed on the base part of a container. After closing the lid (2, 4) the tissue carriers are fixed in position so that transport of medium becomes possible at the luminal and basal sides. Gradient culture experiments under visual control are performed by a special microscope container (5, 6). First the tissue carrier is transferred to the base part (5) of the microscope container. The lid is closed by a special metal clamp (6).

A gradient container is CAD-constructed and CNC-machined out of Makrolon® polycarbonate in a specialized work shop (Minucells and Minutissue, Bad Abbach, Germany). During fabrication the machined surfaces are treated with a specific lubricant. This treatment makes the inner surfaces of the container non-attractive for cell attachment and causing cells to grow exclusively on the inserted substrate.



Figure 116: Illustration of a gradient perfusion culture experiment running under atmospheric air on a laboratory table. Media from storage bottles (left side) are transported (1 ml/h) by a peristaltic pump to a gas expander module (1) and then to a gradient container (2). The waste medium is collected in the bottles on the right side. A thermo plate and a lid (not shown) maintain the correct temperature at 37°C.

In gradient perfusion culture experiments the inserted tissue carrier is continuously held in position between the base part and the lid of the used container (Fig. 115.2,4,6). By this principle of construction the luminal and basal side can be provided with individual media mimicking a tissue-specific environment for epithelia. Fresh culture medium is continuously transported into the gradient container at one side, while the metabolized medium is removed at the other side.

Described perfusion culture experiments with a gradient container are performed under room atmosphere at a constant temperature of 37°C. This temperature can be achieved by placing the gradient perfusion culture container onto a thermo plate (MEDAX-Nagel, Kiel, Germany) covered by a removable lid (Fig. 116).

**[search: gradient perfusion culture container]**

**Self-study: Get informed about the culture of epithelia in a gradient container**

## 29. Fluid environment in gradient culture

Gradient perfusion culture experiments can be performed principally with all culture media. For example, published papers from several groups show that gradient perfusion culture was successfully performed with Hanks' Balanced Salt Solution (HBSS), Dulbecco's Modified Eagle Medium (DMEM), Iscove's Modified Dulbecco's Medium (IMDM) or others. Depending on the experimental design some of the media contain serum as a supplement, while in other series serum free or chemically defined media were applied.

### 29.1. Providing always fresh medium

A typical medium used for gradient perfusion culture in our laboratory is chemically defined (IMDM; order # 21980 - 032; Gibco/Invitrogen, Karlsruhe, Germany). It is used for the generation of renal collecting duct (CD) epithelia. In individual series aldosterone ( $1 \times 10^{-7}$  M; Sigma-Aldrich Chemie, Munich, Germany) is added. To prevent growth of microorganisms 1% antibiotic-antimycotic solution (Gibco/Invitrogen) is used.

First of all a culture medium for perfusion culture has to be prepared that the pH stays constant over an extended period of time.

To equilibrate IMDM for the maintenance of a constant pH under room atmosphere, 1 ml is filled in each well of a 24-well plate, then an increasing concentration between 10 and 50 mmol/l HEPES (Nr. 15630-056, Gibco/Invitrogen) and/or 0.1 to 1.5% Buffer All (Nr. B-8405, Sigma-Aldrich Chemie) is added. For the following 24 hours the culture plate is incubated on a thermo plate at 37°C under atmospheric air. Finally the pH in each well is measured with a pH meter or with a whole blood analyzer such as the Stat Profile 9 Plus (Nova Biomedical, Rödermark, Germany). In that way the HEPES respectively Buffer All concentration, which yields a medium pH between 7.2 and 7.4 under room atmosphere, can easily be determined.



Determination of the pH by visual inspection of the phenol red color is not recommended, since phenol red is not sensitive enough to indicate small pH shifts around the physiological range between pH 7.2 and 7.4.

Medium used in gradient perfusion culture must provide sufficient oxygen. Thus, oxygenation is performed by transporting media through 1 m long highly gas-permeable silicone tubes with 1 mm inner and 3 mm outer diameter to allow optimal exchange of gas. Since the tube is gas-permeable, it guarantees for oxygen optimal diffusion between surrounding atmospheric air, wall of the silicone tube and the contained culture medium.

<b>pH 7.4</b>	<b>pO<sub>2</sub></b> mmHg	<b>pCO<sub>2</sub></b> mmHg	<b>Na<sup>+</sup></b> mmol/l	<b>K<sup>+</sup></b> mmol/l	<b>Cl<sup>-</sup></b> mmol/l	<b>Ca<sup>++</sup></b> mmol/l	<b>Glucose</b> mg/dl	<b>Osmolarity</b> mOsm
<b>Luminal</b> perfusion	171	11	<b>144</b>	3.9	97	0.8	375	297
<b>Basal</b> perfusion	172	10	<b>120</b>	4	86	1.1	370	255
<b>Serum</b>	99	40	142	4	103	2.5	100	290

Figure 117: Physiological parameters of medium at the luminal and basal sides on the inlet of a gradient perfusion culture container including a sealing epithelium. During gradient perfusion culture the epithelium is exposed to a Na<sup>+</sup>-load at the luminal side (144 versus 120 mmol/l Na<sup>+</sup>). As comparison parameters of serum are added.

To allow continuous control of the fluid environment throughout an experiment, medium for analysis is drawn with a sterile syringe through a T- connector in the tube directly before or after the gradient perfusion culture container. Since the epithelia are exposed to a luminal and basal fluid environment, specimens of media are collected additionally from the luminal as well as the basal medium.

Regarding the physiological parameters it illustrates that for example 171 mmHg O<sub>2</sub> are present in gradient perfusion culture, while only 99 mmHg O<sub>2</sub> is found in serum (Fig. 117).

The aim of the present experiments was to expose renal collecting duct epithelia to a NaCl load at the luminal side. In consequence, to the luminal culture medium NaCl was added, until 144 mmol/l Na<sup>+</sup> are detected, while in the basal IMDM medium 120 mmol/l Na<sup>+</sup> were contained. Controlling these parameters over 14 days at the outlet of the gradient container revealed that the luminal-basal NaCl gradient (144 versus 120 mmol/l Na<sup>+</sup>) was maintained by the epithelium for the complete period of culture.

**[search: gradient perfusion culture minuth]**

**Self-study: Inform yourself about gradient perfusion culture**

## **29.2. Presence of gas bubbles during medium transport**

Epithelia kept within a gradient perfusion culture container must be supplied with a constant flow of always fresh and oxygenated medium. Only this kind of medium transport leads to a constant nutrition, avoids unstirred layers of fluid achieving thereby a high degree of functional differentiation.

The transport of culture medium is best performed using a slowly rotating peristaltic pump designed to deliver very low, adjustable and parallel pump rates. It is favorable for the development of epithelia within a gradient culture container to transport medium at a rate of 1 ml/h for a culture period of 2 weeks using an IPC N8 peristaltic pump (ISMATEC, Wertheim, Germany) (Fig. 116).

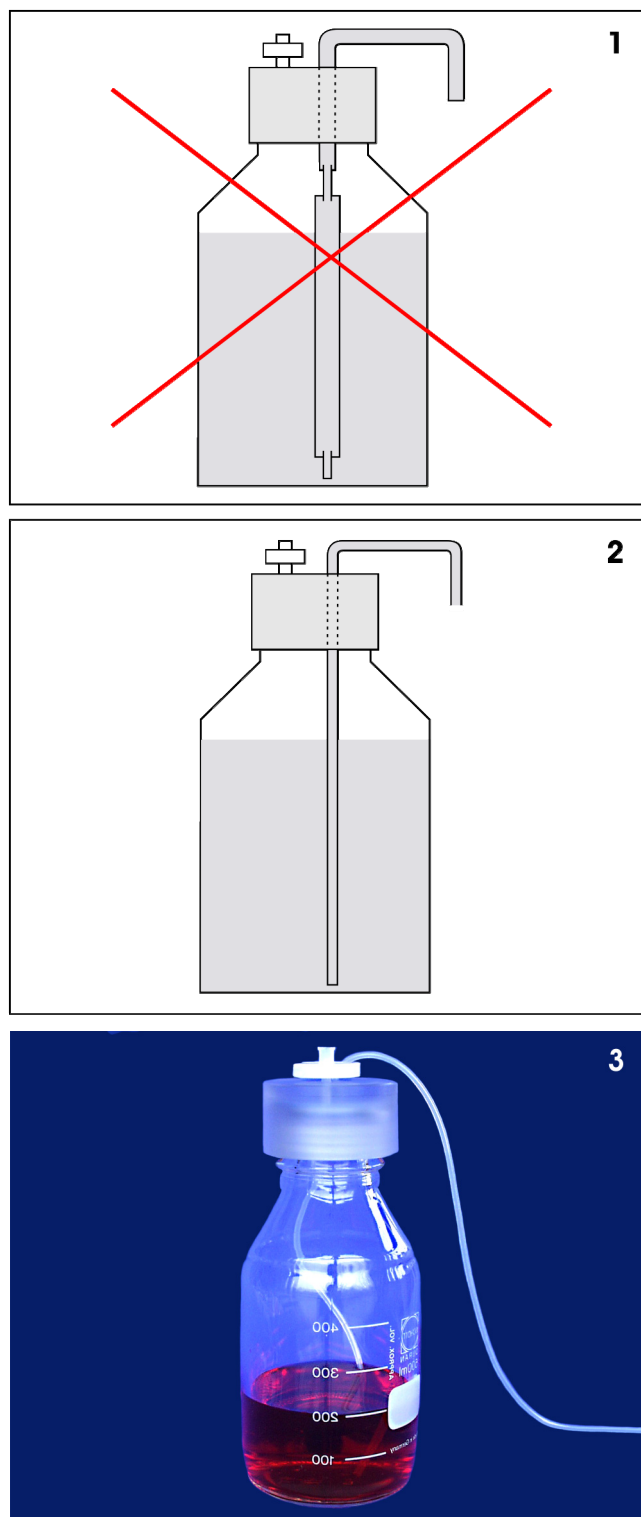


Figure 118: Illustration of unsuitable (1) and suitable (2, 3) bottle screw caps for medium transport. Large dead fluid volume, connectors and various diameters of the tube including the screw cap support formation of gas bubbles during medium transport (1). In contrast, perfect transport of medium with strongly decreased gas bubble formation is obtained by a continuous piece of silicone tube. It is conducted through the screw cap to allow medium transport without connectors (2, 3).

In this culture set up fresh medium is continuously transported from a storage bottle to the gradient container. The metabolized medium is collected in a separate waste bottle and consequently not re-circulated. This procedure guarantees on the one hand a constant nutrition and oxygen supply, while on the other hand a continuous elimination of harmful metabolic products is obtained and paracrine factors can be kept on a constant level. All these factors together are leading to a controlled provision with nutrition and respiratory gas.

Transport normally starts by drawing the medium up from the bottom of a storage bottle through tubes connected with the inside of the bottle cap (Fig. 118). Large dead fluid volume, connectors and various diameters of the tube including the screw cap support formation of gas bubbles during medium transport (Fig. 118.1).

The suction force of the pump has to be high enough in order to surmount the difference in elevation of medium within the storage bottle and the capillary forces occurring in the lumen of thin silicone tubes. Surprisingly, this relatively high negative pressure in combination with a low pump rate of 1 ml/h meets frequently an increased diffusion of gas through the tube wall. These effects in cooperation is causing arise of harmful gas bubbles within the transported medium.

The biological need in gradient perfusion culture is to transport media rich in oxygen by avoiding as much as possible formation of gas bubbles. For this purpose special bottle caps and tubes were designed, which facilitate the transport of medium from the storage bottles to the gradient container and up to the waste bottle.

One small opening in the cap allows a continuous piece of silicone tube to pass through avoiding material transitions along the fluid path (Fig. 118.2,3). This simple construction considerably reduces gas bubble formation during elevation of medium from the storage

bottle. Another small opening in the screw cap holds a sterile filter allowing atmospheric gas to enter the storage bottle as medium is drawn from it (Fig. 118.3).

**[search: perfusion culture gas bubbles]**

**Self-study: Inform yourself about formation of gas bubbles in perfusion culture**

### **29.3. Elimination of gas bubbles during medium transport**

In gradient perfusion culture medium must be transported in two parallel tubes with the identical rate. However, the transport of medium at low pump rates such as 1ml/h in parallel tubes is influenced by surprisingly numerous factors such as tube material, individually capillary forces in the fluid path, hydrostatic pressure, material transitions and randomly occurring gas bubbles. Performing gradient perfusion culture experiments one has to accept that these rheological influences are not identical in both of the parallel transporting tubes.

In consequence, pressure differences arise between the luminal and basal compartment of the gradient perfusion culture container during transport of medium. If this pressure difference is not compensated, this can lead to a loss of the functional seal caused by mechanical damage of the epithelial barrier.

Frequently it is observed that during transport of medium gas bubbles arise in the lumen of afferent tubes connected with the gradient container. Over time the bubbles increase in size. Further they can leave the site of primary accumulation to concentrate unpredictably within the afferent tubes, inside the gradient culture container but also in the efferent tubes. These gas bubbles are harmful, since they lead to pressure differences resulting in a damage of the inserted epithelium. Consequently, gas bubbles have to be eliminated before reaching the gradient container.

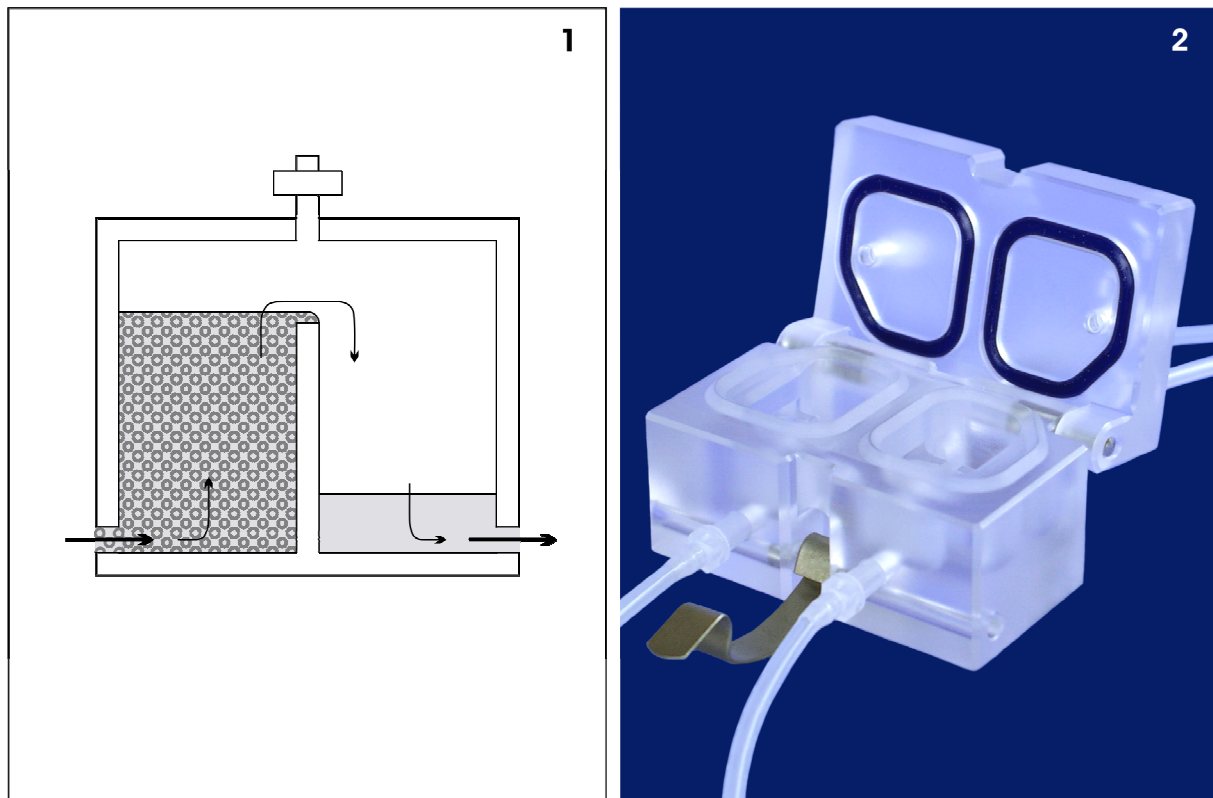


Figure 119: Illustration of a gas expander module. Medium saturated with oxygen enters at one side of the module. Inside the module medium crosses a barrier allowing gas bubbles to separate. At the other side of the module outflow of medium takes place.

In consequence, for the elimination of gas bubbles a gas expander module was constructed, which is linked in front of the gradient container (Fig. 119).

In the gas expander module the culture medium has to cross a barrier leading to a separation of gas bubbles from the liquid phase (Fig. 119.1). During this process gas bubbles are eliminated but the content of solved oxygen within the transported medium remains unaffected.

Further the gas expander module can act as an absorber for pressure differences between the luminal and basal compartment of the gradient perfusion culture container. For that reason it is constructed as a parallel and bicameral module (Fig. 119.2).

Each chamber of the module is ventilated through a port at the top. This port can be used for bridging the gas phase of both chambers. This bridging again results in identical pressures of liquid in the lining tubes before reaching the gradient container.

**[search: perfusion culture gas bubbles elimination]**

**Self-study: Read about elimination of gas bubbles in perfusion culture**

## **29.4. Preventing gas bubbles inside the gradient container**

Not only in the afferent tubes but also within the gradient container presence of gas bubbles can lead to pressure differences leading in turn to damage of the growing epithelium. To avoid the accumulation of gas bubbles to a critical size within the gradient container the technical contribution is to locate the medium inlet and outlet to the tangential aspects of the tissue carrier (Fig. 120.1).

During culture run the gradient container can be turned from its base to its lateral side. Medium enters now at the lowest point, while the outlet is located at the highest point of the fluid compartment facilitating also the outflow of gas bubbles (Fig. 120.1).

Due to this improved flow geometry gas bubbles will not accumulate but are continuously eliminated during medium transport as long they are small. However, problems arise, when they fuse to increase. Further, during transport of medium gas bubble leave at unpredictable times to accumulate at non calculable sites within tubes, at material transitions and within the container. A crucial site for accumulation of gas bubbles are the outlets of a gradient perfusion culture container and the tubes conducting to the waste bottles.

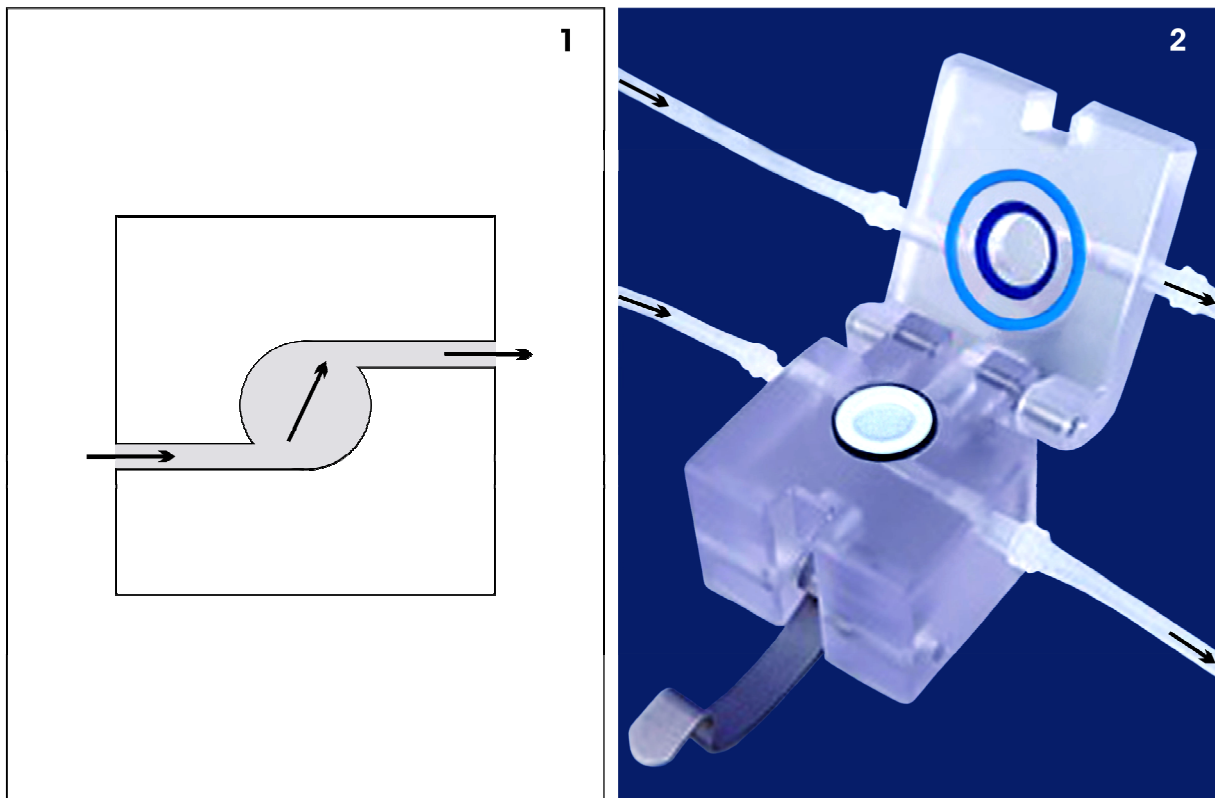


Figure 120: Illustration of a re-designed gradient perfusion culture container. Medium inlet and outlet are located at the tangential aspects of each tissue carrier. When the gradient container is turned to the lateral side, medium enters at the lowest point (left side), while the outlet (right side) is found at the highest point of the fluid reservoir leading to a continuous and efficient elimination of gas bubbles.

Thus, the combination of newly constructed bottle screw caps including special tubes, a gas expander module and a re-designed gradient perfusion culture container with a tangential inlet and outlet for medium will lead to a drastic reduction of gas bubbles throughout the culture period, which in turn increases the harvest of intact epithelia.

**[search: gradient perfusion culture minuth]**

**Self-study: Get informed about gas bubbles in a gradient perfusion culture container**



## 30. Medium flow in gradient perfusion culture

In gradient perfusion culture epithelia have to be provided in parallel at the luminal and basal sides with medium. During a culture period between several days and up to weeks the epithelia must withstand the present rheological forces. In turn they have to maintain a physiological barrier between the luminal and basal compartment in the gradient container as shown for a renal epithelium (Fig. 121).

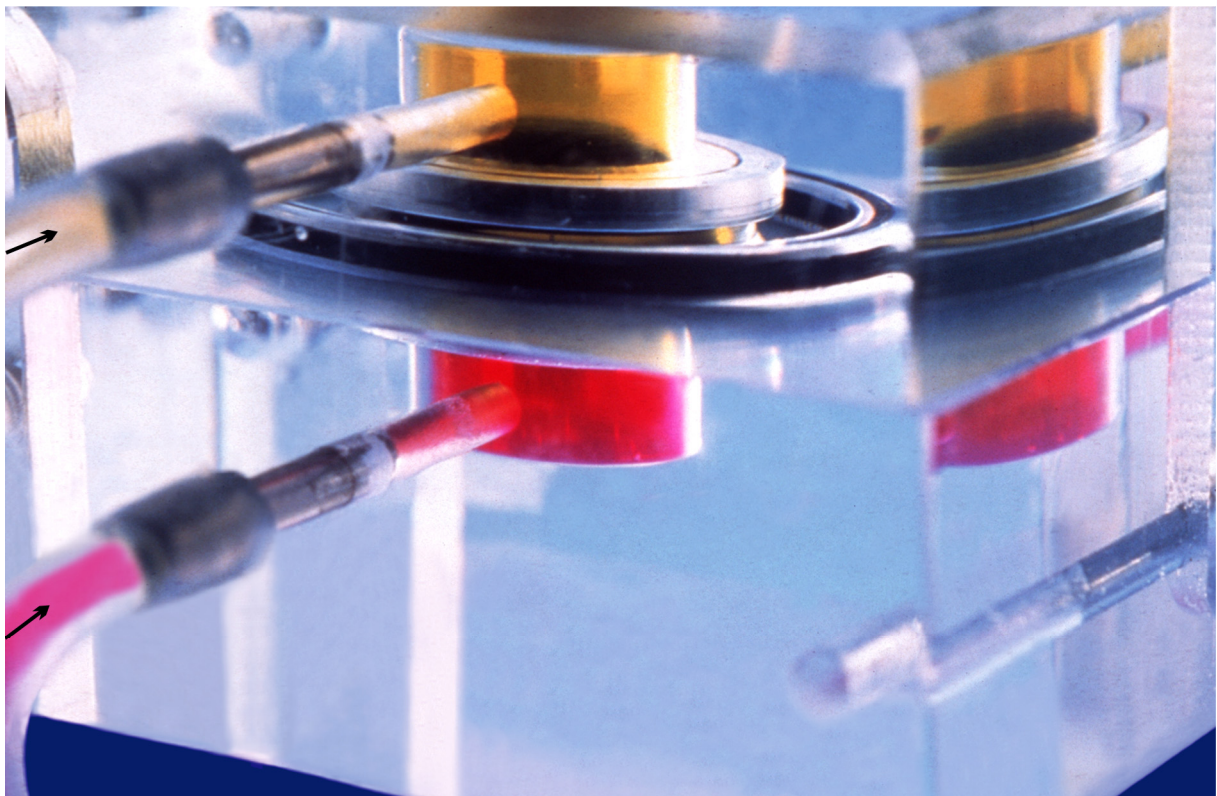


Figure 121: Flow of urine like fluid at the one side and institial fluid at the other side of a renal epithelium kept in a gradient perfusion culture container.

The same barrier functions exhibit epithelia at the individual tissue or organ sites where they derived. In the case of damage, epithelia cannot fulfill their tasks anymore due to a permanent biological short circuit current depressing any unidirectional molecule transport or barrier functions.

### 30.1. Sealing of the epithelium pilots flow

To ensure equal transport rates of medium in gradient perfusion culture experiments parallel channels of a peristaltic pump are used (Fig. 122).

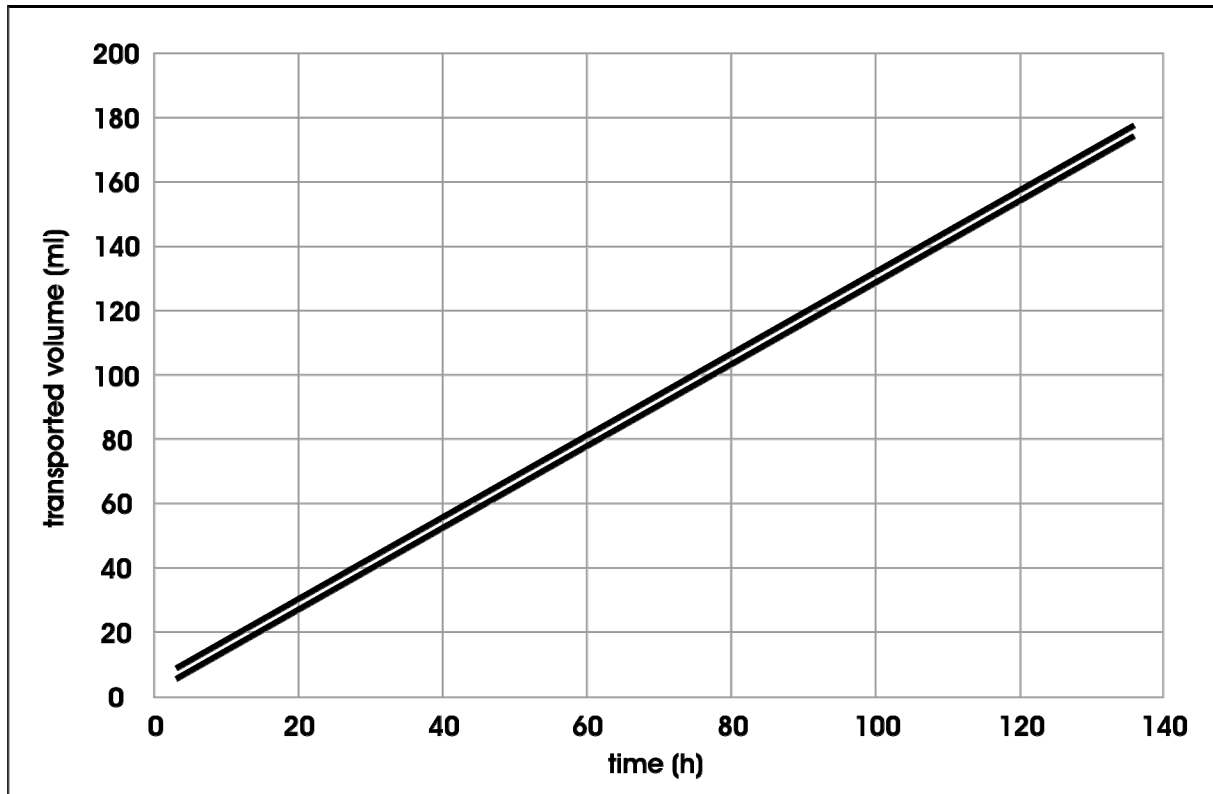


Figure 122: Intact flow of medium inside a gradient perfusion culture container is directed by identical transport rates of medium in parallel channels of a peristaltic pump.

Principally a parallel transport of medium is simply to perform, when two separate channels including two separate tubes are used (Fig. 123.1,2). In this scenario either the same (Fig. 123.1) or different kinds of fluid (Fig. 123.2) can be transported with the identical rate.

However, an identical transport rate of medium is not any more achieved when the parallel tubes are bridged by a gradient container including a non-sealing epithelium (Fig. 123.3,4). In this scenario an uncontrolled mixing of both media occurs inside the gradient container.

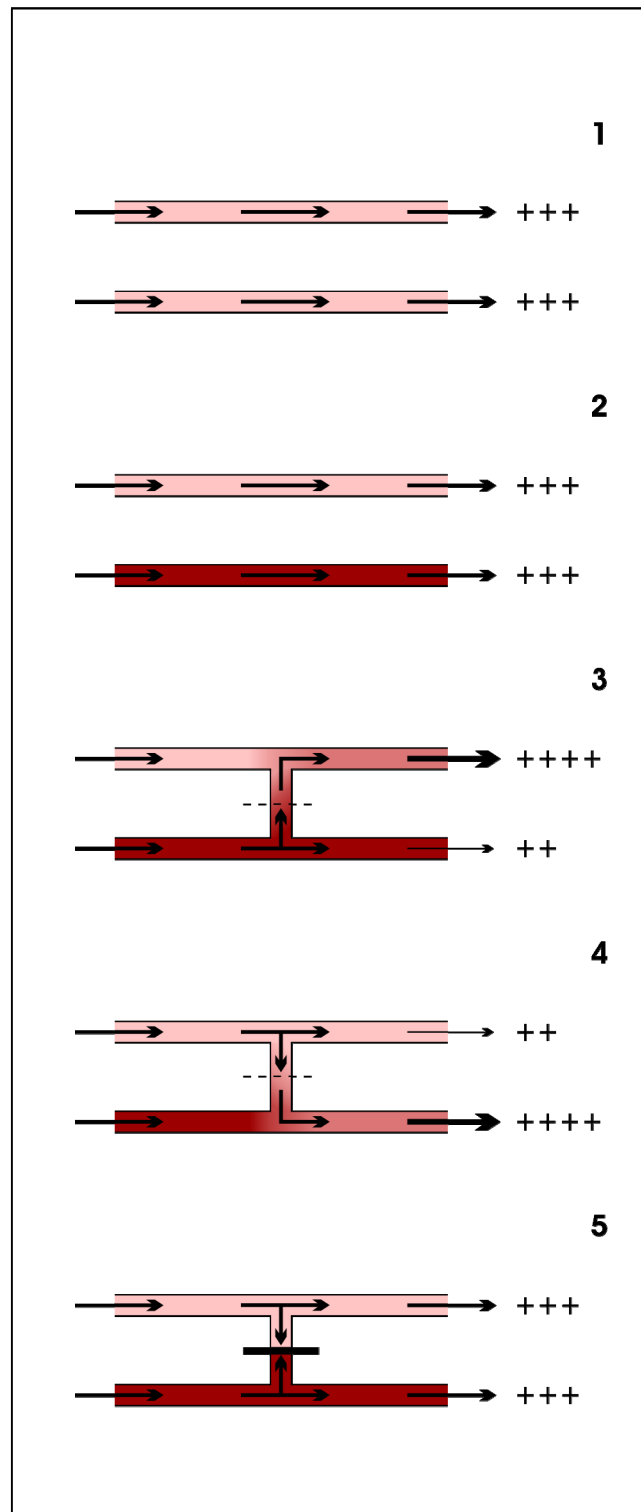


Figure 123: Flow of medium inside a gradient perfusion culture container. Equal transport rates of the same (1) or different (2) media are obtained, when parallel channels are used. Identical transport rates are not achieved, when parallel tubes are bridged by a non-sealing epithelium (3, 4). Mixing of both media is leading to unequal transport to the one (3) or to the other (4) waste bottles. Parallel transport of medium is only obtained when both tubes are separated by a gradient culture container including a perfectly sealing epithelium (5).

However, experimental reality shows that the degree of mixed medium cannot be predicted, which in turn leads to an unequal transport of medium either to the one (Fig. 123.3) or to the other (Fig. 123.4) waste bottle. In consequence, parallel transport of medium is only obtained when both tubes are separated by a gradient culture container including a perfectly sealing epithelium (Fig. 123.5).

**[search: gradient perfusion culture medium flow]**

**Self-study: Get informed of physiological sealing of different epithelia**

## **30.2. Possible leaks within the gradient container**

It has been demonstrated that exclusively the epithelium is responsible for the maintenance of a functional barrier and not the used substrate or the tissue carrier alone (Fig. 124). In consequence, when a tissue carrier with a growing epithelium is inserted in the gradient container in each case crude handling has to be avoided. Wrong manipulation may produce mechanically micro-injuries in the cell layer, which results in leakage of the barrier.

Most important, although the physiological seal is made by the epithelium, a close cooperation with the selected substrate and the tissue carrier is necessary (Fig. 124.1). Only in this case the epithelium maintains a barrier so that different media can be transported at the luminal and basal sides.

Since the epithelial cells contact neighbouring cells but also materials contained in the substrate and the tissue carrier, in turn different kinds of leakage within a gradient perfusion culture container can arise.

An epithelial leak will appear when the cultured cells do not grow in perfect confluence on the substrate or do not develop a complete sealing between neighbouring cells due to imperfect cell to cell contacts (Fig. 124.2).

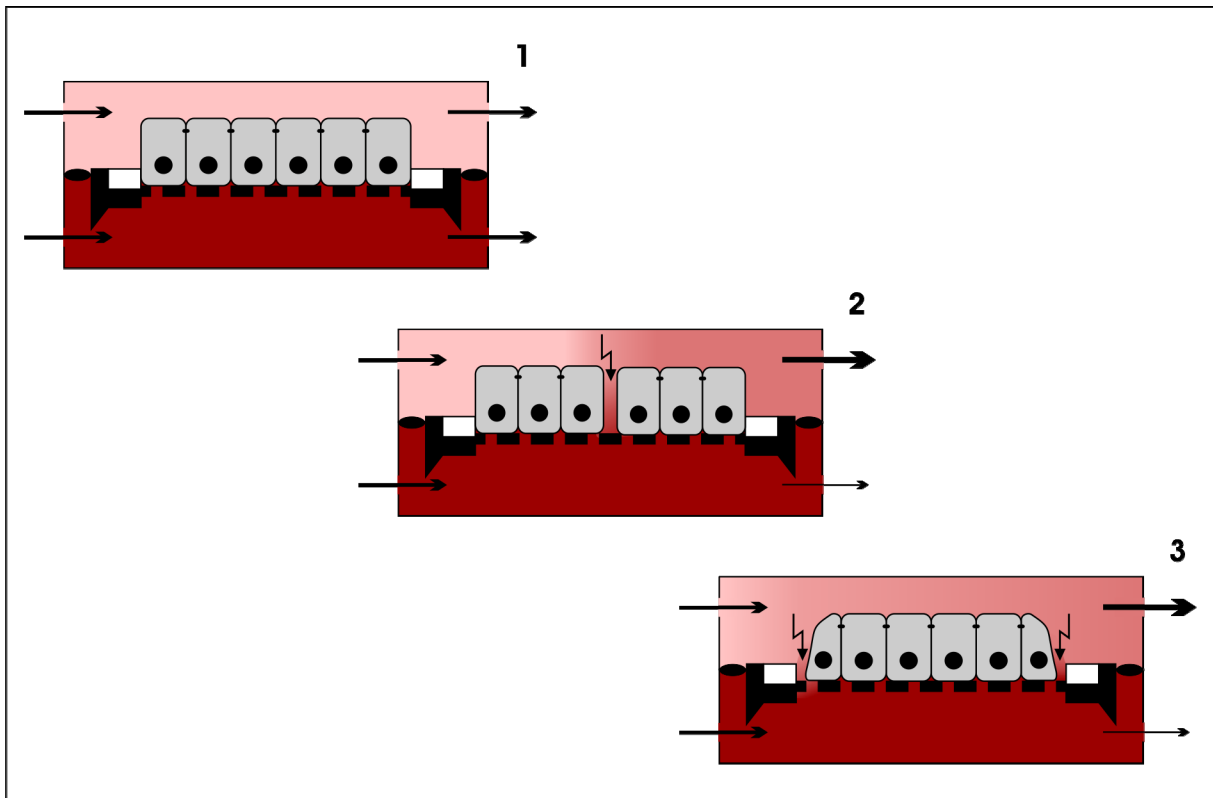


Figure 124: Illustration of epithelial seal and damage during run of gradient perfusion culture. For development of a functional epithelial barrier it is essential that the neighbouring cells produce a perfect seal in combination with the offered substrate mounted in the tissue carrier (1). In contrast, leakage of the seal will occur when incomplete distribution or a non perfect confluence of epithelial cells is present (2). Edge damage occurs at sites, where cells, substrate and tissue carrier do not stand closely in contact with each other (3).

In contrast, edge damage will be found at sites, where epithelial cells, substrate and polymer material of the tissue carrier do not stay in close functional contact (Fig. 124.3).

**[search: gradient perfusion culture edge damage]**

**Self-study: Inform yourself about leaks in epithelia**

### **30.3. Destruction of epithelia due to embolic obstruction**

Damage of an epithelium within a gradient perfusion culture container may occur due to pressure differences by lifting one of the waste bottles higher than the other. In consequence, the fluid meniscus within all of the bottles has always to stand in the height of the tissue carrier mounted within the gradient perfusion container.

Further only a careful mounting of the tissue carrier, a slow transport of medium and a perfect growth of cells on a substrate keeps the gradient container separated in a luminal and basal compartment so that mixing of the two media does not occur (Fig. 121, 123.5, 124.1).

The crucial technical problem in gradient perfusion culture is that the epithelial cells grow on a support, which discontinuously vibrates like the skin of a drum between the luminal and basal compartment. The vibration of the epithelium is depending on the environmental fluid pressure to a more or less extent.

As long as the epithelia are kept on mechanically rigid support materials such as nitrocellulose or polycarbonate filters, the transport of media will not severely affect the barrier function, since a certain mechanical stability is given by compensating the fluid pressure difference.

However, in multiple cases epithelia are kept on mechanically more fragile collagen supports. This experimental set up can only be well preserved in the gradient container as long as a fluid pressure difference between the luminal and basal compartment is not present or is minimized during run of the culture experiment.

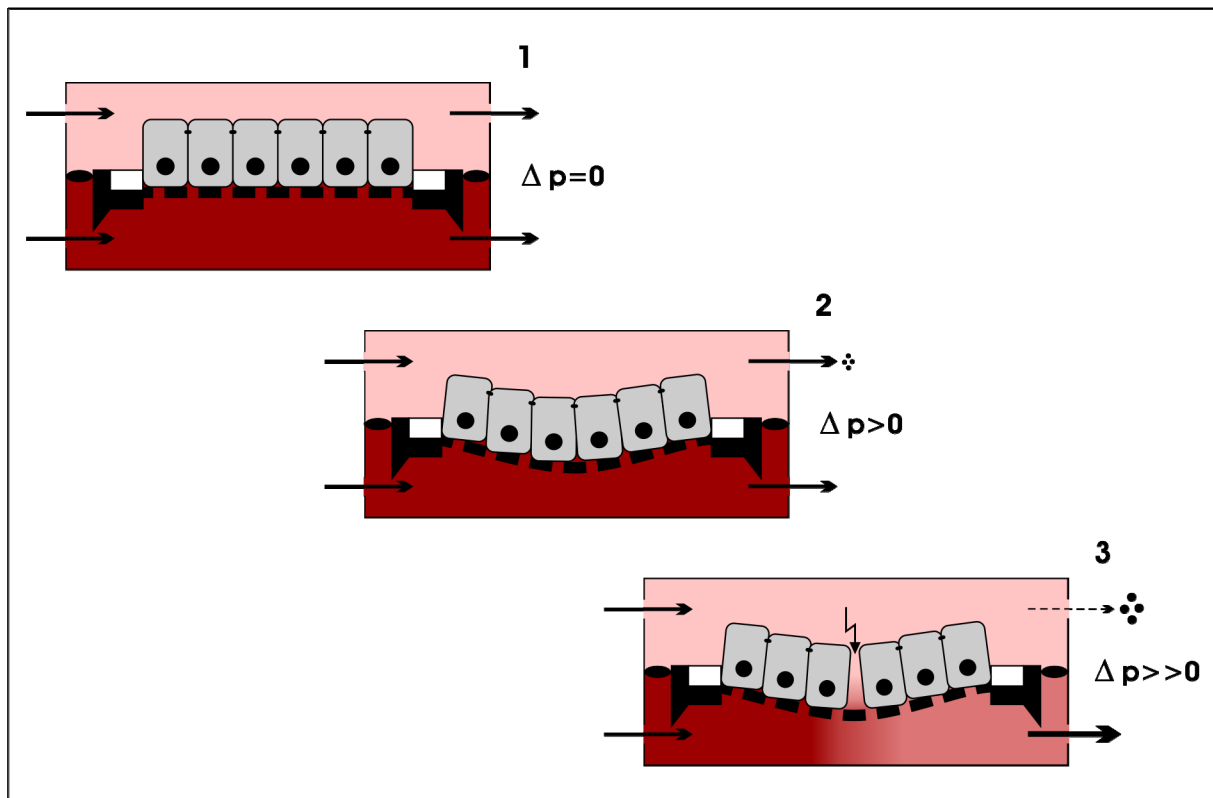


Figure 125: Influence of fluid pressure differences on epithelial integrity during gradient perfusion culture. No damage of the epithelium will occur as long as pressure is identical at the luminal and basal side ( $\Delta p = 0$ ) (1). In contrast, little gas bubbles (little black dots) for example in the luminal efferent tube, will increase the pressure in the luminal compartment ( $\Delta p > 0$ ) resulting in an extension of the epithelium towards the basal side (2). When the number or size of gas bubbles accumulates (big black dots) in the luminal efferent tube a further increase in pressure occurs ( $\Delta p >> 0$ ) so that the epithelium finally disrupts during run of the experiment (3).

Pressure differences are not only present in afferent tubes, but also in efferent tubes. They can easily arise for example by lifting only one of the waste bottles, but also by embolic obstruction. During experimental run gas bubbles are not only imported, but they can also develop within the gradient container due to the respiratory activity of the cultured epithelium.

In contrast, perfect culture in a gradient container only occurs in the absence of a pressure difference between the luminal and basal compartment (Fig. 125.1;  $\Delta p = 0$ ). However, during a long term culture period the number of gas bubbles as well as their diameter can increase.

Part of gas bubbles is then leaving the gradient container towards an efferent tube. This in turn can lead to obstruction of the medium outflow (Fig. 125.2).

The obstruction with gas bubbles randomly affects for example only the basal or the apical medium transport. The resulting fluid pressure difference within the gradient container causes a protrusion of the epithelium towards the side of lower pressure (Fig. 125.2;  $\Delta p > 0$ ). An increasing pressure difference will finally result in the mechanical disruption of the epithelial barrier (Fig. 125.3;  $\Delta p > > 0$ ). The same situation is given, when the obstruction is not found in the apical medium flow but in the basal compartment.

In conclusion, to obtain perfectly sealing epithelia within a gradient perfusion culture container liquid pressure differences including obstruction by gas bubbles has to be avoided.

**[search: gradient perfusion culture gas bubbles]**

**Self-study: Read about performance of gradient perfusion culture experiments**

## **30.4. Monitoring the epithelial barrier over long periods**

After the careful insertion of a tissue carrier into a gradient perfusion culture container both the apical and the basal sides of the epithelium are provided with individual media. Depending on the type of epithelium and the state of development two identical or two different medium compositions are offered. If an immature epithelium is inserted a sealing barrier between the luminal and basal compartment is not established yet. Consequently, an uncontrolled exchange of fluid between the luminal and basal aspect of the epithelium may occur until it has matured during ongoing culture.



Epithelial barrier function can be registered by the online monitoring of Trans Epithelial Electric Resistance (TEER) by electrodes over a short time period within hours. In this case the course of TEER can be analyzed simultaneously to the permeability of sodium fluorescein. However, registration of TEER over several days in a continuous process is less suitable to control the development and maintenance of a sealing barrier during long term gradient perfusion culture experiments. Electrode fouling, resulting in tissue damage and a decreased degree of cellular differentiation were observed.

Addition of radiolabeled molecules to the luminal culture medium in combination with detection of radioactivity in the basal medium path seems possible to investigate the maintenance and integrity of an epithelium. However, an application of radiolabeled molecules is not preferred since non-radioactive fluorescent molecules such as sodium fluorescein can be employed better to monitor the quality of the epithelial barrier throughout a long term gradient perfusion culture period.

A simple and cost saving method to detect non-physiological epithelial leaks is to transport IMDM containing phenol red (order # 21980 - 032; Gibco/Invitrogen) at the luminal compartment, while IMDM without phenol red (order # 21056 - 023; Gibco/Invitrogen) is provided at the basal side of the gradient container (Fig. 121).

Photometrically recorded traces of phenol red indicator in the clear medium indicate mixing of both media and demonstrate the leakage of the epithelium. Only experiments with a perfect parallel transport of red and clear media in the waste bottles indicate a successful barrier function.

A further way of detecting epithelial leakage is to collect medium samples just before and after the luminal and basal compartment of the gradient perfusion container and to analyze these samples in a blood electrolyte analyzer (Fig. 117). As the media used in the luminal

and basal fluid path differ in their electrolyte composition, the stability of the fluid gradient can be controlled over days. In this case the concentrations of key electrolytes (e.g.  $\text{Na}^+$  or  $\text{Cl}^-$ ) or osmolarity in medium are compared in afferent and efferent tubes at the luminal and basal compartment of the gradient perfusion culture container.

**[search: monitoring epithelial barrier]**

**Self-study: Inform yourself about monitoring of epithelia during culture experiments**

## 31. Harvest of differentiated epithelia

A parallel transport of medium during experimental run of gradient perfusion culture illustrates that an intact epithelial barrier is established. The biological seal is produced on the one hand by a tight contact between neighbouring epithelial cells and on the other hand by a close interaction to the adhesion substrate mounted in a tissue carrier.

Regarding the progress of epithelial development it appears as a rather complex cell biological process. It starts in parallel with the formation of cell to cell contacts and the more or less intense binding on adhesion sites at the basal lamina respectively selected substrates. As a result polarization arises exhibiting immature or rather leaky barrier functions.

A complex concert of further influences such as paracrine factors, growth factors, hormones, nutrition, respiratory gas, metabolites and biophysical stress such as hydrostatic pressure or fluid osmolarity stimulates then the development from an immature state towards a physiological epithelial barrier. As a result the junction belt between the luminal and lateral plasma membrane is functionally closed so that the paracellular shunt can be controlled.

From this point of development onward exclusively the epithelial cells control, which electrolytes or other molecules are allowed to pass the barrier. Once the development is finished, the luminal and basal fluid environments are completely separated by the epithelium during gradient perfusion culture.

### **31.1. From embryonic to adult epithelial characteristics**

Experiments with gradient perfusion culture were started years ago in our laboratory to investigate the maturation of embryonic collecting duct epithelia derived from renal stem/progenitor cells.

In this coherence it was found that differentiation of a renal epithelium takes an unexpected long period of 14 days and is dependent on the electrolyte composition offered at the luminal and basal sides.

During a culture period of 14 days the epithelium was provided for instance with 1 ml/h on the basal side with IMDM, while on the luminal side IMDM + 12 mmol/l NaCl was offered (Fig. 126).

It was for example detected that in an electrolyte gradient cell-specific features such as binding for peanut agglutinin (PNA) or binding of monoclonal antibodies mab 503 and mab 703 were up-regulated.

Applying gradient perfusion culture it became for the first time possible to investigate the maturation of an embryonic epithelium under realistic conditions as found during development within an organ.

After gradient perfusion culture immunohistochemistry was performed to analyze on harvested collecting duct epithelia the degree of kidney-specific differentiation (Fig. 126). For example, the occurrence of laminin  $\gamma$ 1 shows, that the epithelium rests on a newly synthesized basal lamina (Fig. 126.1).

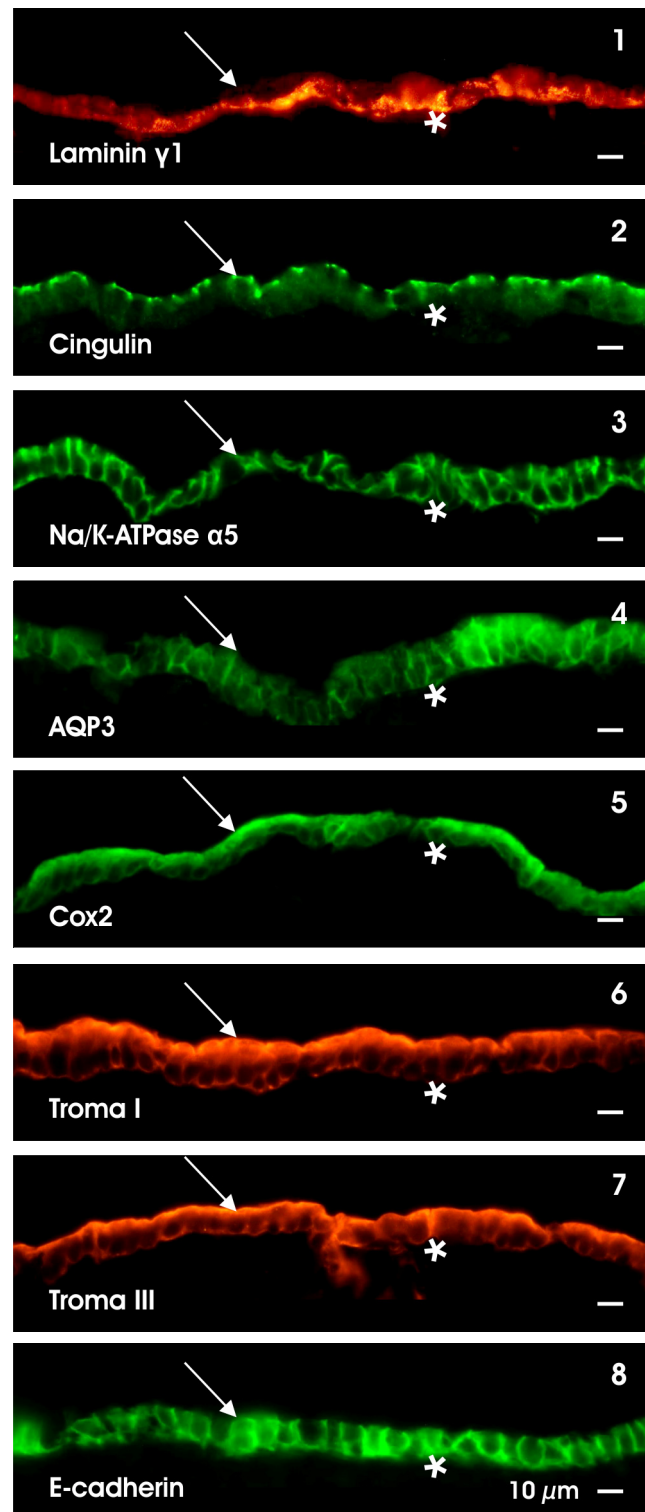


Figure 126: Immunohistochemistry on renal collecting duct epithelia kept for 14 days in gradient perfusion culture with IMDM on the basal and IMDM + 12 mmol/l  $\text{Na}^+$  at the luminal side. Label for laminin  $\gamma 1$  shows a basal lamina (1). Cingulin indicates a junctional complex (2), while label for Na/K-ATPase  $\alpha 5$  (3), aquaporin 3 (AQP 3, (4)) and Cox 2 (5) shows cell-specific features. Troma I / cytokeratin EndoA (6) and Troma III / cytokeratin 19 (7) and E-cadherin (8) presents typical antigens. Basal lamina is marked by asterisk, while lumen is marked by arrow.

The up-regulation of cingulin reveals the formation of a tight junctional complex between the luminal and lateral plasma membrane (Fig. 126.2), while label for Na/K-ATPase  $\alpha 5$  (Fig. 126.3), aquaporin 3 (AQP 3, Fig. 126.4) and Cox 2 (Fig. 126.5) demonstrates the presence of typical functional features. Finally, occurrence of Troma I / cytokeratin EndoA (Fig. 126.6), Troma III / cytokeratin 19 (Fig. 126.7) and E-cadherin (Fig. 126.8) exhibits further kidney-specific characteristics.

**[search: gradient perfusion culture collecting duct epithelium]**

**Self-study: Get informed about features of differentiation in epithelia**

## **31.2. Applications of epithelia kept in gradient perfusion culture**

Up to date more than 200 reviewed papers including patents could be found dealing with Minusheet® perfusion cultures. Among them are over 40 reviewed papers using Minusheet® gradient perfusion culture technique.

Related literature is listed:

[www.biologie.uni-regensburg.de/Anatomie/Minuth/proceedings.htm](http://www.biologie.uni-regensburg.de/Anatomie/Minuth/proceedings.htm).

In a leading opinion paper the related examples are summarized:

Minuth WW, Denk L, Glashauser A (2010) A modular culture system for the generation of multiple specialized tissues. *Biomaterials* 31(11):2945-2954.

Further gradient perfusion culture is described in detail:

Minuth WW, Denk L, Roessger A (2009) Gradient perfusion culture – simulating a tissue-specific environment for epithelia in biomedicine. *Journal of Epithelial Biology and Pharmacology* 2:1-13.

Here selected examples of gradient perfusion culture are given:

**Blood-retina and blood-brain barrier:** For pharmaceutical studies experiments with blood-retina and blood-brain barrier were created in gradient perfusion culture to investigate drug permeation by different methods. The permeation characteristics displayed an intact polarized expression of efflux pumps such as multidrug resistance protein (P-gp) and Multidrug Resistance-associated Protein (MRP).

**[search: blood retina steuer]**

**Blood-air barrier:** Studies on lung alveolar epithelium were performed with gradient perfusion culture. In those experiments cellular interactions and the alveolar metabolism was investigated. When pneumocytes and endothelial cells were co-cultured on a polycarbonate filter within a gradient perfusion container characteristic morphological features were developed and tight junctions were sealing the blood-air barrier. It was concluded that gradient perfusion culture in combination with pneumocytes and endothelial cells is a promising realistic model to investigate dose-controlled exposure of airborne particles, features of barrier transport and repair mechanisms after alveolar injury.

**[search: aerosol tippe]**

**Blood-gas barrier:** Experiments on fish swim bladder gas gland was successfully performed with gradient perfusion culture. Cells of gas gland were cultured at the interface between gas and medium. The epithelium revealed a typical polarity and functionality as observed in the in vivo situation.

**[search: swimbladder prem]**

**Ophtalmology:** Successful perfusion culture in a gradient container was performed with retina. In contrast to static culture it could be shown, for example, that neurons and retinal pigment epithelium maintains a perfect morphology for at least 10 days. This opens new perspectives for safety testing of drugs designed for intraocular application. The new approach opens now the possibility to investigate in vitro the wide field of related regeneration.

**[search: retina culture kobuch]**

**Pharmaceutical application:** New drugs were tested in gradient perfusion culture. It was found for example that the gradient perfusion culture with Caco-2 cells generated sealing epithelia and revealed reproducible results much earlier than traditional 21-day static cultures. In addition, the permeability coefficient of several model compounds across Caco-2 cells was in gradient perfusion culture approximately twofold higher than obtained with static conditions.

**[search: perfusion culture masungi]**

**Regenerating vessels:** Development of micro-vessels was investigated by gradient perfusion culture. In these experiments capillary-like structures were found in the luminal portion of perfused vessel equivalents. It is shown that pulsatile perfusion promotes better the development of a capillary-like network than continuous transport of medium.

**[search: perfusion culture frerich]**

**Regeneration of epidermis:** Epidermis equivalents were generated with gradient perfusion culture. Composite grafts of INTEGRA<sup>®</sup> matrix and human keratinocytes were cultured in a



gradient container in order to evaluate the potential for the cost-effective engineering of full-thickness skin grafts and the treatment of ulcers.

**[search: perfusion culture kremer integra]**

**Non-epithelial barrier:** Experiments related to non-epithelial biomaterial testing were performed with dentin discs in gradient perfusion culture. Polymerized dental resin materials release residual monomers that may interact with pulp tissue. This modified dentin barrier test revealed as an ideal model to investigate described effects. New insights in permeability testing of gelatin membranes were obtained by using fibroblasts in a gradient container.

**[search: perfusion culture schmalz]**

In conclusion, during the last years a versatile modular system for the generation of epithelia within a gradient perfusion culture container was developed. With respect to the numerous factors that influence epithelial development the culture system was designed to allow individual control of a number of important environmental parameters. That way the microenvironment within the gradient perfusion container can be fine-tuned to meet the physiological needs of numerous types of epithelia. An innovative construction principle allows the application of gradient perfusion culture at minimal financial expenditure and the individual modules can be combined as needed to build a number of advanced customized culture set ups. Thus, a variety of epithelia can be kept under tissue-specific conditions to study adherence on new biomaterials, cellular communication, epithelial development and effects of newly developed pharmaceuticals.

**[search: gradient perfusion culture minuth]**

**Self-study: Read about be-cameral system to culture epithelia in a fluid gradient**

## 32. In vitro regeneration of parenchyma

The capability of parenchyma regeneration is limited in patients suffering on chronic or acute diseases such as diabetes, renal failure or heart attack. Regarding the clinical background and the bad prognosis of most of the patients the question arises, which molecular processes hamper a diseased organ to regenerate new parenchyma. Due to these reasons numerous investigations were made in the last years to find new therapeutic strategies promoting the controlled regeneration of parenchyma by drug delivery, cell implantation and tissue engineering. An ideal form of future therapy would be to induce a process of regeneration by morphogenic factors, to guide the spatial growth of parenchyma cells along innovative biomaterials as basal lamina substitute and to stimulate in parallel the necessary microvascularization.

It is imaginable that the therapeutic renewal of parenchyma occurs via pharmaceutical stimulation of non-diseased adult cells found within the organ, by an implantation of stem cells or by an activation of organ specific progenitor cells. Most important, for regenerating cells the succeeding developmental processes into organ-specific structures are of essential importance. During this period the cells have to multiply so that a sufficiently big mass of them is available at the site of regeneration. In a succeeding step the spatial organisation of the cells is leading to organ specific structures. In parallel small vessels must invade so that the regenerating parenchyma is provided with optimal nutrition and respiratory gas. However, most problematic is that the process of regeneration does not occur in an optimal environment, but is influenced by inflammatory processes and reduced vascularisation at the site of organ damage.

Focussing to chronic and acute renal failure sound cell biological information about promoting and hampering parameters influencing spatial tubule development of implanted stem/progenitor cells are scarce. For that reason innovative protocols for a therapeutic

induction and a subsequent guiding of the regeneration process are in the focus of intense research.

**[search: organ regeneration]**

**Self-study: Read about regeneration of organs**

### **32.1. Using a pad for implantation of stem/progenitor cells**

A successful therapeutic application of stem/progenitor cells depends on an optimal site of implantation into the diseased kidney. An implantation can be performed on the one hand by an infusion of stem/progenitor cells via the blood vessel system or by an accidental injection into diseased areas of the kidney. However, the infusion of stem/progenitor cells via the vessel system is unsure, since areas of inflamed parenchyma are not sufficiently supplied by vessels. When punctual implantation is performed, it remains obscure if really all of the areas are supplied with the necessary amount of stem/progenitor cells needed for the process of regeneration. Considering all of these unsolved problems an alternative technique for the implantation of stem/progenitor cells is more and more favored. The idea is to implant stem/progenitor cells within a polyester pad underneath the renal capsule (Fig. 127).

An implantation underneath the renal capsule sounds obscure but is promising. For example, successful subcapsular implantation in the kidney was made with insuline producing pancreatic islet cells. More interestingly, the outer cortex of parenchyma underneath the renal capsule is the site, where nephrogenesis has been terminated at the end of organ growth. In consequence, the idea is to re-activate here the earlier site of nephrogenesis by an implantation of stem/progenitor cells. Further the pouch underneath the capsule is easily accessible for an implantation of a pad harboring stem/progenitor cells by minimal invasive

surgical techniques. According to the individual need multiple polyester pads containing stem/progenitor cells can be implanted underneath the renal capsule by piling and paving.

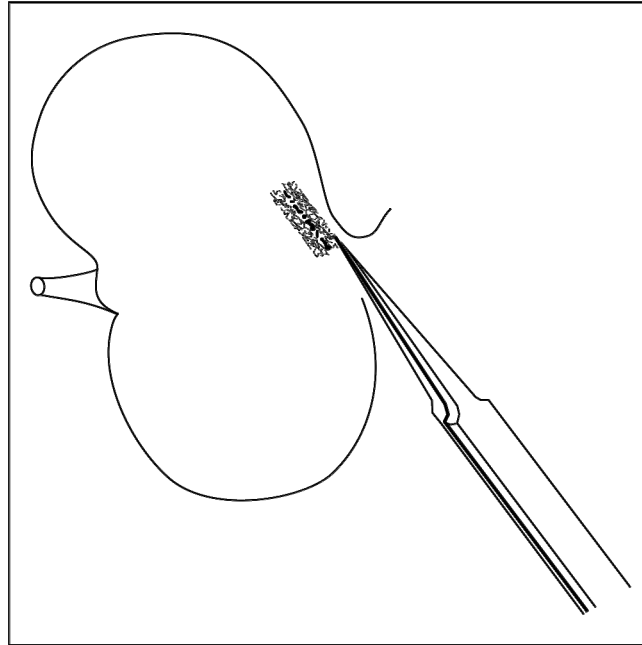


Figure 127: Schematic illustration of implantation site. A polyester pad containing stem/progenitor cells can be implanted underneath the renal capsule. At this specific site nephrogenesis has been terminated, when the growing kidney has reached its final size. The idea is to re-activate the site of nephrogenesis by the tactical implantation of stem/progenitor cells.

Following the idea that stem/progenitor cells are implanted within a polyester fleece pad underneath the renal capsule for the regeneration of parenchyma, intense basic research has to be made within the next decade.

First of all, one has to find out the best composition of biocompatible and biodegradable fleece materials, to analyze the survival of stem/progenitor cells within a polyester pad exposed to low oxygen tension after an implantation has been made. One has to elaborate in how far the site of implantation does support proceeding development in an inflamed environment. Further conclusive data concerning the involved developmental processes leading from stem/progenitor cells to polarized tubule cells have to be elaborated so that the proceedings in regeneration can be controlled step by step.

The aim is to promote physiological development during regeneration, while pathological formation must be early recognized so that damage can be prevented. It is imaginable that during ongoing research a variety of morphogenic drugs will be detected that can be applied to initiate or to accelerate therapeutically the process of regeneration. Such master molecules could be integrated in a fleece fiber to act here as a member of a local drug delivery system. However, only detailed knowledge about organ growth including tubule development will make it possible to find a successful therapeutic protocol for guiding a secure regeneration in future biomedicine.

**[search: regeneration drug delivery system]**

**Self-study: Inform yourself about stem cells within pads**

## **32.2. From stem/progenitor cells to polarized cells**

It is imaginable that regeneration in an organ will be in future therapeutically influenced, when exact information about stem/progenitor cell development into adult tubule cells is available. However, such a development sounds simple but includes very different molecular mechanisms including proliferation, determination, polarization and the fate of functional differentiation. To obtain insights in these multiple molecular cascades, already since years culture experiments with renal epithelial cells were performed.

Some examples are given here to illustrate some of the problems, when renal cells are kept under in vitro conditions. In most of the cases their potency for development is overestimated.

Years ago segments of renal tubules were isolated and placed at the bottom of a culture dish. It appears unbelievable, but the application of culture medium containing foetal bovine serum does not result in elongation of the isolated tubule (Fig. 128.1). Instead, cells leave the interior of the tubule to proliferate on the plane bottom of the culture dish and on the outer surface of its basal lamina. Up to date it is unknown, why the polystyrene at the bottom of the dish or the outer surface of the basal lamina is more attractive for renal cells than to stay in the interior of the tubule. Thus, a piece of a tubule does not regenerate in its original form.

Of special importance is the question, which features are keeping renal cells in close contact with a biomaterial. To investigate the interactive mechanisms between adhesion and a material surface, epithelial renal cells were cultured on various porous materials. In series with filter inserts and tissue carriers it was observed that they reacted unexpectedly sensitive when coming in contact with a biomaterial (Fig. 128.2). It was found that multiple biophysical, biochemical and cell biological properties finally determine if the complete surface of the selected biomaterial is accepted for adhesion or if only part of it is covered by proliferating cells. It is evident that such an unexpected sensitivity of cells is bidirectionally influenced after an implantation by the polyester fleece pad and by the inflamed environment of a diseased organ.

Not only the proliferation and adhesion of renal cells are of importance but also their degree of differentiation after several days of culture. To offer a substitute for the basal lamina renal cells were kept on porous materials in form of hollow fibers enabling exchange of fluid and molecules between the luminal and basal sides (Fig. 128.3). Also these kinds of experiments revealed that on the one hand the surface features of the applied material and on the other hand the distribution and sizes of pores have an enormous influence on the degree of cell proliferation, adhesion, polarization and subsequent maintenance of differentiation. It was further demonstrated that only a fully biocompatible surface as a basal lamina substitute is promoting the molecular processes of functional differentiation.

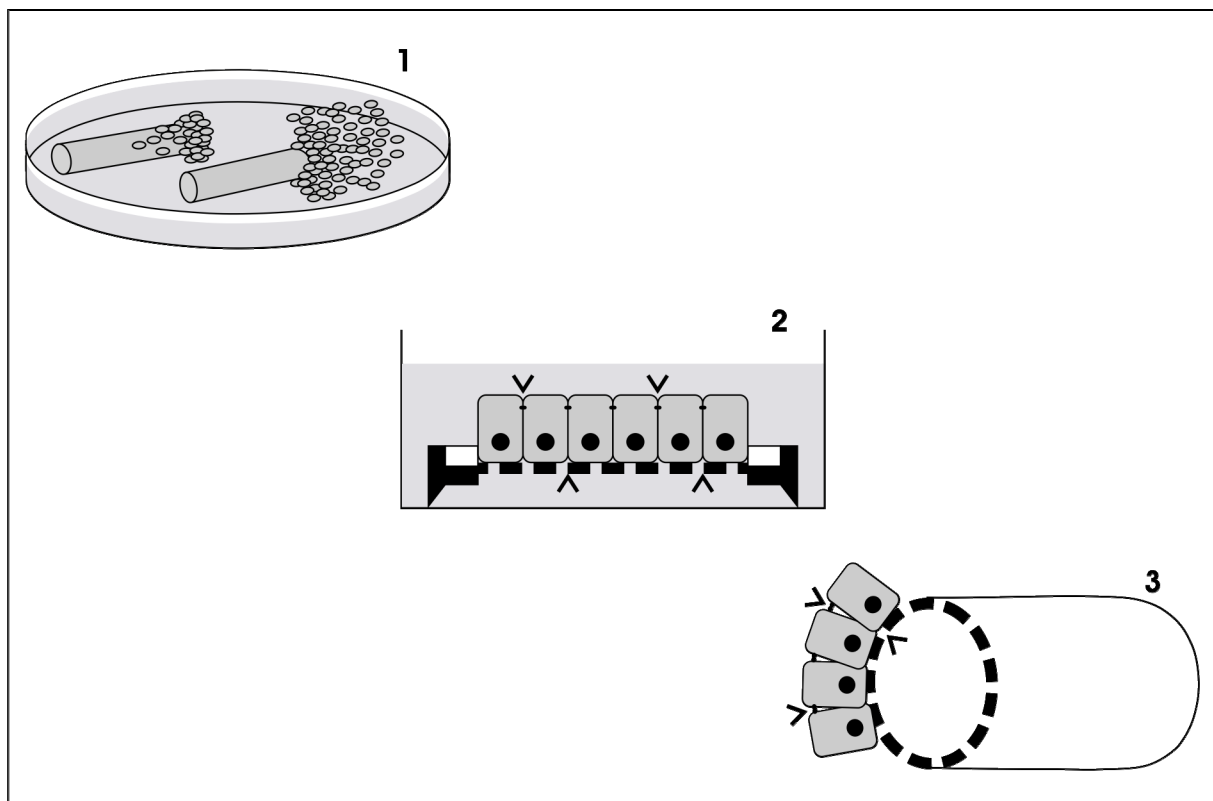


Figure 128: Schematic illustration of culture techniques with renal epithelial cells. When a tubule segment is isolated and cultured in medium containing foetal serum, cells emigrate and spread on the bottom of the culture dish and on the outer side of the tubule basal lamina (1). To offer a substitute for the basal lamina renal cells can be kept on various filter materials promoting exchange of fluid between the luminal and basal sides (2). A biocompatible surface on hollow fibers is needed, when renal cells must develop full functional differentiation so that intense transport of molecules is performed (3).

The culture of a monolayer of cells on the bottom of a culture dish or on a filter membrane is an interesting model to test viability, influences of drugs and transport capability. However, the experimental arrangement is very simple and does not reflect the real situation, when regeneration of parenchyma has to be analyzed. To obtain insights in the development of parenchyma intact metanephric organ anlagen were isolated so that culture on a filter can be performed (Fig. 129.1). To facilitate exchange of respiratory gas the growing organ can be kept near the gas-fluid interface with a minimal overlay of culture medium. It is interestingly to observe that at the start of culture the integrity of the organ anlage promotes spatial development of tubules. However, the continuously increasing layers of parenchyma finally

hinder provision with fresh nutrition and respiratory gas, which in turn limits further extension of the organ. For that reason a glass capillary for perfusion of medium was placed inside the hilus to prolong the period of growth during culture.

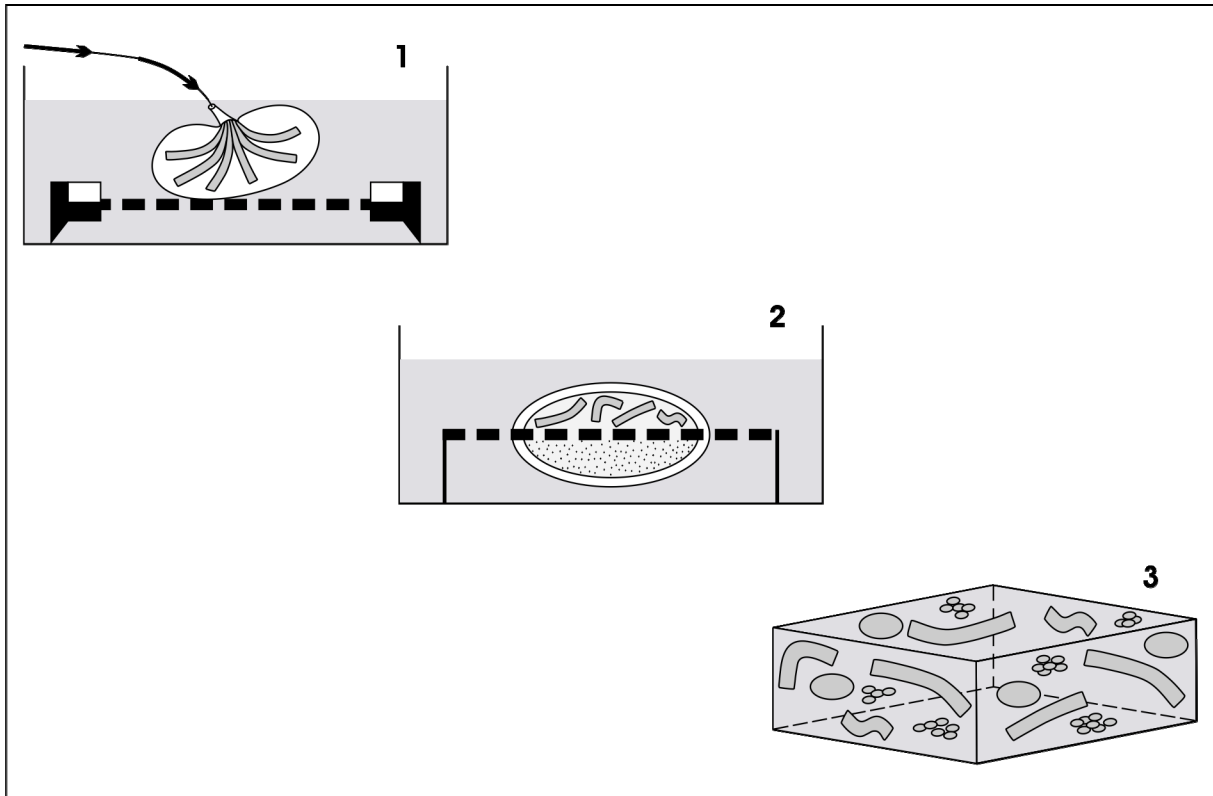


Figure 129: Illustration of advanced techniques to culture epithelial cells. Culture of an intact renal organ anlage within a filter insert promotes spatial development of tubules (1). A glass capillary for perfusion of medium in the hilus provides nutrition during long term culture. Development of structured tubules is obtained in transfilter experiments with isolated nephrogenic mesenchyme coated by agarose (2). Coating of single cells by collagen promotes spatial formation of tubules (3).

To obtain primary information about the development of renal stem/progenitor cells nephrogenic mesenchyme was isolated, placed on a nitrocellulose filter and coated by agarose or collagen (Fig. 129.2). Then the filter was turned to mount living spinal cord from the same foetus as an embryonic inducer. The contact between both tissues through the pores of the filter results in the development of structured tubules within the mesenchymal layer. It was recognized that numerous reciprocal molecular interactions occur during nephron formation resulting in tubules exhibiting specific differentiation.



Finally, beside intact tissue also isolated cells were used to investigate biological mechanisms involved in tubule formation. Cells collected from the urine or MDCK cells were coated before culture by extracellular matrix proteins such as collagen, matrigel®, hydrogel and hyaluronic acid (Fig. 129.3).

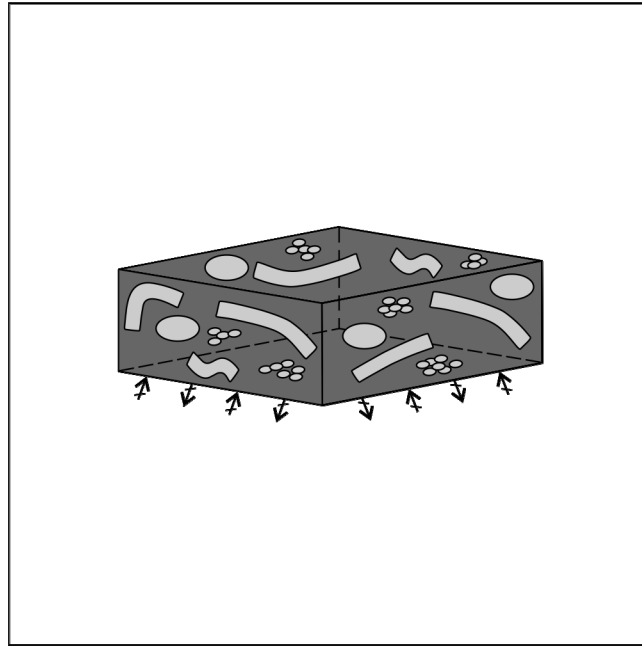


Figure 130: Coating by extracellular matrix proteins. The coat of extracellular matrix does not support maintenance of structures due to the formation of unstirred layers of fluid hindering in turn the provision with fresh nutrition including respiratory gas (arrows).

In all of these experiments it was demonstrated that coating by extracellular matrix proteins promotes the spatial formation of tubules during the initial period of culture (Fig. 130). However, it was also observed that during prolonged periods of culture the coat of extracellular matrix does not further support maintenance of tubules. This unexpected result is due to the formation of unstirred layers of fluid hindering in turn the provision with fresh nutrition including respiratory gas. The situation is leading to a deleterious accumulation of metabolites strongly reducing the period of culture time. In consequence, coating of stem/progenitor cells by extracellular matrix proteins is not the favoured method for the implantation underneath the renal capsule. It is obvious that the described limitations in

conventional cell and tissue cultures coated with extracellular matrix proteins are not only related to renal cells but can also be described for other cell types found in neural tissue, liver, pancreas, heart, intestine or other specialized connective tissues.

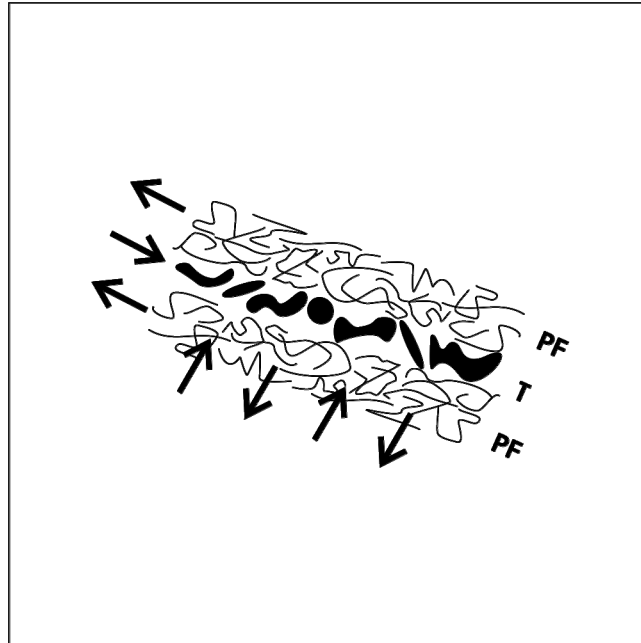


Figure 131: Schematic illustration depicts creation of an artificial interstitium by Polyester Fleeces (PF) covering enclosed tubules (T) at the upper and lower sides. In contrast to coating by extracellular matrix proteins the space between fleece fibers highly support exchange of nutrition and respiratory gas (arrows).

Thus, as an alternative for the coating of cultured cells and tissues has to be found. The idea is to cover cells and tissues by fleeces as a substitute for the coating process (Fig. 131). The fibers of the fleece simulate structural elements of the interstitium, while the space between the fibers highly supports the exchange of nutrition and respiratory gas. As demonstrated later the creation of an artificial interstitium promotes in a high degree the differentiation of cells and spatial development of specialized tissue structures.

**[search: kidney cell culture collagen]**

**Self-study: Read about three-dimensional cell cultures**

### 33. Simulation of the interstitial space

An innovative aim is to create for parenchyma and stem/progenitor cells a spatial environment so that structural and functional features such as lobules, ducts or renal tubules will arise. A classical improvement for the spatial environment is the frequently performed coating by extracellular matrix proteins. However, numerous studies have shown that embedding in extracellular matrix is not an optimal solution, when experiments under in vitro conditions or implantation of stem/progenitor cells into diseased parenchyma shall be made (Fig. 130).

The reason for a minor usability is that the solidification of the linked extracellular matrix proteins is leading to unstirred layers of fluid hampering in turn the provision with nutrition and respiratory gas. That was the reason to think about an alternative to create an artificial interstitium that promotes on the one hand the development of stem/progenitor cells and supports on the other hand provision with nutrition including exchange of respiratory gas.

Further, regarding a classical dish (Fig. 132.1) or a perfusion culture container (Fig. 132.2) shows that a significant dead space around cells or tissues is taken by culture medium. The unnecessarily large fluid filled space causes substantial hydraulic effects and passes pressure differences directly onto the cultured cells and tissues within the container. The dead volume is also the preferred site for the accumulation of gas bubbles. However, as demonstrated earlier an aggregation of them is problematic, since the supply of culture medium is shortened in certain areas leading to differing amounts of nutrition and respiratory gas. Further the presence of gas bubbles is causing surface tensions damaging in close neighbourhood the developing tissue. From the technical sight the dead volume can be reduced by decreasing the height of the container (Fig. 132.3). However, a too intense reduction of the dead volume is contra-productive, since it leads to unpredictable alterations in fluid flow and pressure inside the container due to uncontrolled capillary forces.

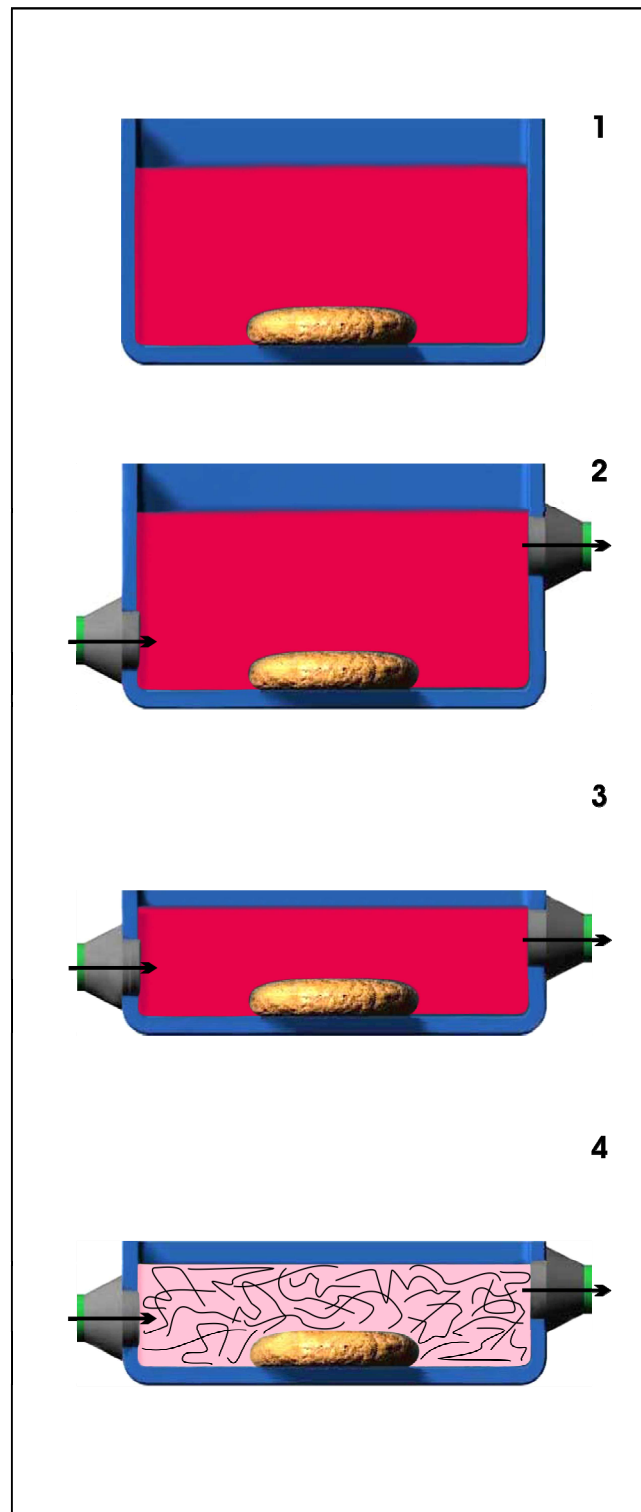


Figure 132: Schematic illustration demonstrates the reduction of dead volume in cell and tissue cultures to create an artificial interstitium for improved spatial development. In a classical dish cells are surrounded by an extended dead volume of medium (1). The same situation of an unnecessary bid dead volume of fluid is found within a classical perfusion culture container (2). As a consequence the dead volume can be reduced altering in turn the fluid dynamics (3). The technical solution is to fill the dead volume by a fleece to create an artificial interstitium (4).

Thus, the experimental aim was to create such an artificial interstitium in culture experiments so that an optimal development of stem/progenitor cells can be achieved (Fig. 132.4).

The best idea for the creation of an ideal environment for the development of stem/progenitor cells comprising the exchange of fluid and the structural stiffness is coming from nature. All of the adherent cells within tissues and organs are embedded in an interstitial space. It consists on the one hand of structural elements of the extracellular matrix and on the other hand of the interstitial fluid (Fig. 133.1).

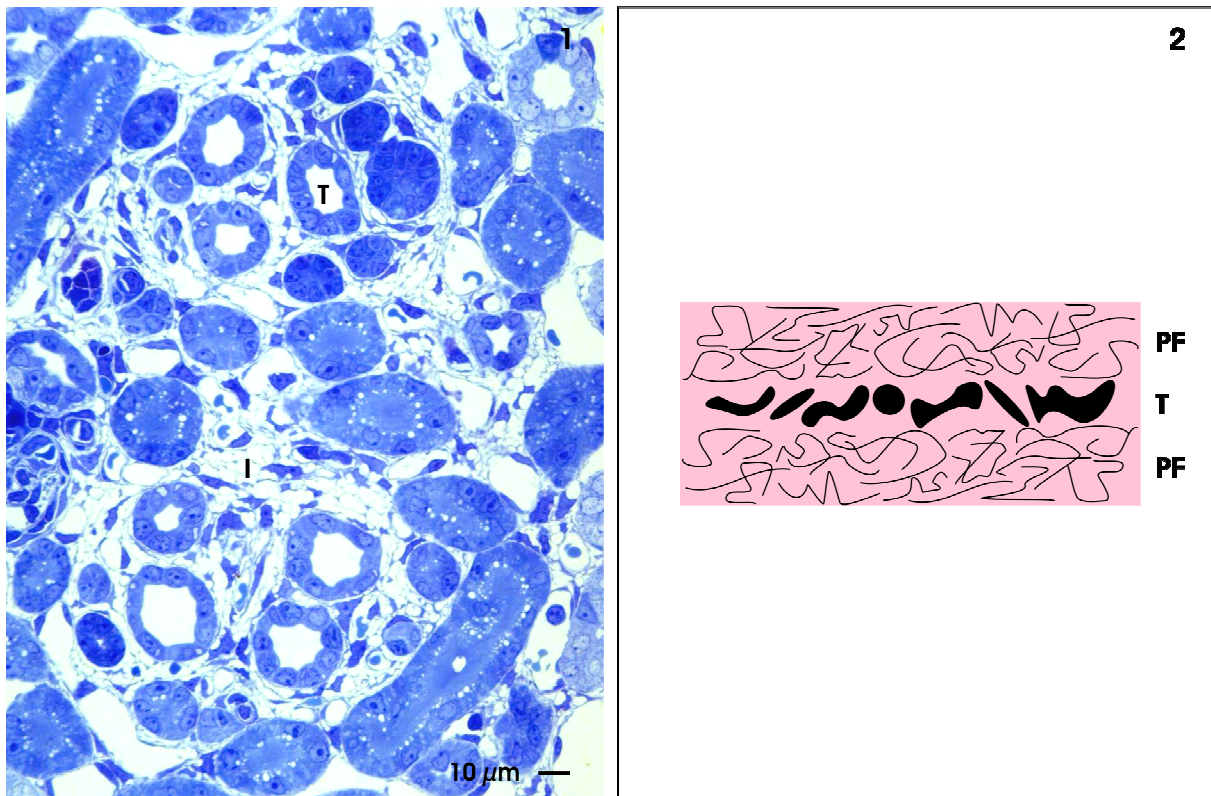


Figure 133: Light microscopic view to the interstitium within the kidney. All of the tubules (T) within the kidney are surrounded by an interstitium (I). Creation of an artificial interstitium by mounting stem/progenitor cells respectively tubules (T) between layers of polyester fleece (PF) (2).

Light and electron microscopy for example of the outer surface of a renal tubule depicts thin collagen fibers spanning from the basal lamina into the interstitium. However, the structural elements within an interstitium are not comparable with the condensed collagen found within

connective tissue but are recognized as tiny fibrils spanning in a spider like net fashion around tubules and vessels. The space between them is filled with interstitial fluid (Fig. 133.1).

The technical solution is to place a polyester fleece as an artificial interstitium at the interface between cells and the culture medium (Fig. 133.2). The fleece compensates alterations in fluid flow by capillary effects, minimizes dead volume and improves the environment for spatial development. It will provide further mechanical protection to the developing tissue. Such a material can be in direct contact with growing cells supporting thereby their spatial distribution.

Further the interface between the fleece fibers and the cells can be experimentally defined in form of a surface contour. Finally the fibers of the polyester fleece can be coated or micro-dotted with defined extracellular matrix proteins, morphogens or growth factors. During development these factors will be released in immediate vicinity to the spatially extending tissue.

**[search: artificial interstitium minuth]**

**Self-study: Inform yourself about the in vivo features of the interstitium**

## 34. Creation of an artificial interstitium

The original aim was to find a technical ambience that meets on the one hand the physiological and structural needs of implanted stem/progenitor cells so that they develop into spatially organized tubules. On the other hand the arrangement has to stand the harmful environment of the inflamed parenchyma and has to support regenerating parenchyma after an implantation was made. In the following text the development of renal stem/progenitor cells as a model system is described.

The idea was to cover stem/progenitor cells with a polyester fleece to simulate an artificial interstitium (Fig. 133.2, 134). In the case of an optimal development of tubules the resulting construct might be implanted later in form of a pad into the subcapsular space of the kidney. Since data about the potential development of stem/progenitor cells within an artificial interstitium were lacking at that time, primary sophisticated culture experiments had to be performed.

The aim in this coherence was to perform culture experiments under exactly defined conditions. For that reason addition of foetal bovine serum to the medium, use of undefined morphogenetic factors from tissue extracts or coating by unknown extracellular matrix proteins was not made. Instead the experiments were run in perfusion culture with always fresh medium and under chemically defined conditions. Only these conditions made it possible to learn about the environment influencing in a good or bad sense the development of contained stem/progenitor cells while developing into spatially structured tubules.

**[search: cell culture artificial interstitium]**

**Self-study: Get informed about the creation of an artificial polyester interstitium**

### 34.1. Covering stem/progenitor cells with polyester fleeces

An artificial interstitium can be simply created by placing isolated embryonic renal tissue between two punched out layers of polyester fleece (I-7, Walraf, Grevenbroich, Germany) measuring 5 mm in diameter and up to 250 µm in height (Fig. 134). This arrangement results in a basic sandwich set-up with the freshly isolated embryonic tissue in the middle and layers of polyester fleece covering the outer sides. As demonstrated later, the interface between the layers exhibits biophysical stimuli promoting the spatial formation of tubules due to up to date unknown reasons.

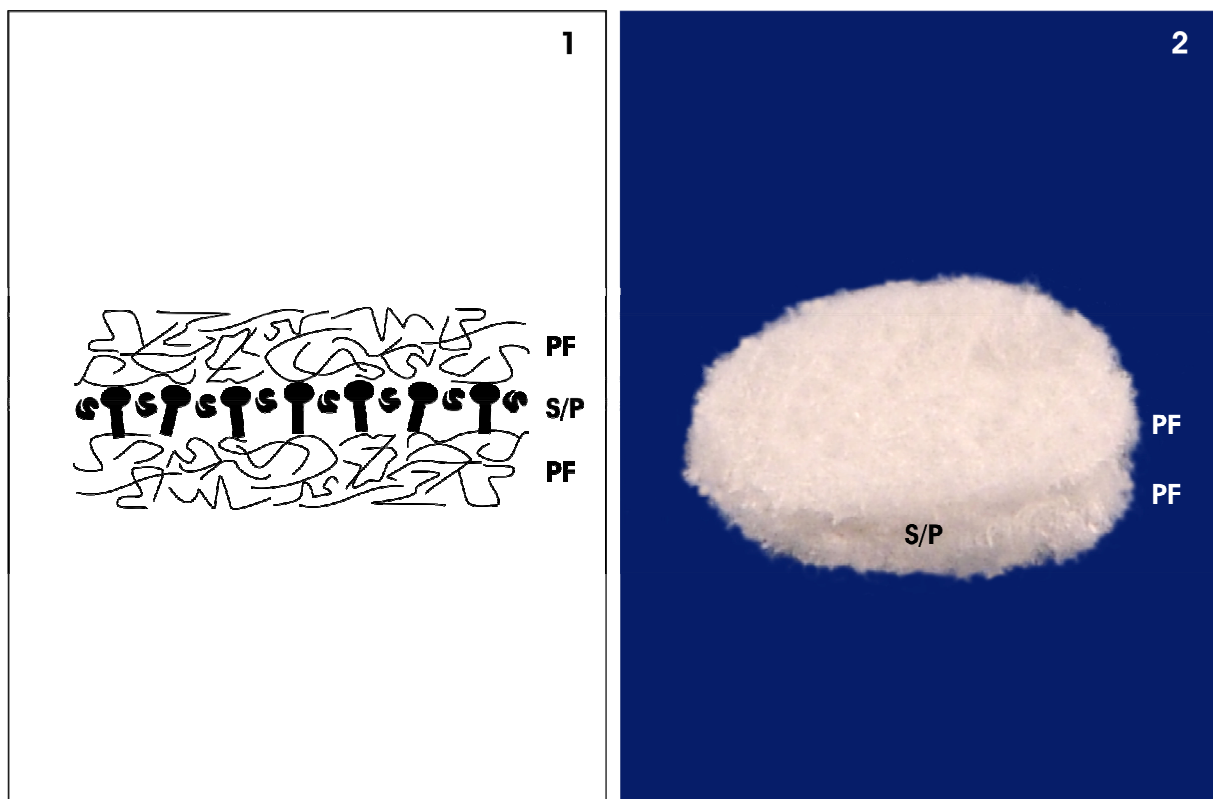


Figure 134: Schematic (1) and photographic (2) illustration of an artificial interstitium covering renal embryonic tissue. An artificial interstitium is made by mounting the isolated embryonic tissue containing Stem/Progenitor cells (S/P) between two layers of polyester fleece (PF) (1). Micrograph depicts a basic sandwich set-up containing stem/progenitor cells between layers of polyester fleece (2).

To prevent damage during perfusion culture the basic sandwich set-up of 5 mm in diameter containing renal stem/progenitor cells is mounted in a tissue carrier (Fig. 135.1). First a layer



of polyester fleece measuring 13 mm in diameter is placed in a base ring of a Minusheet® (Minucells and Minutissue, Bad Abbach, Germany). Then the sandwich set-up containing stem/progenitor cells is mounted and covered by a further layer of polyester fleece measuring 13 mm in diameter.

**[searc2h: cell culture artificial interstitium kidney]**

**Self-study: Read about fleeces suitable for perfusion culture experiments**

## **34.2. Keeping stem/progenitor cells within an artificial interstitium**

For long term culture experiments over 13 days and more the tissue carrier is then transferred to a perfusion culture container with horizontal flow characteristics (Minucells and Minutissue) (Fig. 135.2).

After closing the lid of the container the basic sandwich set up is held in an exact position. For medium supply the container is connected over silicone tubes with a storage and waste bottle to ensure provision with always fresh nutrition and respiratory gas (Fig. 135.3).

To maintain a constant temperature of 37°C, the container is placed on a thermo plate (Medax-Nagel, Kiel, Germany) and covered with a transparent lid.

During an experimental run over 13 days constant transport of medium is performed (Fig. 136). Always fresh and chemically defined IMDM (Iscove's Modified Dulbecco's Medium including phenol red, GIBCO/Invitrogen, Karlsruhe, Germany) is transported by a rate of 1.25 ml/h with an IPC N8 peristaltic pump (Ismatec, Wertheim, Germany).

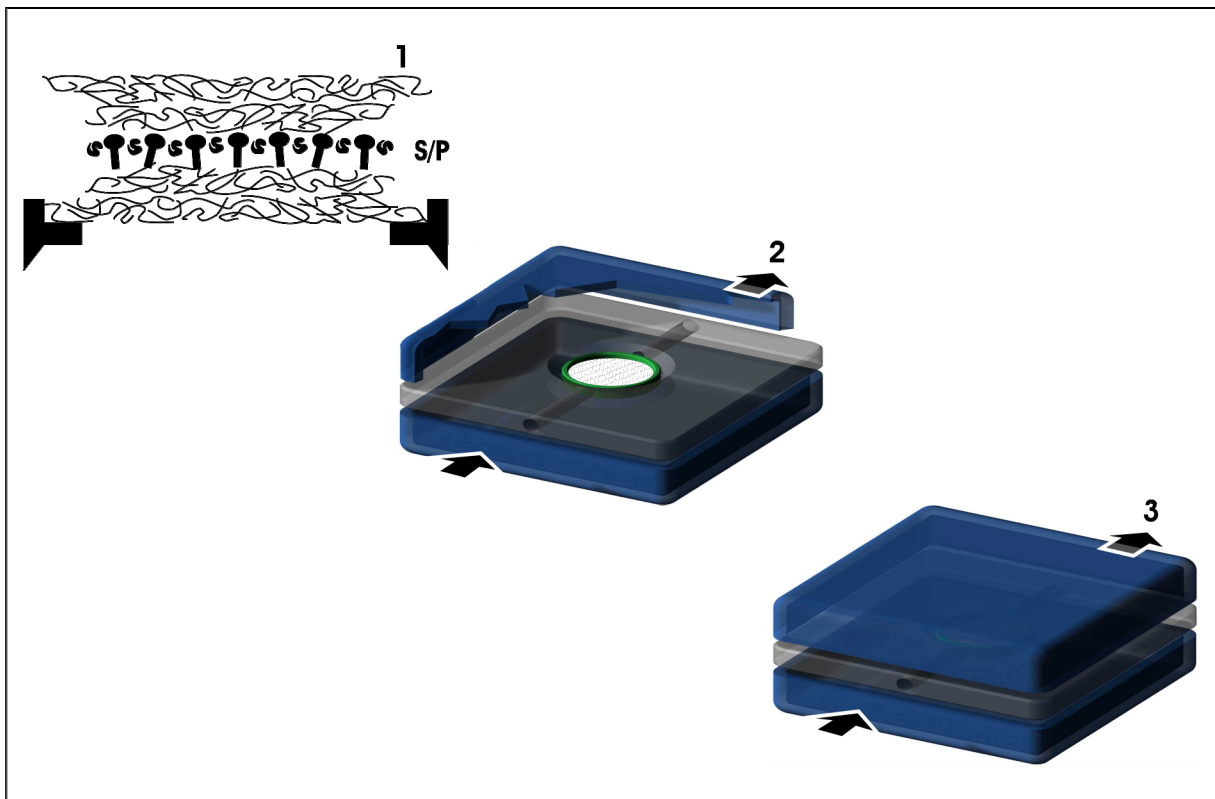


Figure 135: Perfusion culture of renal stem/progenitor cells kept within an artificial interstitium. The basic sandwich set-up containing renal stem/progenitor cells (S/P) is mounted in a tissue carrier (1). For long term culture the tissue carrier is placed at the base of a perfusion container (2). After closing the lid the tissue is held in position (3).

When this kind of perfusion culture technique is applied, medium is saturated to 160 mmHg oxygen during transport. This high content of oxygen in the medium is reached by a long and thin-walled silicone tube, which is highly gas-permeable and ensures optimal diffusion between culture medium and surrounding atmosphere.

It guarantees an optimal supply of respiratory gas for the growing tissue. In this way it is possible to adjust the gas partial pressures within the medium under absolutely sterile conditions. In order to maintain a constant pH of 7.4 under atmospheric air containing 0.3% CO<sub>2</sub>, one has to add HEPES (50 mmol/l, GIBCO) to the culture medium.

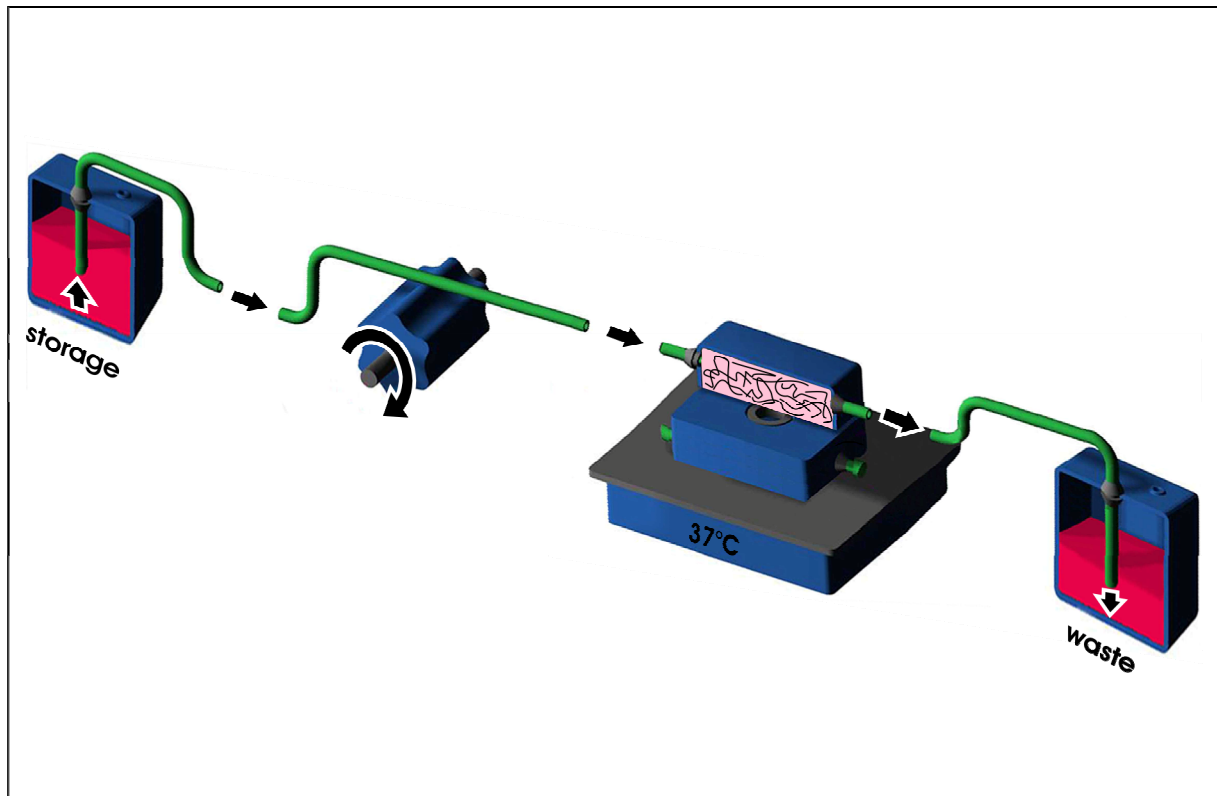


Figure 136: Schematic illustration of perfusion culture with renal stem/progenitor cells covered by an artificial interstitium. Perfusion culture is performed with a peristaltic pump transporting (arrow) always fresh medium (1.25 ml/h) from the storage bottle to the culture container. Used medium leaving the culture container is not recycled but is collected in a waste bottle.

For stimulation of tubulogenic development of renal stem/progenitor cells during the whole culture period aldosterone ( $1 \times 10^{-7}$  M, Fluka, Taufkirchen, Germany) is administered to the medium. Infections are prevented by adding an antibiotic-antimycotic cocktail (1%, GIBCO).

**[search: perfusion culture artificial interstitium]**

**Self-study: Get informed about stem cells within biomatrices**

## 35. Tubule growth in an artificial interstitium

After a perfusion culture period of 13 days the artificial polyester interstitium can be opened by tearing off the layers of fleece. Yet several histochemical, immunohistochemical and electron microscopic techniques have to be applied so that the distribution, number and quality of generated tubules can be determined.

### 35.1. Whole mount label of generated tubules

To analyze the spatial extension of tubules specimens are fixed in 70% ethanol, labeled by Soybean Agglutinin (SBA) and analyzed in a fluorescence microscope (Fig. 137).

For better understanding, at begin of culture isolated renal stem/progenitor cells do not exhibit cellular SBA-label. In contrast, after a culture period of 13 days intense binding of SBA is recognized on numerous generated tubules (Fig. 137). The up-regulation of SBA-label signals development and a step in differentiation as it was described for the maturing collecting duct in neonatal kidney.

The surface view of SBA labelled specimens further depicts that numerous tubules are growing in a spatial manner (Fig. 137.1). Part of generated tubules exhibits a straight growth, while others reveal curling or dichotomous branching. All of the them show polarized cells, a visible lumen and a basal lamina.

The number of generated tubules can be determined using for example a WCIF ImageJ program (Bethesda, Maryland, USA). To take into account each of the SBA labeled tubules is marked by a white dot (Fig. 137.2).

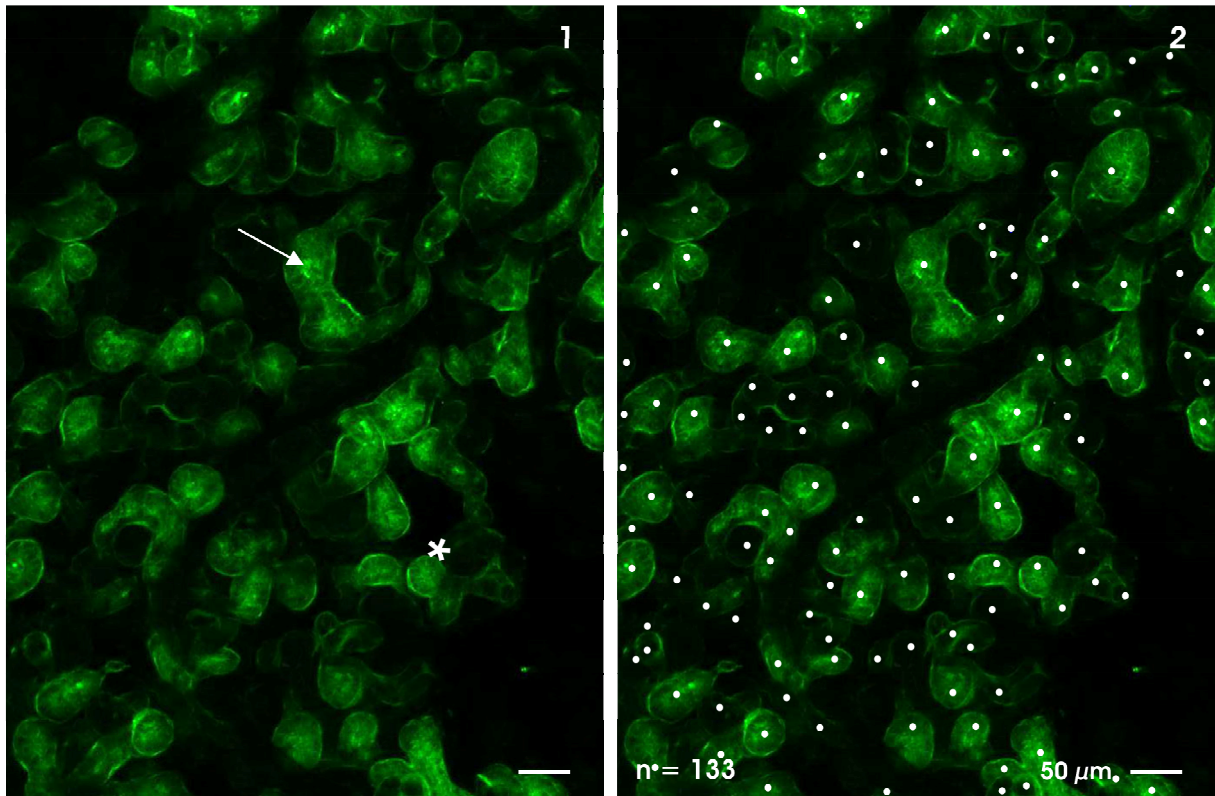


Figure 137: Illustration of whole mount labelled renal tubules generated at the interface of an artificial polyester interstitium after 13 days of perfusion culture. Fluorescence microscopy depicts distribution of whole mount specimens labeled by SBA (1). Tubules exhibit a lumen (arrow) and a basal lamina (asterisk). The white dots indicate that within the specimen 133 tubules were generated (2).

Counting specimens by the morphometric program revealed that for example in an individual sample 133 tubules can be detected within a microscopic opening of 579 x 769 μm (Fig. 137.2). When the tubules are not leaving the optical plain, it is possible to follow their longitudinal growth over a distance between 300 and 400 μm.

**[search: soybean lectin kidney]**

**Self-study: Inform yourself about development of specialized tissues**

## 35.2. Cell biological differentiation on growing tubules

When a polyester fleece as an artificial interstitium is tested, the degree of acquired cell biological differentiation within generated tubules is of fundamental importance. In consequence, to analyze features of differentiation histochemistry was performed (Fig. 138).

First, to obtain a surface view generated tubules were stained by Toluidine blue. Yet the spatial distribution of developed tubules (T) covered by layers of polyester fleece (PF) can be recognized (Fig. 138.1).

View to a SBA labeled specimen in higher magnification shows the growth of tubules in a spatial arrangement (Fig. 138.2). A longitudinal view to SBA labeled tubules demonstrates a distinct lumen (arrow) and a basal lamina (asterisk) (Fig. 138.3). The vertical view illustrates in the centre a lumen, while at the basal aspect a basal lamina can be seen (Fig. 138.4).

To obtain further information about the degree of cell biological differentiation, further analysis was performed on cryosections by immunohistochemistry (Fig. 139).

The immunohistochemical data further demonstrate that an intense label for cyclooxygenase 2 (Cox2) (Fig. 139.1), cytokeratine endoA (Troma I) (Fig. 139.2), cytokeratine 19 (CK 19) (Fig. 139.3) and E-cadherin (Fig. 139.4) is present within all cells of the tubules (T). Label for cingulin (arrow) demonstrates the development of a tight junctional belt recognized as faint label in the luminal portion of generated tubules (Fig. 139.5). The label for Na/K-ATPase  $\alpha 5$  depicts an intensive fluorescence at the basolateral aspect of cells (Fig. 139.6). Finally, labeling the tubules for laminin  $\gamma 1$  exhibits an intensive reaction at the basal lamina (asterisk) (Fig. 139.7). In conclusion, all of the immunohistochemical data indicate that the generated tubules contain a polarized epithelium with functional features resembling the adult collecting duct tubule within the kidney.

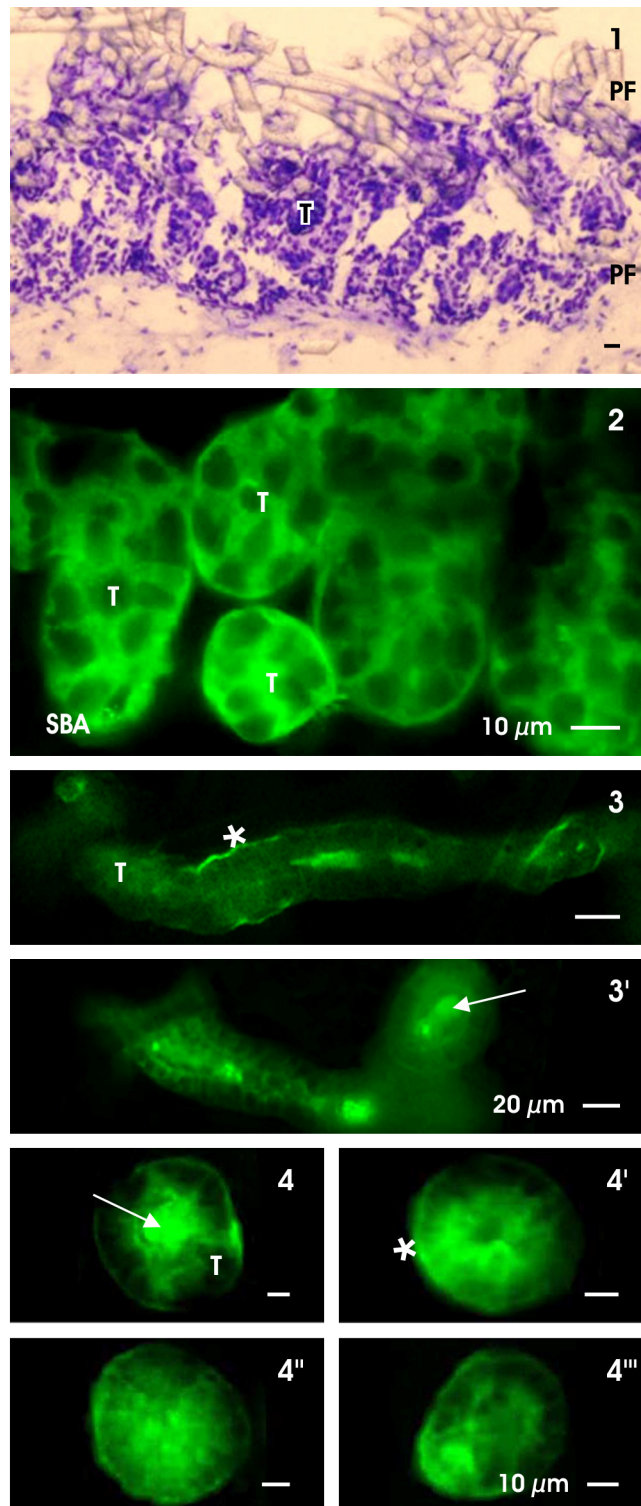


Figure 138: Toluidine blue staining and SBA label on renal tubules (T) generated at the interface of an artificial polyester interstitium after 13 days of perfusion culture. Distribution of generated tubules can be recognized after Toluidine blue staining (1). They are covered by layers of polyester fleece (PF) on the upper and lower side. View to specimens labelled by SBA in higher magnification illustrates tubules in a spatial arrangement (2). In longitudinal view labeled tubules show a distinct lumen (arrow) and a basal lamina (asterisk) (3). Also a vertical view illustrates a lumen, while at the basal aspect a basal lamina can be detected (4).



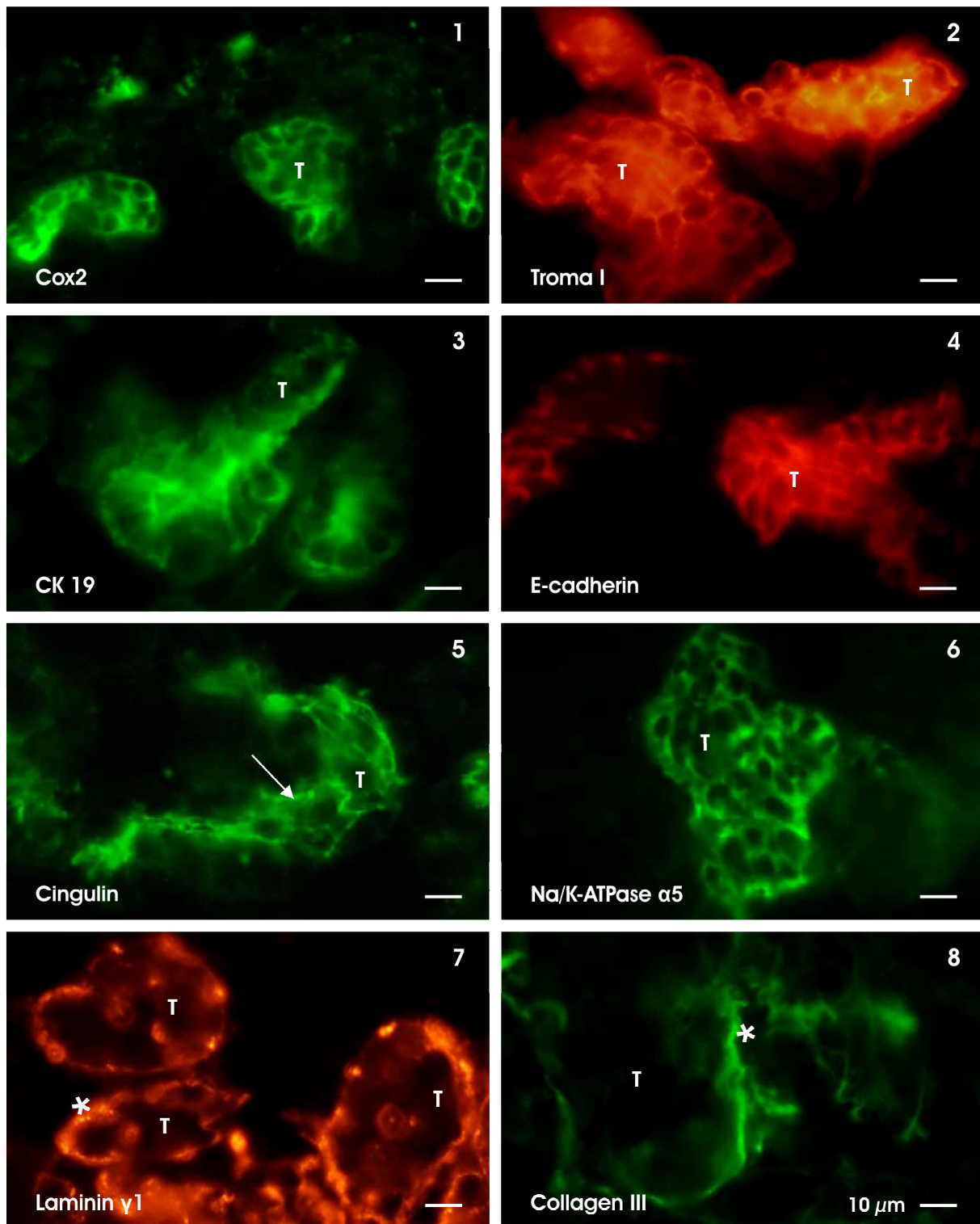


Figure 139: Immunohistochemistry on cryosections of renal tubules generated at the interface of an artificial polyester interstitium after 13 days in perfusion culture. Label for cyclooxygenase 2 (Cox2) (1), cytokeratine endo A (Troma I) (2), cytokeratine 19 (CK 19) (3), E-cadherin (4), occludin (arrow) (5), Na/K-ATPase  $\alpha 5$  (6), laminin  $\gamma 1$  (7) and collagen III (8) reveals intensive reaction on generated tubules (T). Arrow indicates a lumen, while the asterisk depicts the basal lamina.



Immunohistochemical label on cryosections further reveals that collagen type III is contained in both the basal lamina of generated tubules (T) and in the surrounding interstitial space standing in contact with polyester fibers of the fleece (Fig. 139.8). This result speaks in favour that the differentiation of tubules appears strongly correlated with the synthesis of an intact basal lamina containing laminin  $\gamma 1$  and a typical interstitial protein such as collagen type III.

**[search: laminin collagen III kidney]**

**Self-study: Read about polar differentiation in epithelia**

### **35.3. Electron microscopic view**

Since the specimens are generated without coating by extracellular matrix proteins within the artificial interstitium, it became for the first time possible to analyze the basal aspect of developing tubules by Scanning Electron Microscopy (SEM) (Fig. 140). Thus, the interference of proteins derived from a coating process does not disturb the view to generated tubules.

SEM analysis of the used polyester fleece (PF) shows that numerous fibers are running in a longitudinal, transversal and oblique course (Fig. 140.1)). The fibers appear to be of homogeneous composition and exhibit a smooth surface without recognizable protrusions or roughness. The average diameter of a polyester fiber is 10  $\mu\text{m}$ . Chemical cross-linking between them cannot be observed.

The area of the polyester fleece used for tissue development illuminates numerous tubules (T) (Fig. 140.2). Part of them grows in a parallel fashion in the vicinity of the polyester fibers, some of them show a curling course, while others exhibit a dichotomous branching.

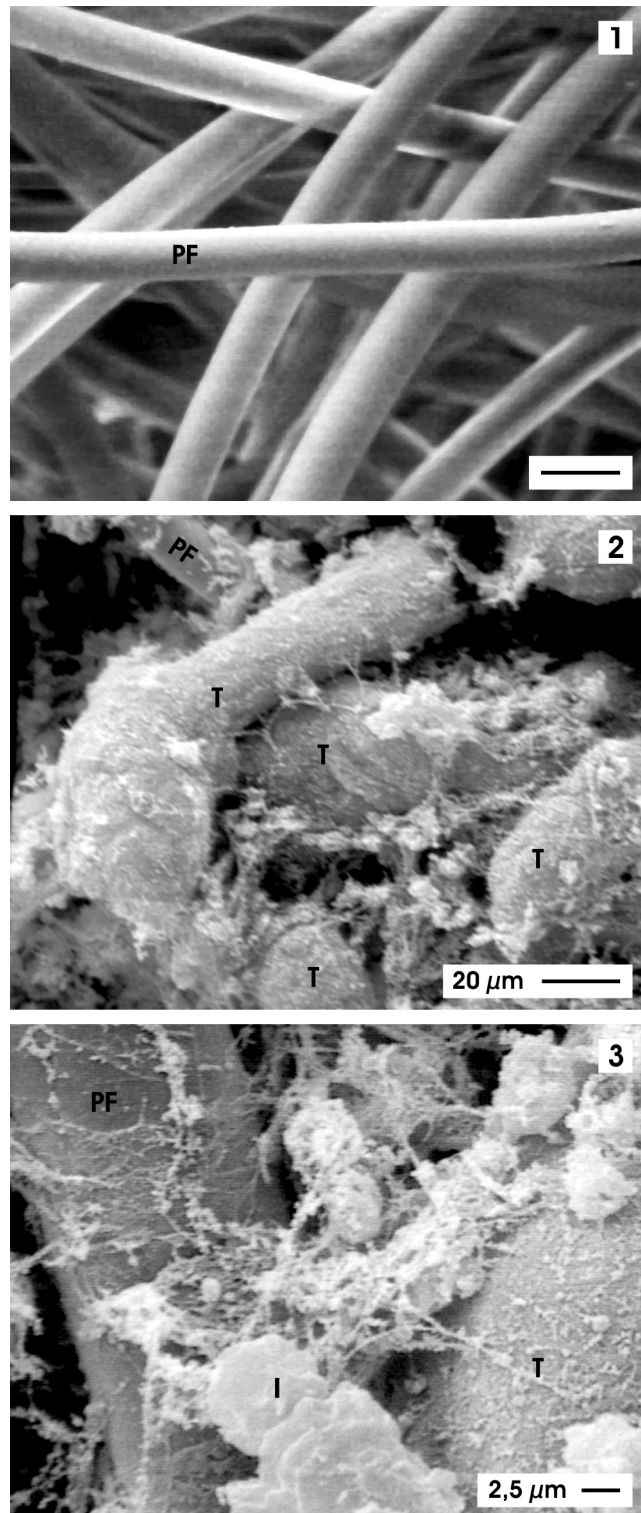


Figure 140: SEM of tubules (T) generated with an artificial interstitium. The polyester fleece shows numerous fibers (PF) running in a longitudinal, transversal and oblique course (1). Tubules are growing in close vicinity of fibers (2). On the surface of tubules single interstitial cells (I) and extracellular matrix are recognized (3).

All of the generated tubules are covered by a continuously developed basal lamina. Higher magnification further demonstrates that the tubules have an apparently light contact to the

fibers of the polyester fleece. On the surface of the tubules single interstitial cells (I) and bundles of newly synthesized extracellular matrix proteins are recognized (Fig. 140.3).

To obtain further insights in generated tubule cells Transmission Electron Microscopy (TEM) was performed (Fig. 141). Low magnification reveals that generated tubules contain a lining epithelium with a clearly visible lumen (arrow) and a constantly developed basal lamina (asterisk). In the surrounding of tubules synthesized extracellular matrix, single interstitial cells and some debris is noticed.

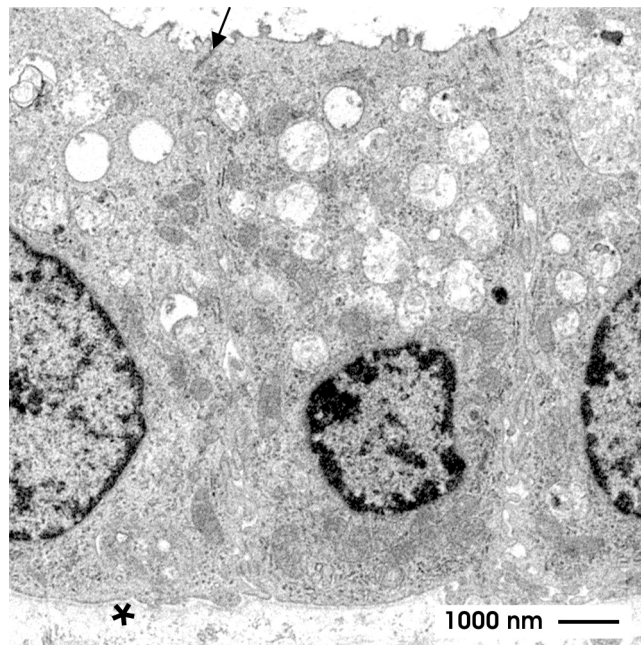


Figure 141: Transmission electron microscopy of tubules generated at the interface of an artificial interstitium. Illustration depicts that generated tubules contain a polarized epithelium. The apical side borders a lumen and a tight junction (arrow), while the basal side contains a basal lamina (asterisk). Between the apical and lateral plasma membrane a typical tight junctional complex is developed. This feature speaks for a physiological sealing of the epithelium.

Higher magnification reveals in transmission electron microscopy that an isoprismatic epithelium is established within generated tubules (Fig. 141). Most convincingly, the luminal and lateral plasma membranes are separated by a typical tight junctional complex. It consists of a zonula occludens, zonula adhaerens and a desmosome. The cells exhibit a large

nucleus, which is located in the middle of the cytoplasm. In the apical and basal cytoplasm numerous lysosomal elements are found. Within the cytoplasm small, medium-sized and large vacuoles are filled to a various degree with electron-dense material. The vacuoles may suggest that the containing material has been phagocytosed. At the basal aspect of the epithelium a basal lamina is found consisting of a lamina rara, a lamina densa and an extended lamina fibroreticularis.

All of the demonstrated ultrastructural data point out that during culture a polarized and an obviously sealing epithelium is developing within generated tubules.

**[search: tubule minuth electron microscopy]**

**Self-study: Get informed about typical features of functional epithelia**

## 36. Tubulogenic effect of aldosterone

When culture experiments with renal stem/progenitor cells mounted in an artificial interstitium were started, different media lacking aldosterone were applied. However, the results were disappointing, since structured tubules could not be observed. Instead a disintegration of the isolated embryonic tissue was noticed (Fig. 142.1). This result was indicating that a special growth factor in the medium inducing tubule development was lacking. In consequence a variety of growth factors, hormones and supplements was tested.

### 36.1. Inducing tubulogenic development

Performing a multitude of culture experiments it was finally detected that aldosterone administration ( $1 \times 10^{-7}$  M) to chemically defined Iscove's Modified Dulbecco's Medium (IMDM) is leading to a complete change in the developmental pattern of renal stem/progenitor cells (Fig. 142.2). Fully unexpectedly, it was elaborated that the steroid hormone induces the formation of numerous SBA-labeled tubules within a culture period of 13 days.

For a better insight in the tubulogenic effect of aldosterone, the hormone was applied in different concentrations ranging from  $1 \times 10^{-10}$  M to  $1 \times 10^{-5}$  M. A first run of experiments revealed that a low dose of aldosterone ( $1 \times 10^{-10}$  M) does not stimulate the development of tubules, while in higher concentrations such as  $1 \times 10^{-9}$  M and  $1 \times 10^{-8}$  M outgrowth of SBA-labeled cells is observed forming long rows and clusters but not structured tubules. However, intact formation of tubules was obtained after the application of  $1 \times 10^{-7}$  M and  $1 \times 10^{-6}$  M aldosterone. Surprisingly, an administration of a higher dose such as  $1 \times 10^{-5}$  M does not better stimulate the development of tubules.

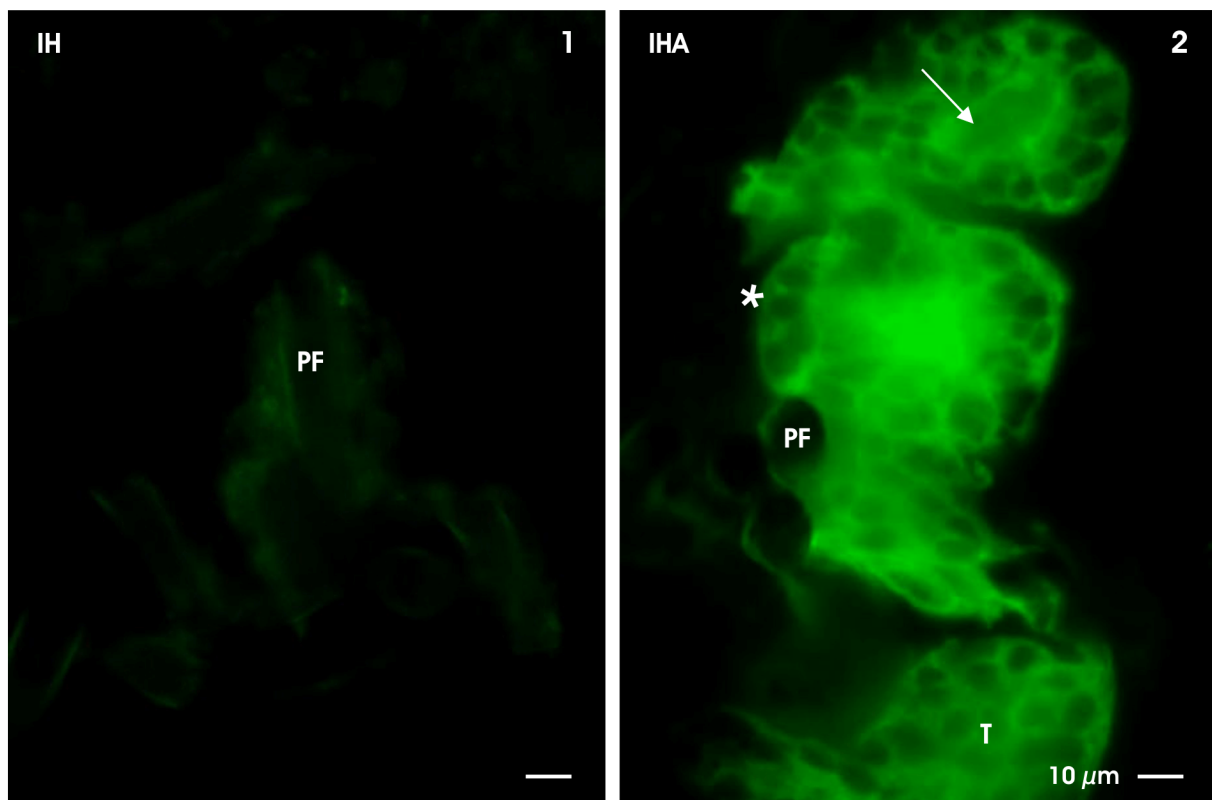


Figure 142: Tubulogenic action of aldosterone. Generation of SBA-labeled tubules cannot be recognized, if aldosterone is lacking in IMDM (1). In contrast, numerous tubules (T) are observed after application of aldosterone ( $1 \times 10^{-7}$  M) (2). Arrow shows lumen, while asterisk depicts basal lamina.

From the adult kidney it is known that not only aldosterone but also some of its molecular precursors reveal a high affinity to the Mineralocorticoid Receptor (MR) influencing thereby physiological functions. Experiments on adult kidney showed for example that 11-deoxycorticosterone is as effective as aldosterone, while corticosterone is 100 times less potent.

In consequence, molecular precursors of aldosterone were applied in current experiments. It was detected that application of cholesterol or pregnenolone does not induce the formation of any SBA-positive tubule. Treatment with progesterone is leading to the development of single tubules with low SBA-label. When 11-deoxycorticosterone is used, only few tubules with a faint SBA-label can be detected. In contrast, administration of corticosterone does not exhibit any development of tubules. Instead, numerous SBA-labeled but atypical cell clusters

are observed in close contact to polyester fibers. Data for 18-hydroxycorticosterone are missing, since this substance was not commercially available. Only the administration of aldosterone is leading to numerous SBA-labeled tubules exhibiting a distinct lumen and a clearly recognizable basal lamina.

Finally, the tubulogenic action of aldosterone might be triggered in cooperation via the Glucocorticoid Receptor (GR). Consequently, the glucocorticoid dexamethasone instead of aldosterone was applied. This series of experiments shows that administration of aldosterone ( $1 \times 10^{-7}$  M) results in the development of numerous SBA-labeled tubules, while the use of dexamethasone ( $1 \times 10^{-7}$  M) produces widely distributed clusters of non-polarized cells. In conclusion, the process of tubule development is highly dependent on the administration of aldosterone ( $1 \times 10^{-7}$  M). Precursors of aldosterone do not induce the formation of tubules.

**[search: cell culture minuth aldosterone]**

**Self-study: Inform yourself about steroid hormones**

## **36.2. Interfering the tubulogenic action of aldosterone**

Previously performed experiments demonstrated that aldosterone is required to induce a tubulogenic development in isolated renal stem/progenitor cells kept at the interface of an artificial interstitium. However, the data do not reveal if the tubulogenic effect of aldosterone is mediated via the Mineralocorticoid Receptor (MR) or if the action of the steroid hormone is related to a more or less unspecific side effect.

First of all it was investigated if renal stem/progenitor cells really contain the mineralocorticoid receptor (MR). Thus, embryonic renal tissue was isolated to perform tissue fractionation, SDS electrophoresis followed by Western blot analysis. Probing the

nitrocellulose sheet with monoclonal antibody anti-rMR1-18 1D5 revealed that renal stem/progenitor cells in deed contain MR recognized by a typical 96 kDa band.

In consequence, to obtain more information about the binding of aldosterone on MR, the influence of antagonists such as spironolactone and canrenoate was tested. For control, missing administration of aldosterone does not show generation of tubules (Fig. 142.1), while application of aldosterone ( $1 \times 10^{-7}$  M) induces numerous tubules (Fig. 142.2). Addition of a low dose of spironolactone ( $1 \times 10^{-7}$  M) in the presence of aldosterone ( $1 \times 10^{-7}$  M) does not affect the development of SBA-labeled tubules. However, administration of a higher concentration of spironolactone ( $1 \times 10^{-5}$  M) in the presence of aldosterone demonstrates inhibitory effects leading to a switch in development. In this case the number of structured tubules is strongly reduced and SBA-labeled cells start to form extended cell clusters. Finally, application of  $1 \times 10^{-4}$  M spironolactone in the presence of aldosterone completely prevents the development of SBA-labeled tubules (Fig. 143).

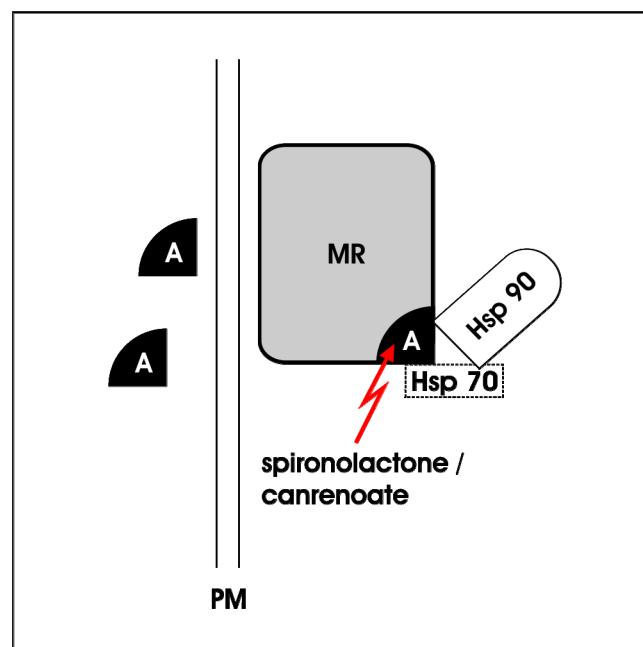


Figure 143: Schematic illustration of action of aldosterone (A) on renal stem/progenitor cells. The effect of aldosterone depends on applied concentration and is mediated via the Mineralocorticoid Receptor (MR). The administration of aldosterone with spironolactone or canrenoate antagonizes the tubulogenic effect.



In a further experimental series the antagonist canrenoate was tested. The results show the same concentration-dependent effect on the tubulogenic action of aldosterone as it was observed with spironolactone. Application of  $1 \times 10^{-7}$  M canrenoate in the aldosterone-containing medium does not affect the development of tubules, while administration of  $1 \times 10^{-6}$  M and  $1 \times 10^{-5}$  M canrenoate drastically reduces SBA-labeled cells. Finally, the use of  $1 \times 10^{-4}$  M canrenoate results in a complete lack of SBA-labeled cells and tubules (Fig. 143). Thus, the simultaneous administration of aldosterone in combination with spironolactone or canrenoate demonstrates that the tubulogenic effect is inhibited in a dose dependent manner. This result also clearly illustrates that the tubulogenic effect of aldosterone is not an unspecific reaction or a side effect on renal stem/progenitor cells but is mediated specifically via the mineralocorticoid receptor.

A further set of experiments was made to illuminate the intracellular processing of the tubulogenic effect of aldosterone. At that time no information was available if MR is randomly distributed within the cytoplasm of stem/progenitor cells or if MR is in narrow molecular contact with heat shock proteins (hsp) 90 and 70 (Fig. 144). In consequence, the tubulogenic effect of aldosterone must be abolished, when renal stem/progenitor cells are incubated with drugs that disrupt these interactions.

To investigate the contact between MR and hsp 90 renal stem/progenitor cells kept within an artificial interstitium were cultured in IMDM. For control a first set of experiments was made without administration of aldosterone (Fig. 144.1). In this case the formation of tubules was absent. In the next set culture was performed in IMDM containing geldanamycin ( $3,6 \times 10^{-6}$  M) in combination with aldosterone ( $1 \times 10^{-7}$  M) for 13 days (Fig. 144.2). It is known that geldanamycin specifically binds to hsp 90 thereby blocking the ATP-binding site due to its higher affinity compared to ATP. In this way, geldanamycin hinders the contact between hsp 90 and activated MR.

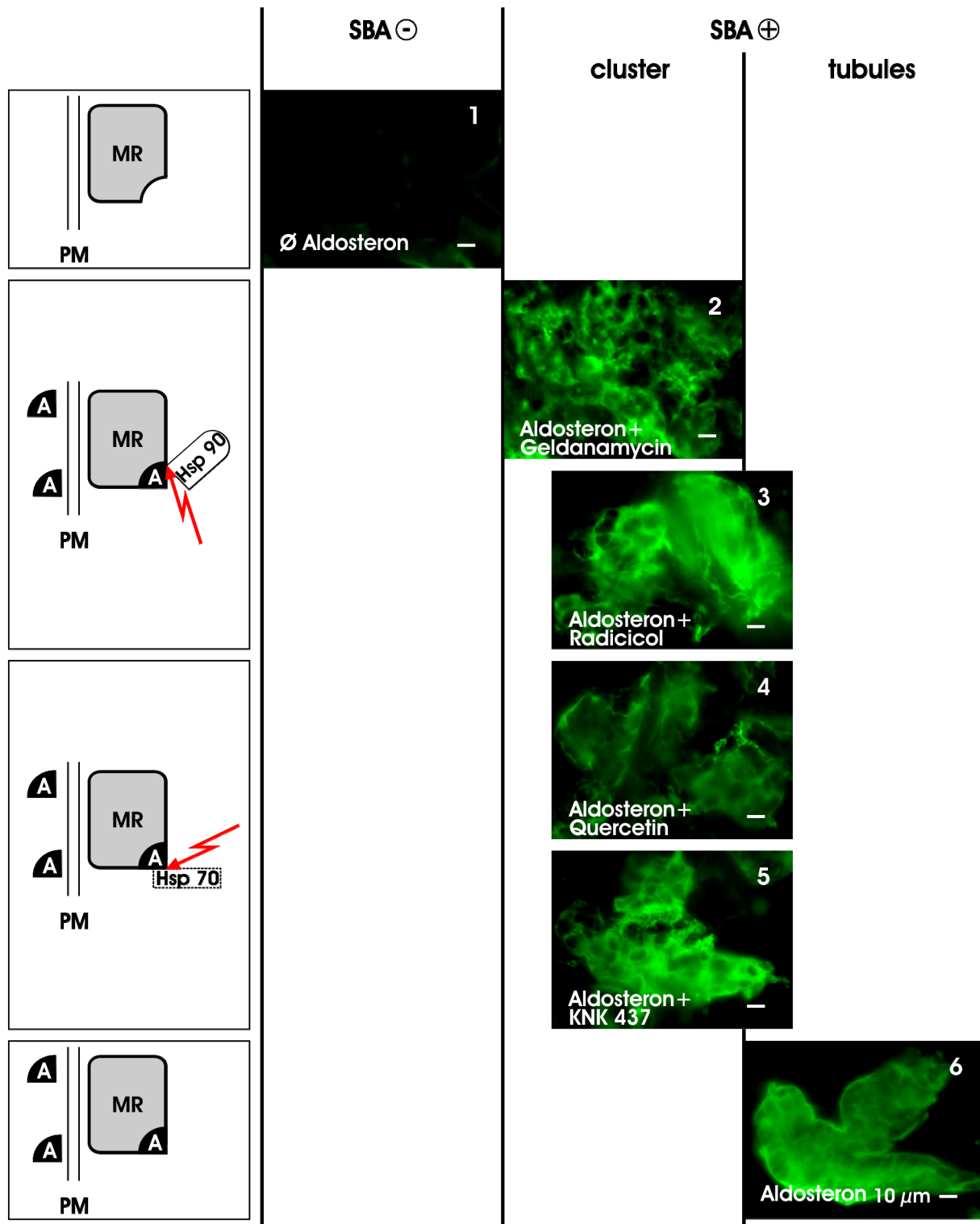


Figure 144: Generation of SBA-labeled tubules versus cell clustering by the interference of the tubulogenic signal between the mineralocorticoid receptor and heat shock proteins. Histochemistry demonstrates that renal stem/progenitor cells without hormone administration do not exhibit SBA-label (1). Formation of extended SBA-labeled cell clusters is observed after treatment with aldosterone ( $1 \times 10^{-7}$  M) in combination with geldanamycin ( $3,6 \times 10^{-6}$  M) (2). Application of aldosterone ( $1 \times 10^{-7}$  M) in combination with radicicol ( $2 \times 10^{-6}$  M) is leading to cell clustering, while minimal formation of SBA-labeled tubules was observed (3). Both quercetin ( $2 \times 10^{-4}$  M) (4) and KNK 437 ( $1 \times 10^{-4}$  M) (5) in combination with aldosterone ( $1 \times 10^{-7}$  M) produce numerous SBA labeled cells within

Advanced culture experiments with adherent cells / Minuth and Denk 2015

extended clusters, while only single tubules are found. Formation of intact tubules was observed after aldosterone ( $1 \times 10^{-7}$  M) administration, while occurrence of cell clusters is lacking (6).

Thus, performed culture experiments revealed that structured tubules are not found, when treatment with geldanamycin was performed. Instead numerous SBA-labeled cells are localized within extended clusters.

The binding of MR and hsp 90 can be further investigated with radicicol. It is a macrocyclic antifungal drug binding in the same way as geldanamycin and hindering ATP-dependent conformational changes that are required for cytoplasmic interactions with target proteins such as MR. In consequence, culture of renal stem/progenitor cells with radicicol ( $1 \times 10^{-6}$  M) in combination with aldosterone ( $1 \times 10^{-7}$  M) produced only few structured tubules, but numerous SBA-labeled cells in form of extended clusters (Fig. 144.3). In so far experiments with both geldanamycin and radicicol show that the tubulogenic effect of aldosterone is missing, when the molecular contact between MR and hsp 90 is disturbed.

When the tubulogenic signal was interfered at the level of hsp 70 by the administration of quercetin ( $2 \times 10^{-4}$  M) in combination with aldosterone ( $1 \times 10^{-7}$  M) numerous SBA-labeled cells are contained within extended clusters (Fig. 144.4). However, only a minimal development of tubules can be detected. Same results were obtained with KNK 437. It is a benzylidene lactam molecule that inhibits heat shock factor activity. The effect results in a decreased expression of heat shock proteins interfering thereby activity of MR. Finally, performing culture experiments with KNK 437 ( $1 \times 10^{-4}$  M) in combination with aldosterone ( $1 \times 10^{-7}$  M) illustrates that development of tubules is very low, but numerous SBA-labeled cells are found within extended clusters (Fig. 144.5).

Regarding this series of experiments all of the presented data point out that aldosterone is able to stimulate the formation of tubules, when an intact contact between MR and related chaperons hsp 90 and 70 is given (Fig. 144.6). In contrast, when the contact between MR

and related chaperons is disrupted, the tubulogenic effect of aldosterone is shifting to the formation of extended but untypical SBA-labeled cell clusters (Fig. 144.1-5). One has to accept that the development of cell clusters instead of tubules reflects a malformation. Such a development has to be prevented in each case, when stem/progenitor cells are applied as an implant to promote a process of regeneration in a diseased kidney.

**[search: cell culture minuth heat shock protein]**

**Self-study: Read about steroid hormone receptors**

## **37. Steroid hormones and stem/progenitor cells**

A challenge in future biomedicine is the repair of diseased renal parenchyma by implanted stem/progenitor cells. Following this strategy stem/progenitor cells are transferred from a more or less comfortable but healthy in-vivo respectively in-vitro environment to the harmful atmosphere within a diseased kidney. Here the implanted cells are surrounded by the interstitial fluid, where they are exposed to a multitude of biologically active molecules including a mixture of steroid hormones.

However, up to date sound cell biological knowledge is lacking about an optimal implantation site of stem/progenitor cells and about compounds within the interstitial fluid possibly influencing the renewal of tubules within a diseased kidney. Of special interest are molecules especially steroid hormones occurring all the time in the interstitial fluid. At the begin of experiments it was unknown, if they have promoting or hampering effects on the development of stem/progenitor cells.

To obtain first information about a possible influence of steroid hormones on the development of renal stem/progenitor cells, the present culture experiments were performed. The data provide new information that only aldosterone is promoting the development of tubules, while a set of other steroid hormones interferes this process leading to malformation in form of cell islets and clusters. Although up to date barely investigated, this harmful effect has to be considered when stem/progenitor cells are implanted into a diseased organ.

### **37.1. Culture conditions as near as possible to organ-specific sites**

To investigate the influence of steroid hormones on regenerating tubules a realistic culture system is of great importance. Due to the limited size of embryonic mouse or rat specimens,

neonatal rabbit kidney was selected as a favorite model, since still after birth the cortex of the kidney contains numerous stem cell niches within their original extracellular environment. Underneath the organ capsule two different kinds of stem/progenitor cell populations can be recognized. Within the tip of the collecting duct ampulla epithelial stem/progenitor cells are found. Around each ampulla numerous mesenchymal nephrogenic stem/progenitor cells are present. Advantageous is further that due to the lack of strong extracellular matrix fibers the embryonic tissue containing stem/progenitor cells is easily accessible for isolation.

Stripping off the capsula fibrosa with fine forceps a thin layer of stem/progenitor cells can be isolated (Fig. 145.1). Applying this simple method a layer of up to 1 cm<sup>2</sup> in square can be harvested. Up to date no other species is known for the isolation of renal stem/progenitor cells in such an amount necessarily needed for subsequent cell biological analysis.

For the described culture experiments the isolated layer containing stem/progenitor cells was placed between two punched out pieces of polyester fleece (I-7, Walraf, Grevenbroich, Germany) (Fig. 145.2). As shown earlier this arrangement resulted in a sandwich set up measuring 5 mm in diameter. To prevent damage the sandwich set up was placed between two polyester fleeces measuring 13 mm in diameter for mounting in a base ring of a Minusheet<sup>®</sup> tissue carrier (Minucells and Minutissue, Bad Abbach, Germany). The tissue carrier was then transferred to a perfusion culture container with horizontal flow characteristics (Minucells and Minutissue). By closing the lid of the perfusion container the sandwich set up was fixed in an exact position during culture.

For the maintenance of constant temperature of 37°C the perfusion culture container was placed on a thermoplate (Medax-Nagel, Kiel, Germany) (Fig. 145.3). For a period of 13 days always fresh medium was continuously transported at a rate of 1.25 ml/h with an IPC N8 peristaltic pump (Ismatec, Wertheim, Germany). Applying this method medium is saturated up to 190 mmHg oxygen during transport. This rather high content of oxygen is reached by a

long thin-walled silicone tube, which is highly gas-permeable. It guarantees optimal diffusion of respiratory gas between culture medium and surrounding atmosphere.

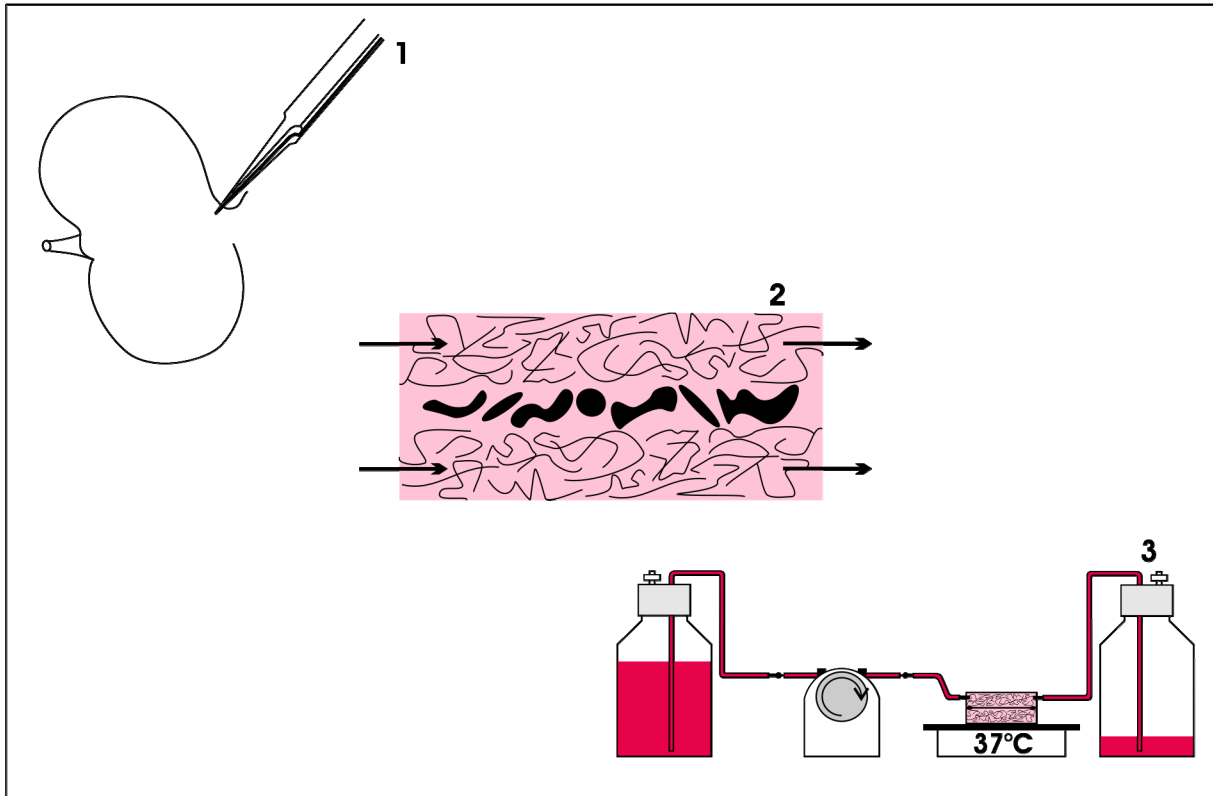


Figure 145: Creation of an artificial interstitium for the long term culture of renal stem/progenitor cells. Stripping off the capsula fibrosa of neonatal rabbit kidney a thin layer containing numerous stem/progenitor cells can be isolated (1). For the generation of tubules during culture the isolated tissue is mounted between two layers of a polyester fleece creating an artificial interstitium (2). A peristaltic pump transports always fresh medium (1.25 ml/h) from the storage bottle to the culture container and then to the waste bottle (3).

In the described experiments chemically defined IMDM (Iscoe's Modified Dulbecco's Medium) including Phenol red (GIBCO/Invitrogen) was used. Infections are prevented by adding an antibiotic-antimycotic cocktail (1%, GIBCO/Invitrogen). To induce tubulogenic development aldosterone ( $1 \times 10^{-7}$  M, Fluka, Taufkirchen, Germany) has to be administered.

All the other tested steroid hormones and metabolites were applied in the same concentration ( $1 \times 10^{-7}$  M). Cholesterol, pregnenolone, progesterone, 11-deoxycorticosterone,

corticosterone, 17- $\alpha$ -hydroxyprogesterone, dihydrotestosterone, testosterone, 17- $\beta$ -estradiol and dexamethasone were obtained from Sigma-Aldrich, Taufkirchen, Germany.

**[search: artificial interstitium aldosterone]**

**Self-study: Get informed about hormone receptors in stem/progenitor cells**

## **37.2. Comparing the effect of aldosterone with its precursors**

Stem/progenitor cells are promising candidates for the regeneration of diseased renal parenchyma. However, after an implantation is performed, they are exposed to the interstitial fluid containing a multitude of bioactive molecules including various steroid hormones.

Since the influence of steroid hormones on renal stem/progenitor cell development is not known, several sets of culture experiments were performed.

After a culture period of 13 days the artificial interstitium was opened by tearing off the fleece layers. The spatial area for tissue development between the fleeces was 5 mm in diameter and up to 250  $\mu$ m in height. To recognize extension of tubule development, cryosections were made to incubate them with antibodies and to register cell distribution and differentiation.

From the adult kidney it is known that not only aldosterone but also the precursors of aldosterone synthesis show an affinity to the Mineralocorticoid Receptor (MR) influencing thereby physiological functions.



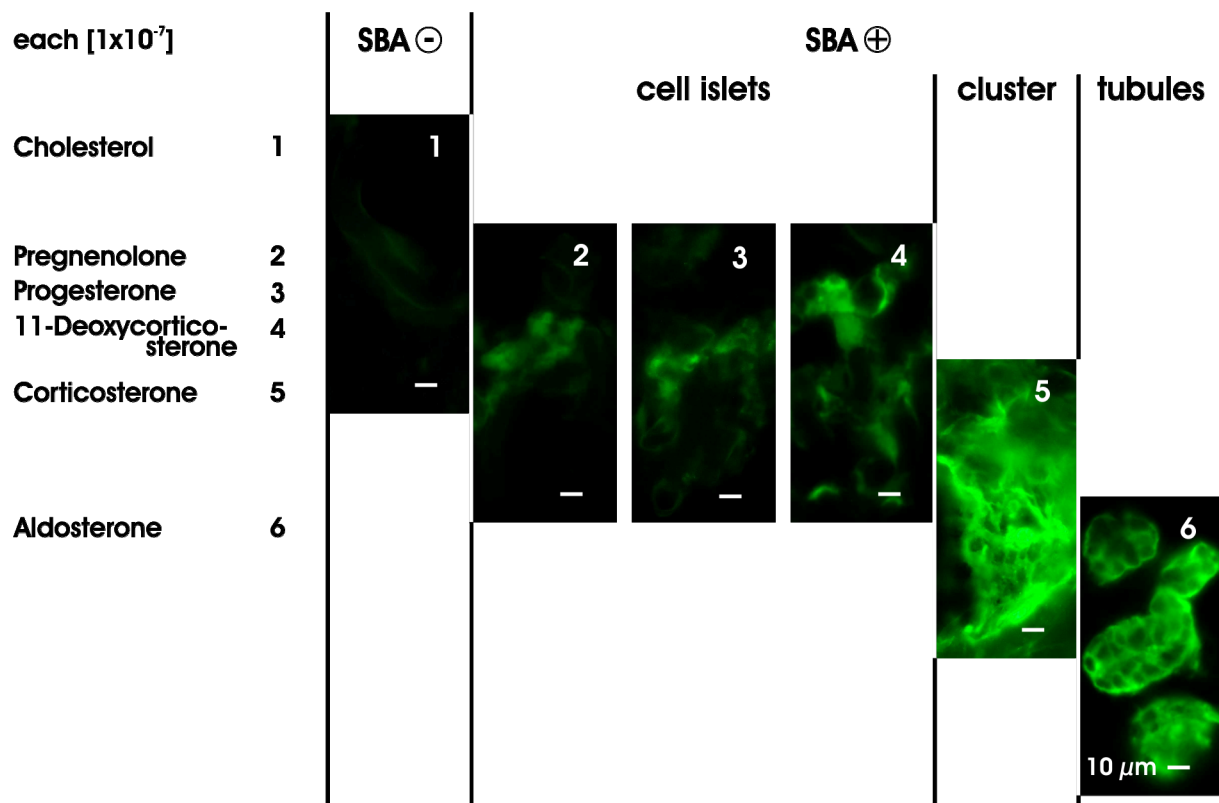


Figure 146: Action of aldosterone and its precursors on renal stem/progenitor cells kept for 13 days in an artificial polyester interstitium. SBA-label illustrates that administration of cholesterol (1) lacks tubulogenic activity, while application of pregnenolone (2), progesterone (3) and 11-deoxycorticosterone (4) produces cell islets. Administration of corticosterone (5) induces numerous SBA-labeled cell clusters. In contrast, application of aldosterone results in numerous SBA-labeled tubules exhibiting a distinct lumen and a clearly recognizable basal lamina (6).

The experiments revealed that administration of cholesterol does not show SBA-label (Fig. 146.1), while application of pregnenolone (Fig. 146.2), progesterone (Fig. 146.3) and 11-deoxycorticosterone (Fig. 146.4) shows small SBA-labeled islets. Use of corticosterone does not stimulate development of tubules, instead numerous and extended SBA-labeled cell clusters were seen in close contact to polyester fibers (Fig. 146.5). Data for 18-hydroxycorticosterone are lacking, since this substance is not commercially available. For control, administration of aldosterone results in numerous SBA-positive tubules exhibiting a distinct lumen and a clearly recognizable basal lamina (Fig. 146.6). This series of experiments clearly demonstrated that precursors of aldosterone synthesis do not stimulate

intact development of tubules. In contrast, untypical structures in form of cells islets and clusters arise.

**[search: renal stem/progenitor cells aldosterone]**

**Self-study: Get informed about the action of steroid hormones as growth factors**

### **37.3. Action of aldosterone versus other steroid hormones**

It is obvious that not only aldosterone and its precursors are contained in the interstitial fluid but also other kinds of steroid hormones. In consequence, one could assume that the tubulogenic development can be evoked beside aldosterone also by other steroid hormones showing affinity to both the Glucocorticoid Receptor (GR) and MR. However, administration of 11-deoxycorticosterone ( $1 \times 10^{-7}$  M) produced SBA-labeled cells within atypical islets (Fig. 146.4), while in corticosterone ( $1 \times 10^{-7}$  M) treated specimens SBA-labeled cells within extended atypical clusters were noticed (Fig. 146.5). Also application of dexamethasone ( $1 \times 10^{-7}$  M) produced atypical aggregation of SBA-labeled cells in form of clusters, while development of intact tubules was not observed (Fig. 147.6).

Beside glucocorticoids the influence of further steroid hormones on renal stem/progenitor cells was tested. In this set of experiments the pattern of reactions could be asorted to four different categories of reactions.

1. Administration of 17- $\alpha$ -hydroxyprogesterone ( $1 \times 10^{-7}$  M) for example does not evoke SBA-binding on cells and does not stimulate the formation of tubules (Fig. 147.1).
2. Application of dihydrotestosterone ( $1 \times 10^{-7}$  M) for example produces SBA-labeled cells within small islets (Fig. 147.2).

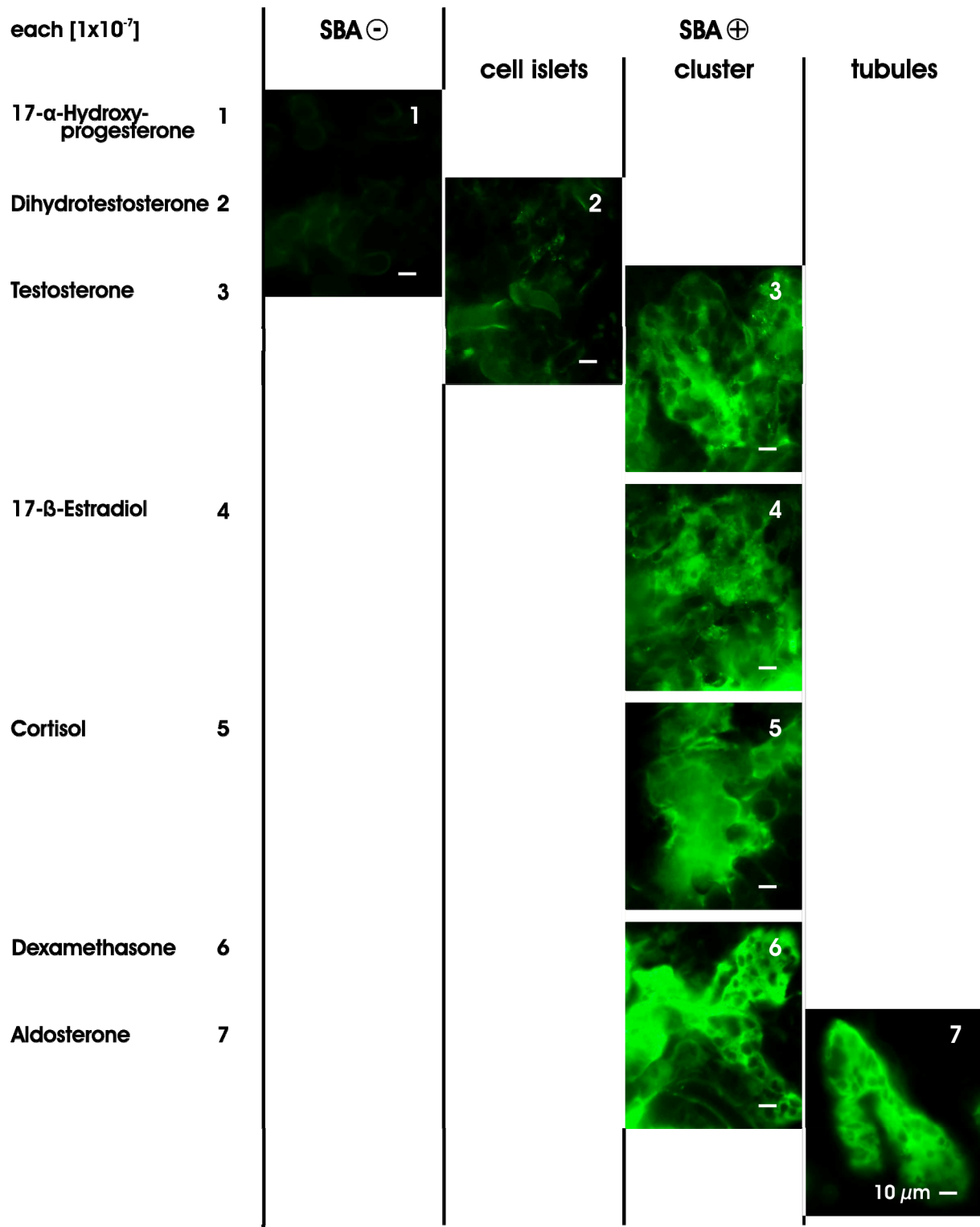


Figure 147: Action of steroid hormones on renal stem/progenitor cells kept for 13 days in an artificial polyester interstitium. 17-α-hydroxyprogesterone does not reveal a positive effect on renal stem/progenitor cell development (1). Formation of SBA-labeled cell islets is found after treatment with dihydrotestosterone (2). Extensive formation of SBA-labeled cell clusters can be detected after application of testosterone (3), 17-β-estradiol (4), cortisol (5) and dexamethasone (6). SBA labeled tubules can only be observed after aldosterone application (7).

3. Administration of testosterone ( $1 \times 10^{-7}$  M) (Fig. 147.3), 17- $\beta$ -estradiol ( $1 \times 10^{-7}$  M) (Fig. 147.4), cortisol ( $1 \times 10^{-7}$  M) (Fig. 147.5) and dexamethasone ( $1 \times 10^{-7}$  M) (Fig. 147.6) results in the formation of extended SBA-labeled cell clusters.
4. Application of aldosterone ( $1 \times 10^{-7}$  M) is leading to the development of intact SBA-labeled tubules, while formation of cell islets and clusters does not occur (Fig. 147.7).

Most interestingly, up to date no information is available about the molecular mechanisms switching between typical tubule development (Fig. 148.4) and atypical formation of cell islets (Fig. 148.2) or extensive clusters (Fig. 148.3). A plausible explanation might be that unspecific binding of steroid hormones may destabilize MR or that different steroid hormones may selectively modulate the mineralocorticoid function. A further idea is that aldosterone is acting over a classical MR and a non-classical receptor. Finally, proteins may interact with the mineralocorticoid receptor in the cytosol depending on the presently binding ligand. An alternative pathway for MR activation may be Rac1, a small GTP-binding protein.

In conclusion, a challenge in future biomedicine is to implant stem/progenitor cells to initiate regeneration of diseased parenchyma within the kidney. It appears most probable that stem/progenitor cells are not injected as a cell suspension but are implanted within a nano-structured fleece material to concentrate at the site of damage and to facilitate the start of regeneration.

Following this strategy one has to consider that both the artificial interstitium and a variety of steroid hormones occurring in the interstitial fluid may influence the starting process of regeneration.

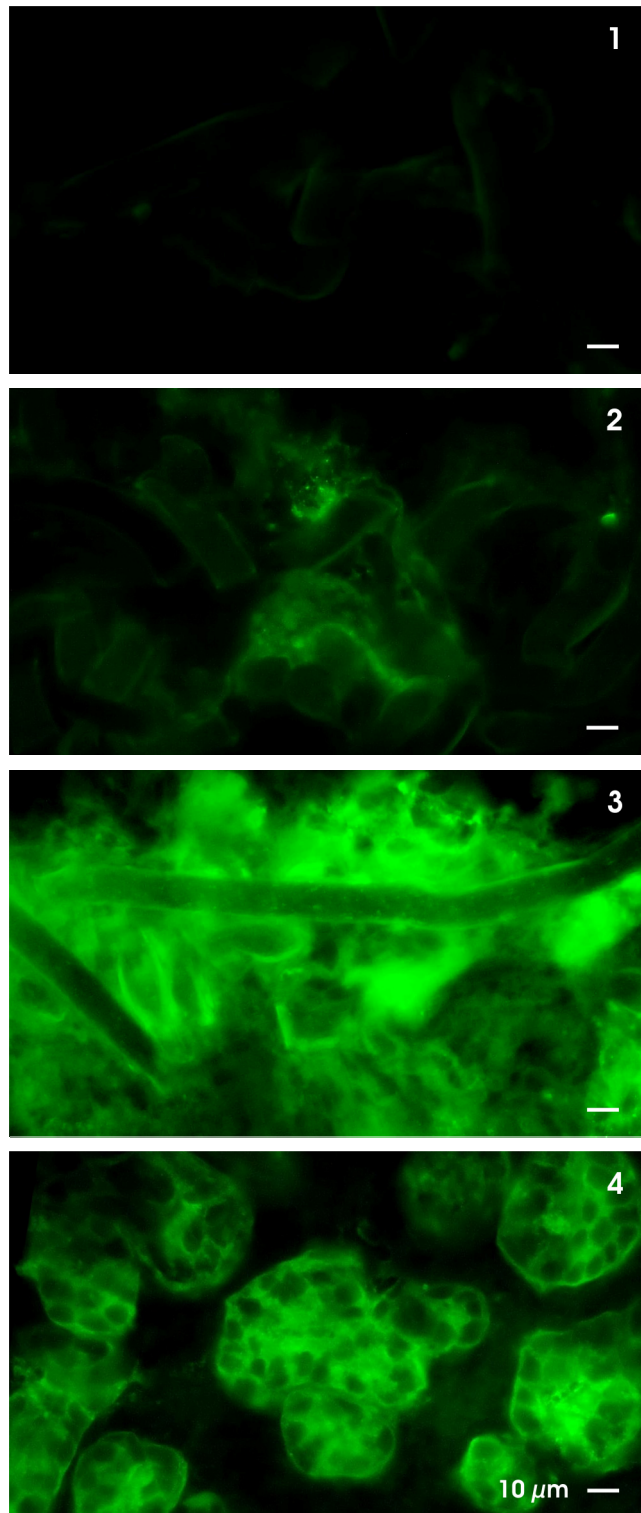


Figure 148: Action of different steroid hormones inducing malformations or tubules. Renal stem/progenitor cells were kept for 13 days in an artificial polyester interstitium. The effect of steroid hormones can be divided into four groups: Lack of SBA-label (1), formation of cell islets (2), extensive cell clusters (3) and tubule development (4).

It was the first time that an interference of steroid hormones on development of stem/progenitor cells could be demonstrated in the presented experiments. The harmful

influence of individual steroid hormones contained in the interstitial fluid of the kidney may be one of the reasons that implanted stem/progenitor cells show up to date a limited survival after implantation. In so far experiments are in progress to compensate harmful effects of steroid hormones and to elaborate the molecular mechanisms switching between solid development of SBA-labeled tubules and atypical formation of cell islets respectively clusters (Fig. 148).

**[search: renal stem/progenitor cells steroid hormone]**

**Self-study: Read about malformations in embryonic tissues induced by steroids**

## 38. Interface between tubules and fleece fibers

Recovery from acute or chronic liver, pancreas or kidney failure requires a substitute of injured tissue with new cells that restore epithelial integrity and functionality within tubules. An increasing number of investigations is therefore dealing with strategies for repair of parenchyma by the help of stem/progenitor cells. Up to date the results show that an effective therapy is still far away from a widespread clinic application.

Regarding kidney regeneration unsolved issues in renal tissue engineering are the concentration of stem/progenitor cells at the site of damage, their integration in a diseased environment, the controlled steering of the process of differentiation into nephron-specific cells and the spatial regeneration of tubules within the kidney parenchyma.

An innovative technical concept is to incorporate stem/progenitor cells in a polyester fleece and to implant it as a pad underneath the capsule of a diseased kidney (Fig. 149). Following this strategy the therapeutic aim is that stem/progenitor cells are able to regenerate intact parenchyma. In this context the diseased parenchyma must not have a harmful influence on the ongoing development of implanted stem/progenitor cells. Further the implanted fleece consisting of polyester fibers must not have a harmful influence on diseased parenchyma, on intact parenchyma and on implanted stem/progenitor cells. In so far it must be fully biocompatible.

Regarding the schema it can be recognized that regeneration does not depend on one but on several and very different influences (Fig. 149). All of the involved partners of the process must positively interact so that intact parenchyma can arise. In each case diseased parenchyma and applied fleece materials must not have a harmful influence.

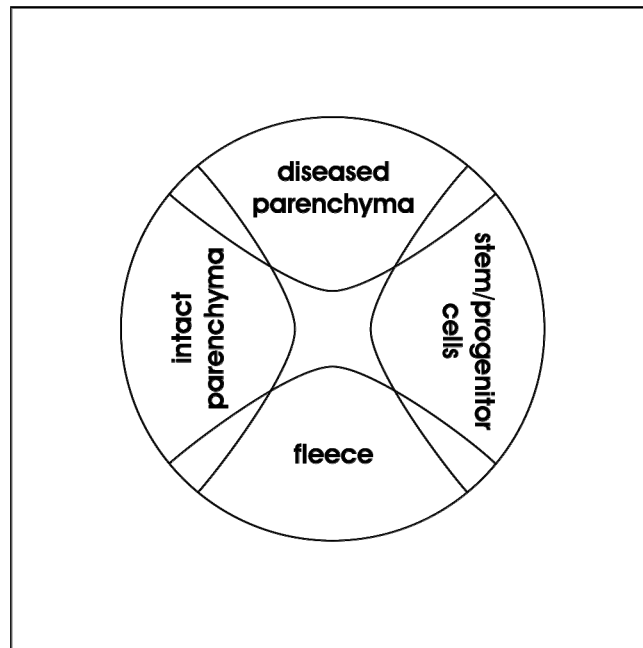


Figure 149: Schematic illustration of various interactions between stem/progenitor cells, implanted fleece material, diseased and intact parenchyma.

A further unsolved issue in this coherence is the organization of a special area in the diseased organ that signals stem/progenitor cells to regenerate tubules at this specific site and in the necessary amount urgently needed for the repair.

Further incorrect fusion of tubules must be prevented but spatial development of them in close neighbourhood must be supported. For example, in the embryonic kidney can be seen that tubules do not exhibit a close contact to each other so that they are separated by an astonishingly wide interstitial space during the first phase of development (Fig. 150).

In contrast, during proceeding development it can be observed that the interstitial space between tubules becomes closer and closer. The essential question in this coherence is, which mechanisms are determining the primary site of arise and the cell biological processes steering the spatial orientation including distance between generating renal tubules.



Up to date it is an unsolved question if the distance between generating tubules occurs accidentally or if it can be influenced for example by the selection of an advanced fleece texture or by a better adapted culture environment.

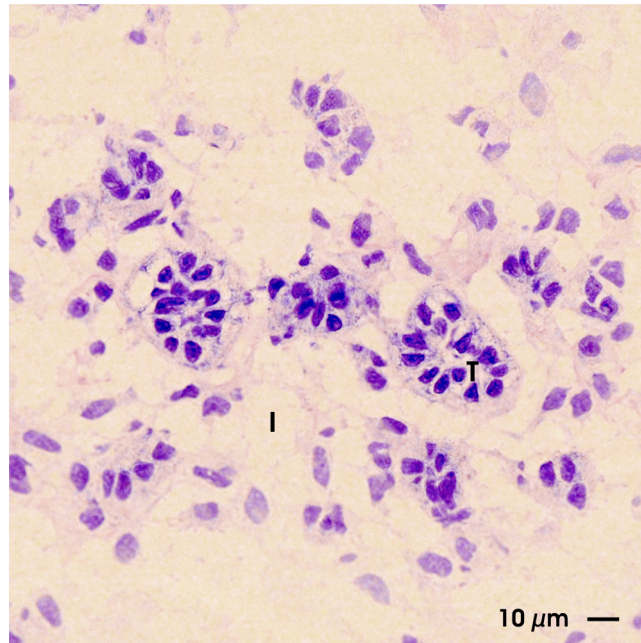


Figure 150: Semithin section of tubules in a developing kidney. The illustration shows that the growing tubules (T) are surrounded by an astonishingly wide interstitial space (I).

Thus, to obtain an idea about the mechanisms steering the distance between tubules, renal stem/progenitor cells were placed between two layers of an I-7 polyester fleece to simulate an artificial interstitium for the spatial extension of tubules. As earlier described the space between the fibers promotes the exchange of nutrition and respiratory gas, when always fresh and chemically defined medium is transported to the perfusion culture container during a period of 13 days.

Earlier performed experiments demonstrated that the application of a polyester fleece influences to a high degree the transition of stem/progenitor cells into polarized epithelial cells during spatial growth. Consequently it was assumed that the fibers of the fleece may play also a key role in spacing the regenerating tubules.

Thus, the focus of further experiments was directed to the distance of them. Most astonishingly, the actual data reveal that generating tubules avoid on the one hand a direct contact to the fleece fibers and on the other hand they keep a surprisingly minimal distance to neighbouring fleece fibers and arising tubules.

**[search: kidney stem cell regeneration]**

**Self-study: Inform yourself about the interface between tubules and fleece fibers**

### **38.1. Site to site orientation of generated tubules**

To obtain a more detailed insight in the spacing process of regenerating tubules, perfusion culture was performed as described before. Always fresh medium was transported with 1.25 ml/h by an IPC N8 peristaltic pump during a period of 13 days. For culture chemically defined IMDM (Iscove's Modified Dulbecco's Medium including phenol red, GIBCO/Invitrogen, Karlsruhe, Germany) was used and 50 mmol/l HEPES (GIBCO) was added for maintenance of a constant pH of 7.4 under atmospheric air. As tubulogenic factor aldosterone ( $1 \times 10^{-7}$  M, Fluka, Taufkirchen, Germany) was added.

To analyze the spatial development of generated tubules the artificial interstitium was opened at the end of culture by separating the polyester fleece layers with fine forceps. In a first set of experiments whole mount specimens were labeled by SBA to visualize the spatial distribution and distance of generated tubules. Fluorescence microscopy demonstrates that numerous labeled tubules can be seen in a longitudinal, transversal and oblique course (Fig. 151). All of them exhibit a continuously developed basal lamina, lining epithelial cells and a visible lumen.

To register the density of generating tubules WCIF ImageJ (Bethesda, Maryland, USA) was used as morphometric computer program. The distance between the basal lamina of neighbouring tubules was measured on magnified DIN A4 illustrations. Independently from their length the individual distance between SBA-labeled tubules within a microscopic opening of 579 x 579  $\mu\text{m}$  was analyzed (Fig. 151).

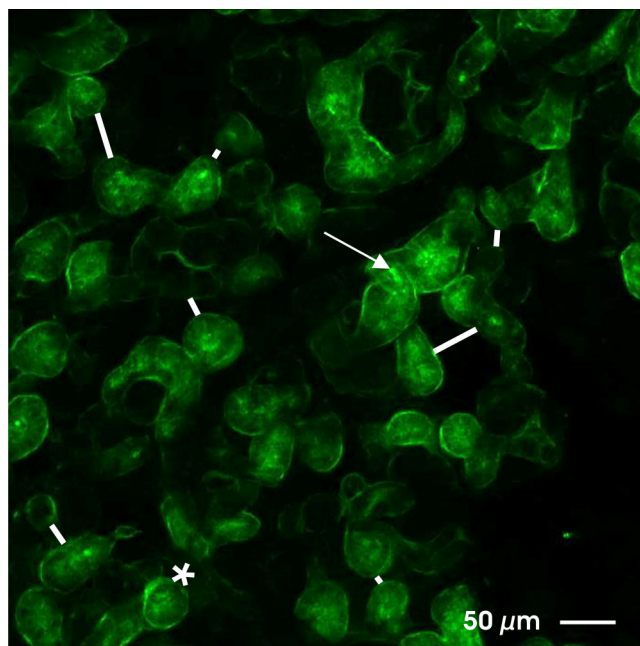


Figure 151: Fluorescence microscopy of SBA-labeled tubules generated over 13 days. All of the tubules exhibit a basal lamina (asterisk) and a lumen (arrow). On a microscopic opening of 579 x 579  $\mu\text{m}$  numerous developed tubules can be recognized. The example demonstrates generated tubules in small-, medium- and big-sized distances (white lines).

Beside the spatial distribution of special interest in this series of experiments was the distance between developing tubules.

To determine the number of SBA-labeled tubules in a microscopic opening of 579 x 579  $\mu\text{m}$ , fluorescent specimens were analyzed by morphometry. In the presented example numerous tubules can be seen (Fig. 151).

Surprisingly, the surface view depicts that generated tubules do not contact each other. In contrast, most of them show small and medium distances, while wide ones are more seldom found as indicated by white bars (Fig. 151).

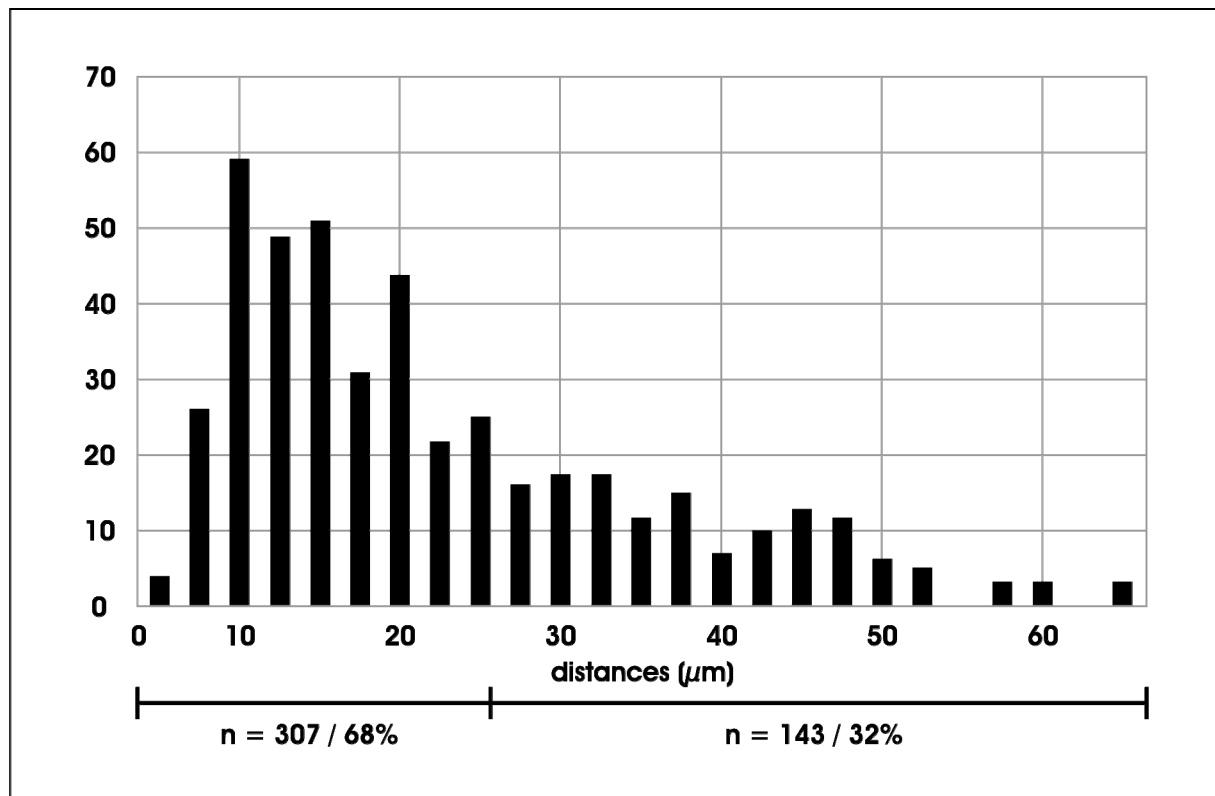


Figure 152: Performance of morphometric measures on numerous recordings depicts that around 68% of tubules exhibit a distance between 5 and 25  $\mu\text{m}$ , while 32% are separated by a distance between 26 and 65  $\mu\text{m}$ .

To obtain exact information about the distance between generated tubules, numerous recordings were analyzed by a morphometric computer program (Fig. 152). Most obvious is that a distance between tubules smaller than 2.5  $\mu\text{m}$  does not occur.

In contrast, in the majority of cases (68%) distances between 5 and 25  $\mu\text{m}$  were found. It appears that the most frequently found space is between 10 and 15  $\mu\text{m}$ . In contrast, distances between 26 and 65  $\mu\text{m}$  are less frequently detected (32%).

Thus, the presence of distances in a range between 5 and 25  $\mu\text{m}$  is a clear indication that the spatial formation of generated tubules does not occur accidentally but appears to be organized by the surrounding fleece so that tightly packed parenchyma can regenerate.

**[search: artificial interstitium miess]**

**Self-study: Read about development of renal tubules within polyester fleeces**

## **38.2. View to the outer surface of tubules**

Of special interest is to find out the cell biological reason that regenerated tubules exhibit such a close neighbourhood as detected in the present experiments (Fig. 150, 151). For a more detailed analysis the interstitial space between them was investigated in a further set of experiments by immunohistochemistry. Surprisingly, label for collagen type III is found in both the basal lamina and in the crevice between generated tubules (Fig. 153.1).

This observation shows that at the basal aspect of generated tubules not only epithelial but also interstitial matrix is synthesized. The experiments further demonstrate that laminin  $\gamma 1$  is co-localized with collagen type III not only in the basal lamina, but also in the space between neighbouring tubules (Fig. 153.2).

The immunohistochemical results depict that both collagen type III and laminin  $\gamma 1$  molecules appear as candidates piloting the distance between generating tubules.

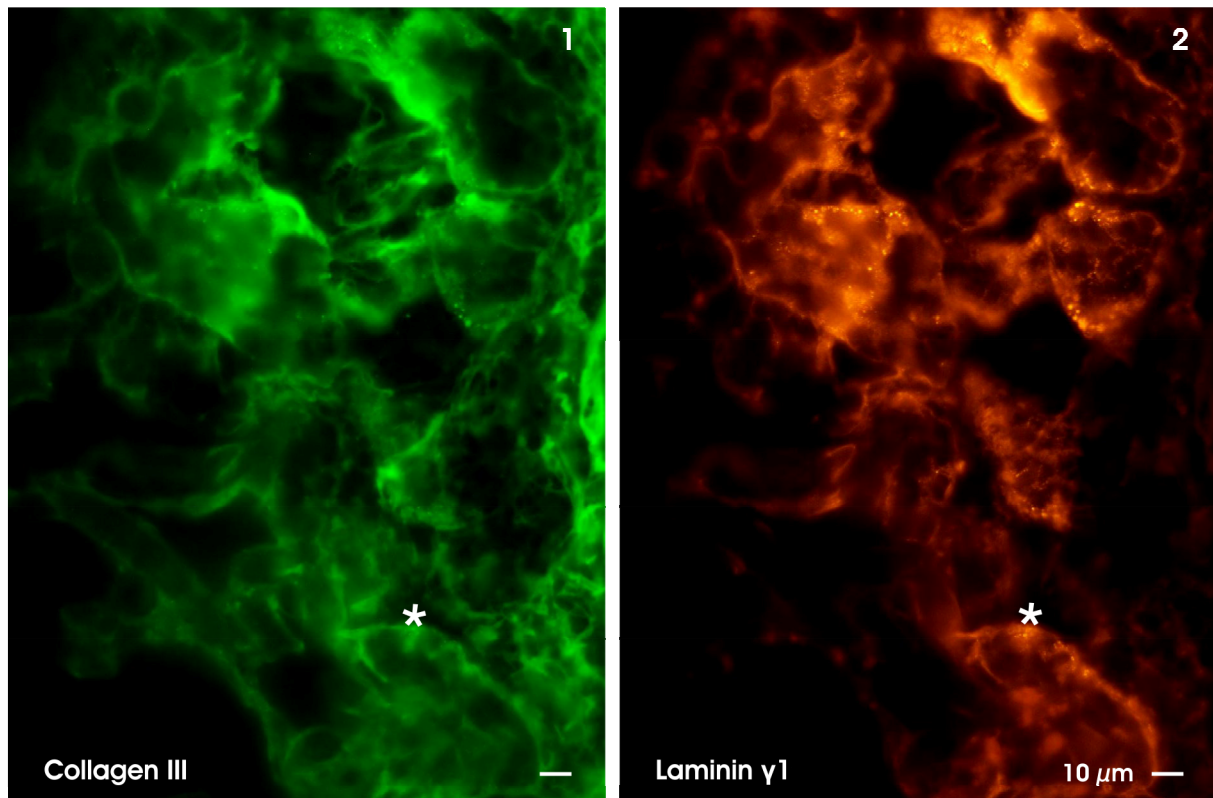


Figure 153: Immunohistochemical analysis of tubules generated over 13 days at the interface of an artificial interstitium. Label for collagen type III is found at the basal lamina (asterisk) and within the gap between the tubules (1). Label for laminin  $\gamma 1$  is located within the basal lamina (asterisk) and between tubules (2).

To obtain a more detailed view from the outer surface of tubules in the next set of experiments Transmission Electron Microscopy (TEM) was performed (Fig. 154). The surface view shows that generated tubules exhibit a polarized epithelium. As shown earlier the luminal plasma membrane of epithelial cells borders a clearly visible lumen. In all of the cases the luminal and lateral plasma membranes are separated by a tight junction complex, while the basal plasma membrane is in intense contact with a basal lamina.

Higher magnification of TEM view illustrates that the basal lamina is composed of several layers as it is known from the kidney. A lamina rara faces the basal plasma membrane of epithelial cells, while a lamina densa and a lamina fibroreticularis cover the tubules at all sides of the outer surface.



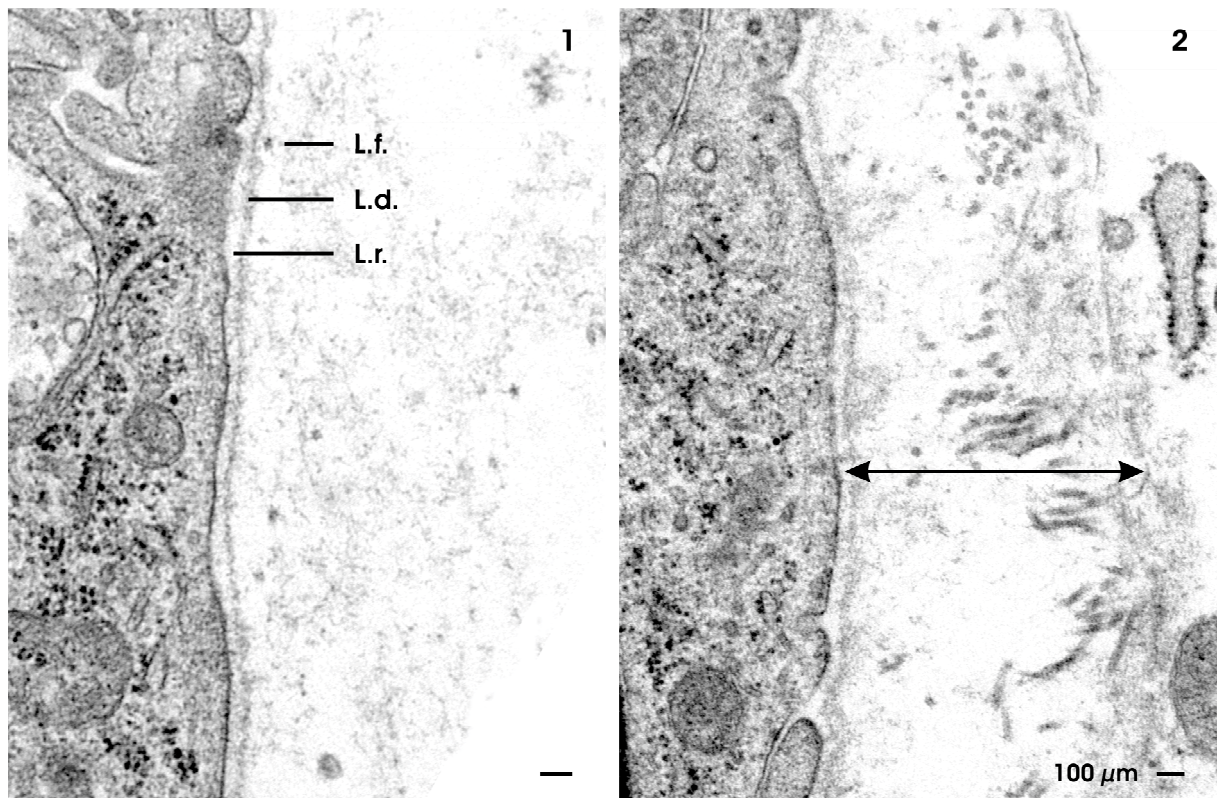


Figure 154: Electron microscopy on generated tubules after 13 days of culture at an artificial interstitium. Transmission electron microscopy depicts that the basal side of the tubule epithelium rests on a basal lamina (asterisk) consisting of a Lamina rara (L.r.), Lamina densa (L.d.) and a Lamina fibroreticularis (L.f.) (1). A varying thickness of the lamina fibroreticularis may cause the individual distance between generated tubules (arrow) (2).

It is obvious that the lamina fibroreticularis acts as a connecting element between the outer side of tubules and the interstitial space (Fig. 154). This area contains numerous collagen fibers. Surprisingly, in some cases the lamina fibroreticularis appears light and thin (Fig. 154.1), while in other cases it is thickened by collagen fibers (Fig. 154.2). Thus, a varying thickness of the lamina fibroreticularis may be the reason that tubules grow in a more or less wide distance to each other.

**[search: artificial interstitium fleece]**

**Self-study: Read about the basal lamina of renal tubules and glomeruli**

### 38.3. Bridging elements within the artificial interstitium

In a further set of experiments Scanning Electron Microscopy (SEM) was performed to receive three-dimensional information about structures spacing respectively connecting generated tubules within an artificial interstitium (Fig. 155). Regarding the fleece layer the polyester fibers exhibit an average diameter of 10  $\mu\text{m}$  and a smooth surface without recognizable roughness.

A surface view by SEM further demonstrates that tubules do not seek a close neighbourhood but are contacting the polyester fleece fibers from time to time but without integrating them into the epithelium. Thus, development of tubules does not occur in really close vicinity of polyester fibers (Fig. 155.1).

SEM micrographs further depict that a basal lamina is covering the complete outer surface of tubules. In addition, the basal lamina of generated tubules is covered over wide areas by a network of extracellular matrix proteins obviously synthesized by interstitial cells (Fig. 155.2). Surprisingly, newly synthesized collagen fibers are spanning between the basal lamina of tubules and the neighbouring fibers of the polyester fleece.

It can be recognized that on the basal lamina of generated tubules dispersed interstitial cells are found (Fig. 155.2). They exhibit a more or less round shape. In some cases their surface appears smooth, while in other cases a network of pseudopodia respectively filopodia and extracellular matrix fibers is protruding from the interstitial cells to neighboring polyester fibers. However, looking by SEM to filopodia of interstitial cells it cannot be decided, where a cell is ending and at which point the extracellular matrix is starting.



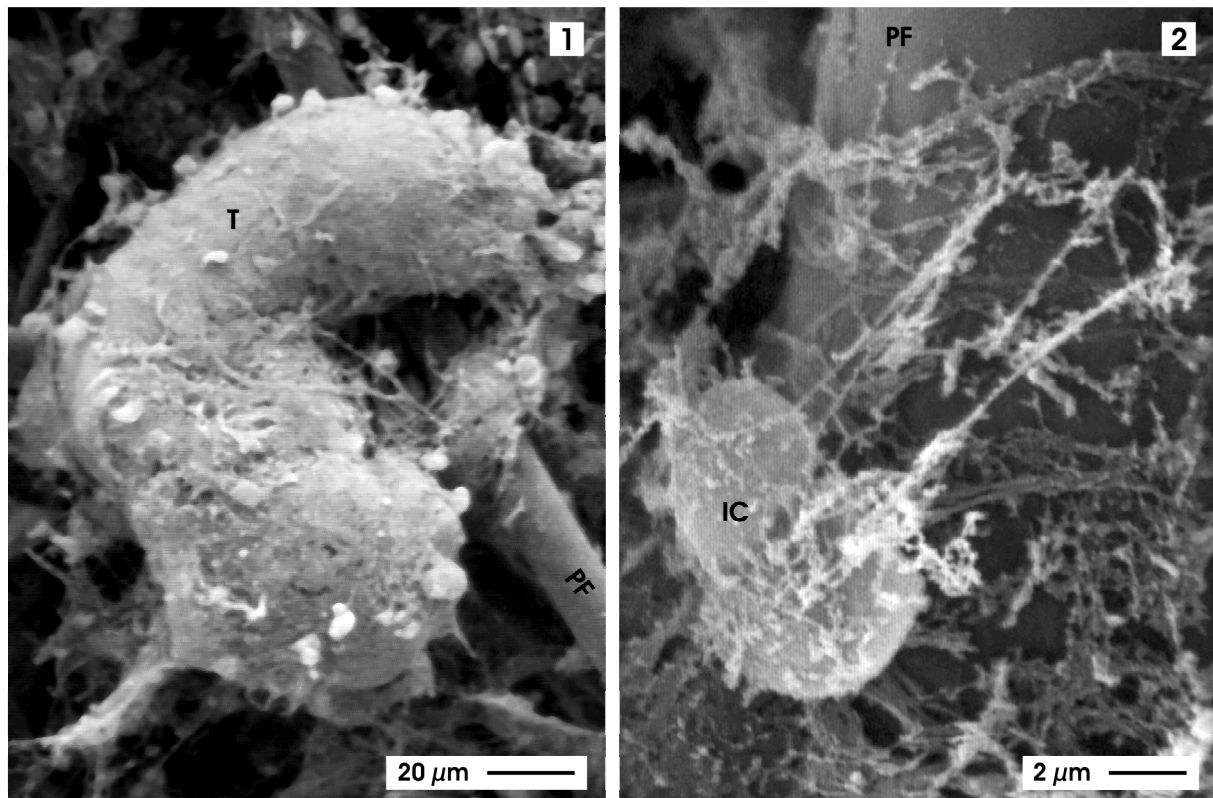


Figure 155: Scanning electron microscopy on generated tubules after 13 days of culture at an artificial interstitium. Analysis shows that fibers of the polyester fleece (PF) are detected in a longitudinal, transversal and oblique course. They exhibit a homogeneous composition and a smooth surface without recognizable protrusions or roughness. The diameter of them is 10  $\mu\text{m}$  in average. Development of tubules (T) occurs in the space between the fibers (1). A basal lamina is covering completely the outer surface of tubules. On this outer surface dispersed interstitial cells (IC) are found exhibiting a round shape (2). On the surface of interstitial cells a network of filopodia and extracellular matrix is recognized lining to polyester fibers (PF).

To obtain more information about the connections between interstitial cells and extracellular matrix in a final set of experiments transmission electron microscopy was carried out (Fig. 156). It can be seen that always a close contact exists between protrusions of interstitial cells and attached fibers consisting of synthesized extracellular matrix. Surprisingly, at the contact site the plasma membrane appears solubilized and the present microstructure is barely recognizable. Instead amorphous material is protruding through the plasma membrane into the cytoplasm.

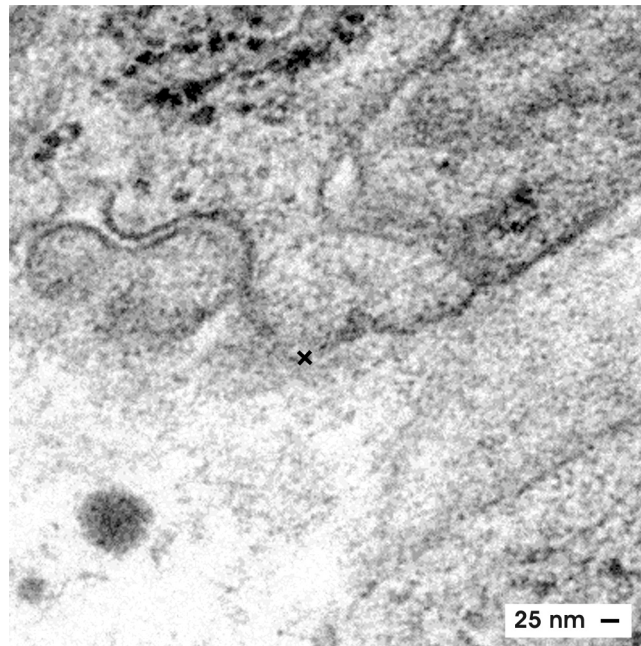


Figure 156: Transmission electron microscopy on generated tubules after 13 days of culture at an artificial interstitium. Analysis illustrates that on the surface of interstitial cells a network of filopodia and extracellular matrix is recognized that is lining to polyester fibers. At the contact site the plasma membrane appears solubilized (cross). Instead amorphous material is protruding through the plasma membrane into the cytoplasm.

In conclusion, the surface view on whole mount SBA labeled specimens illuminates that generated tubules do not fuse, but are separated by a discrete distance to each other (Fig. 151, 152). Morphometric analysis further elucidates that the majority of tubules generated at an artificial interstitium exhibits a distance in the range between 5 and 25  $\mu\text{m}$ . Up to date an exact reason for this discrete distance is not known.

Moreover, generated tubules show a co-localization of collagen type III and laminin  $\gamma 1$  along the basal lamina. The same situation is found within the developing kidney. A clear signal for collagen type III is located in the interstitium of generated tubules. This result illuminates that collagen type III appears as an important master molecule linking the artificial polyester interstitium with the basal lamina of generated tubules.

**[search: kidney interstitium regeneration]**

**Self-study: Read about the interstitial space during regeneration of parenchyma**

## 39. Searching a strategy for implantation

When comparisons are made with patients suffering on liver and kidney diseases, a magnitude of clinical experiences demonstrates that regeneration of parenchyma is limited in patients with chronic or acute renal failure. Thus, due to the different capabilities in regeneration the principal question must be raised, which kind of molecular processes hinder a diseased kidney to regenerate nephrons.

### 39.1. Finding a strategy for stem/progenitor cell application

Beside kidney transplantation and haemodialysis an alternative strategy for the repair of parenchyma is under present investigation. The idea is to implant stem/progenitor cells and to stimulate the process of regeneration in combination with tissue engineering techniques, morphogenic drugs, hormones and growth factors.

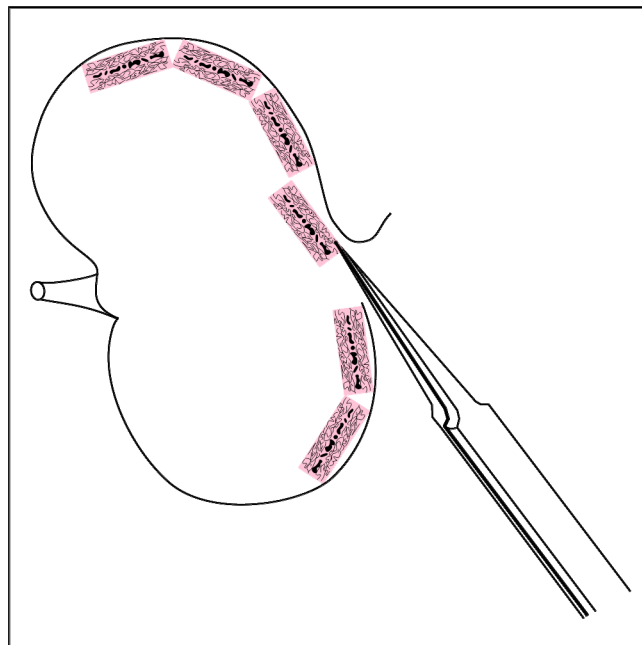


Figure 157: Schematic illustration of subcapsular tactical implantation of stem/progenitor cells respectively maturing tubules within polyester fleeces.

Since accidental injection into diseased parenchyma or infusion of stem/progenitor cells via blood vessels was up to date not leading to successful results, the implantation of stem/progenitor cells underneath the renal capsule in form of a pad is in the focus of actual research (Fig. 157).

Independently from the site of implantation one has to consider that regenerating cells are exposed to an irritating environment within the interstitium of a diseased kidney. It is obvious that in a diseased parenchyma environment tubule cells undergo necrosis, apoptosis and detachment including denudation of the basal lamina. In so far the interstitial fluid of a diseased kidney does not appear as a suitable medium guaranteeing survival of implanted cells. In consequence, at this unfriendly site stem/progenitor cells have first to terminate the process of degradation and then turn it into a process of regeneration. One of the presuppositions for regeneration is therefore the recreation of an optimal microenvironment within the interstitium suppressing inflammation but promoting cell proliferation and differentiation with subsequent spatial development of functional tubules.

Presented experiments reveal that culture of renal stem/progenitor cells at the interface of an artificial interstitium appears on the one hand as an ideal model to investigate stimulating and inhibiting influences of morphogenic drugs and innovative biomaterials on the spatial development of renal tubules. On the other hand exact knowledge about the growth of tubules at an artificial interstitium is the base for a fruitful subcapsular implantation of stem/progenitor cells into the kidney to regenerate parenchyma.

Although first successful culture experiments have been performed, the way to a therapeutic realization needs much more experimental attention. The implantation of stem/progenitor cells within an artificial interstitium is a complex task and has to be realized in a multidisciplinary approach. First for a future therapeutic application one has to find out the best source and an optimal embedding of human stem/progenitor cells.

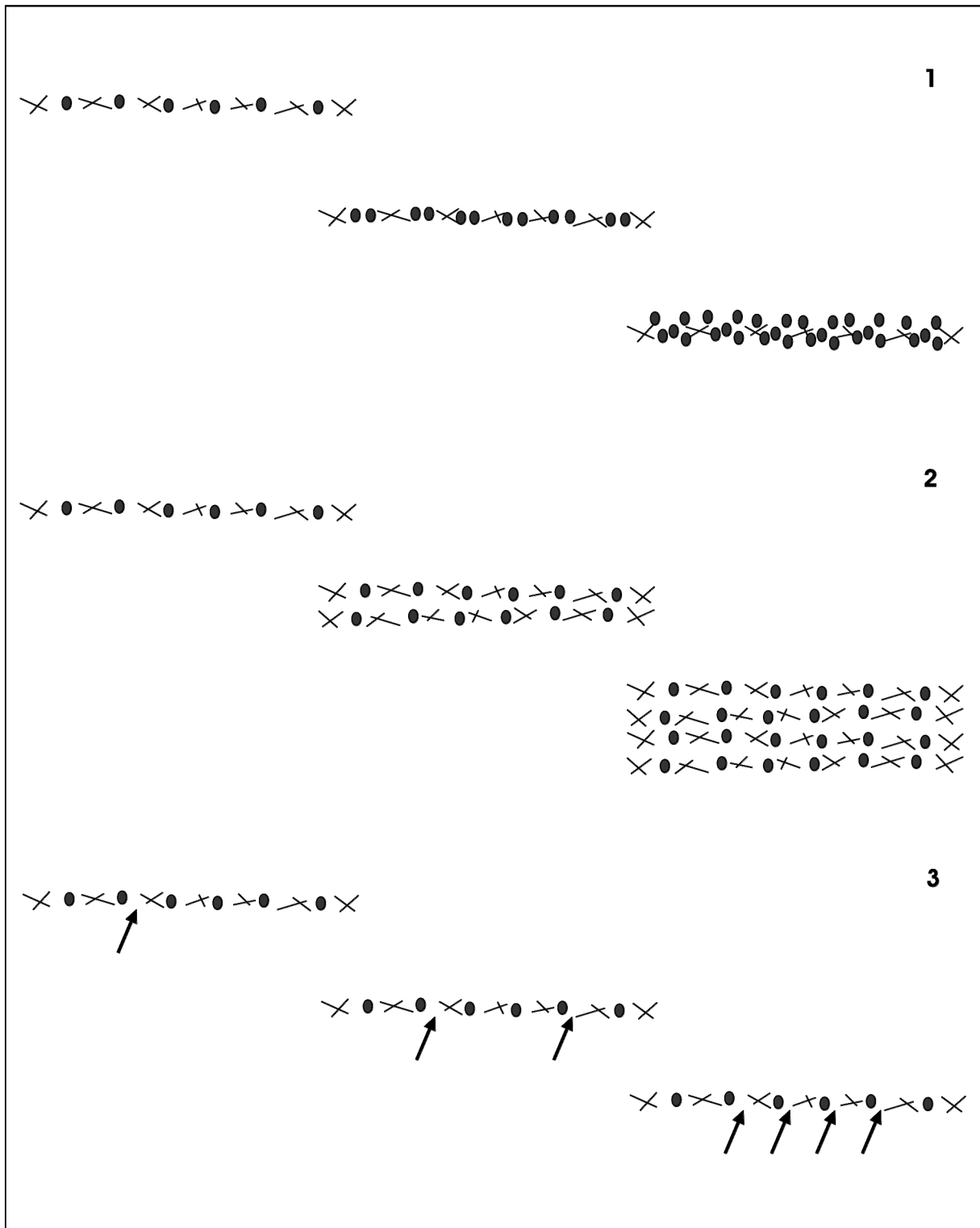


Figure 158: Schematic illustration of interactions between a subcapsular implant and diseased renal parenchyma. For mounting the optimal concentration of stem/progenitor cells on a biodegradable fleece material one has to consider standardized procedures (1). To learn about an optimal integration of implantation one has to elaborate the correct square size and fleece layers harbouring stem/progenitor cells (2). One has to find a suitable fleece material promoting rapid microvascularization (black arrows) supporting regeneration of parenchyma (3).

Following this strategy much more basic research in the area of spatial tubule formation must be performed. For mounting the optimal concentration of cells on a biodegradable fleece material one has to consider a standardized procedure (Fig. 158.1). To know about the optimal situation of stem/progenitor cells after implantation one has to elaborate the correct square sizes and necessary number of the fleece layers (Fig. 158.2). In turn one has to find out a suitable method to promote rapid microvascularization in parallel to the starting regeneration of parenchyma (Fig. 158.3). Finally the implantation must guarantee that the process of degeneration is turned into a process of successful regeneration.

**[search: kidney stem cell implantation]**

**Self-study: Read about implantation of stem cells into organs**

## **39.2. Piling and paving of stem/progenitor cells**

To find first answers for the mentioned problems experiments were performed to concentrate the amount of stem/progenitor cells by piling and paving. Pilot experiments demonstrated that tubules can be generated between two layers of a fleece (Fig. 159.1). However, for an implantation this relatively small amount of stem/progenitor cells appears less suitable for surgical application. That was the reason to concentrate the amount of them within fleeces.

The technical solution is to place stem/progenitor cells between several layers of fleece. The earlier applied sandwich set up configuration (Fig. 159.1) makes it possible to increase systematically the amount of stem/progenitor cells by piling (Fig. 159.2) and/or by paving (Fig. 159.3). These combinations finally result in a pad of increasing thickness harbouring stem/progenitor cells in increasing concentrations.

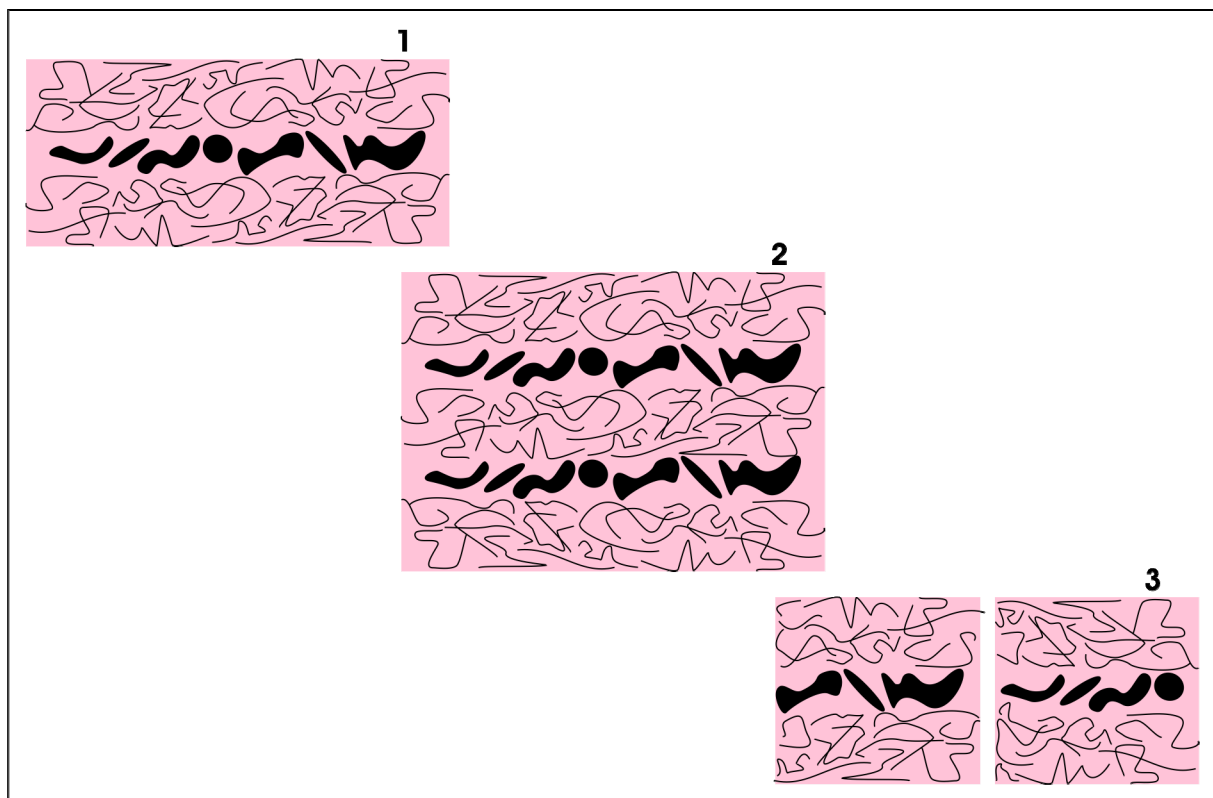


Figure 159: Schematic illustration depicts piling and paving of stem/progenitor cells respectively maturing tubules. A basic sandwich set up by mounting stem/progenitor cells between layers of polyester fleece (1). The layers can be increased by piling (2). The basic sandwich set up can also be used to extend the area of growth by paving (3).

To demonstrate the feasibility of extending systematically the spatial expansion of tubules, in a first issue of experiments renal stem/progenitor cells were isolated and piled between layers of polyester fleece at the begin of perfusion culture (Fig. 159.2). After 13 days of culture the spatial development of tubules was investigated.

A cryosection of piled specimens stained with Toluidine blue shows development of two parallel rows of tubules (Fig. 160). Each of the rows is separated at the top and base by layers of polyester fleece. The experiment clearly depicts that piling makes it possible to concentrate in a spatial orientation renal stem/progenitor cells.



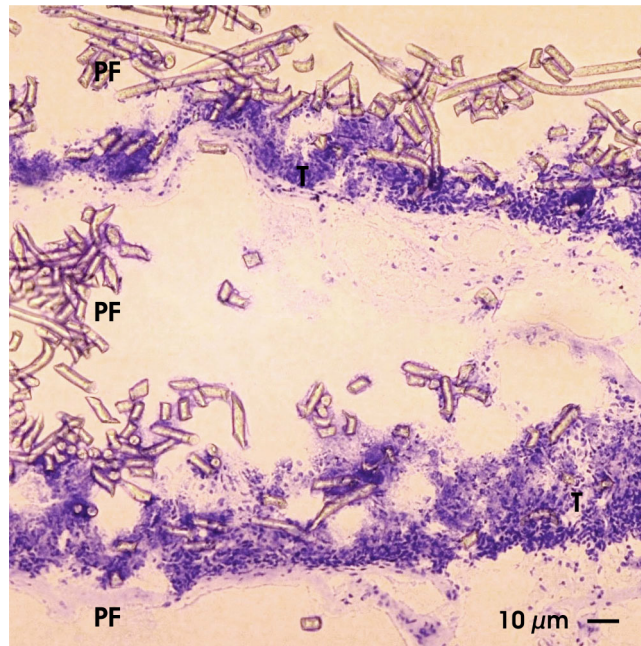


Figure 160: Illustration of piled tubules separated by polyester fibers. Toluidin blue staining of a cryosection demonstrates two rows of tubules (T) between polyester fleece fibers (PF).

Labeling of the generated tubules by SBA (Fig. 161), cyclooxygenase 2 (Cox2), Troma I and E-cadherin further illustrates that a high degree of cell specific differentiation is found to be developed in specimens put on a pile.

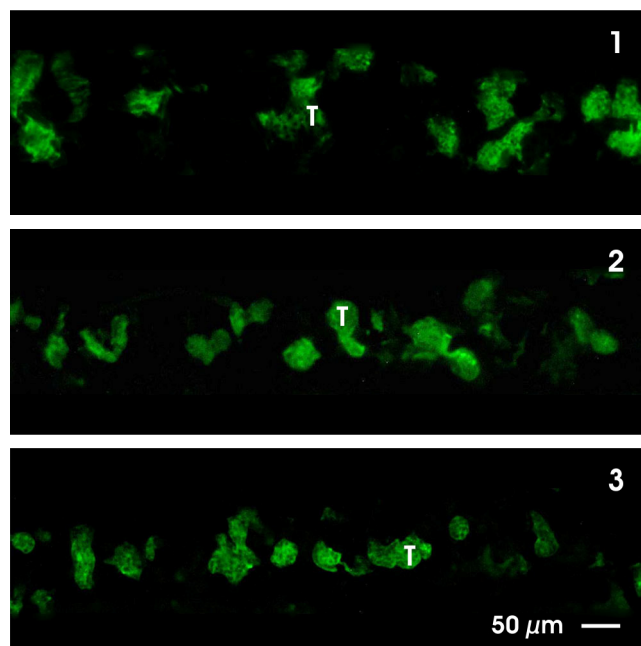


Figure 161: Illustration of SBA- label on three specimens (1-3) of tubules (T) generated under piled conditions.



In so far presented experiments exhibit that piling of basic sandwich set ups like bricks is principally feasible and appears as a fruitful perspective for increasing the amount of stem/progenitor cells and to support the spatial growth of tubules, when a subcapsular implantation into the kidney is taken under consideration. It is obvious that in a similar way other types of stem/progenitor cells can be concentrated in form of piling and paving within fleeces.

**[search: kidney stem cell piling]**

**Self-study: Get informed about the minimal mass of stem cells for development**

### **39.3. Finding a path for microvascularization**

When piling and paving for an implantation is considered, stem/progenitor cells are mounted between layers of polyester fleece. Thus, each row of them is separated by the height of the selected fleece layer. Although a relatively wide spatial separation takes place in this experimental set up, the single tissue rows are connected over an extended network of fluid within the artificial interstitium. In consequence, medium is transported in the space between the fleece fibers guaranteeing at each site constant provision with nutrition and respiratory gas (Fig. 162.1).

Moreover, the highly networked space between the fibers can be used to promote the proliferation of endothelial cells to build up the necessary microvascularization. For example, after mounting of stem/progenitor cells endothelial cells could be added to the culture medium. During run of the experiments always fresh medium is transported along the fluid network with included endothelial cells (Fig. 162.2). When this experimental set up is made after a subcapsular implantation in the kidney, a widely distributed net of capillaries can

develop that promotes further intact vessel formation within shortest time and in parallel to a starting regeneration of tubules.

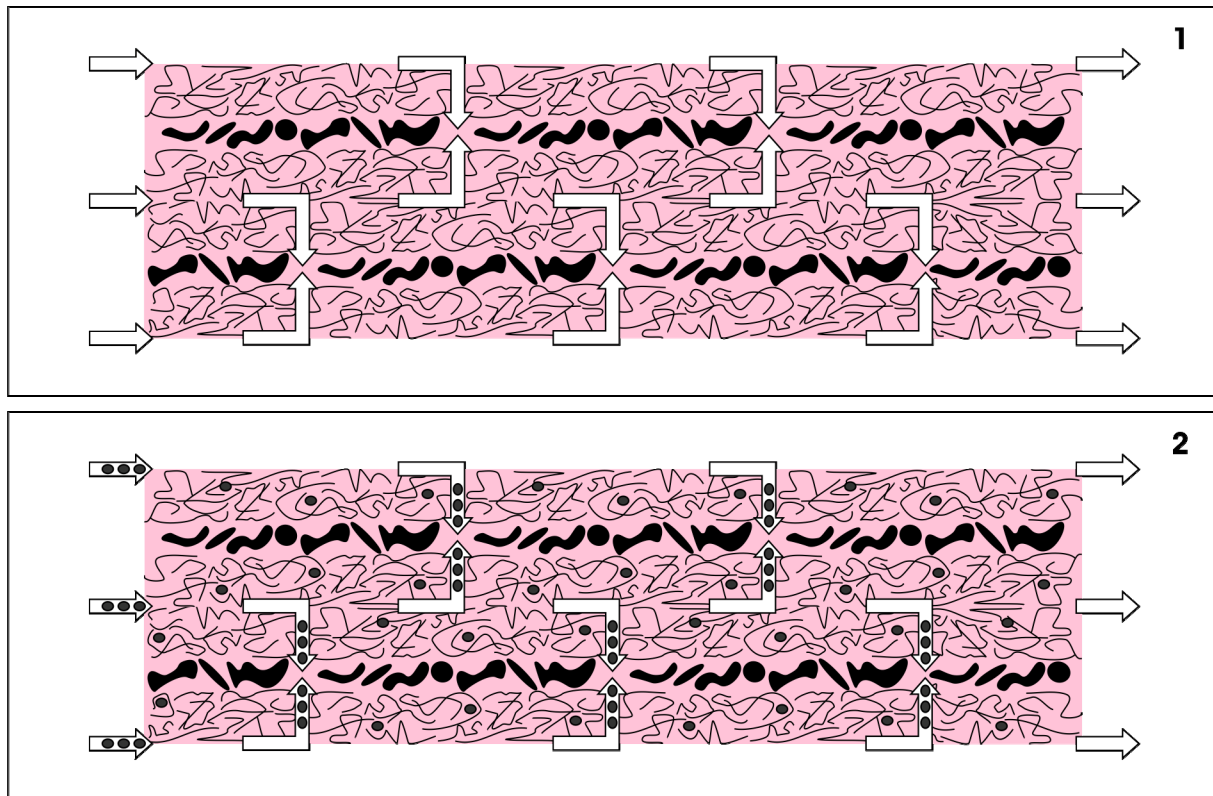


Figure 162: Schematic illustration of fluid path (white arrows) and possible microvascularization (white arrows with dark spots) within piled and paved polyester fleeces. Exchange of fluid is promoted via the highly networked space between the polyester fibers (1). The same path can follow endothelial cells to build up the necessary network of microvascularization (2).

In conclusion, the aim of presented experiments was to collect basic data dealing with the regeneration of renal tubules. Stem/progenitor cells were isolated from the embryonic cortex of neonatal rabbit kidney and mounted between several layers of polyester fleece to create an artificial interstitium. Perfusion culture was performed with chemically defined IMDM containing aldosterone ( $1 \times 10^{-7}$  M) as tubulogenic factor. The experiments demonstrated that renal superstructures within an artificial interstitium can be generated (Fig. 160). Most advantageous for the concentration of stem/progenitor cells and for the spatial development of tubules is, when specimens are piled (Fig. 161). Applying this innovative approach culture medium is transported through the space between the fleece fibers supporting provision with

nutrition and respiratory gas. Following this strategy unstirred layers of fluid are prevented, while exchange of nutrition including respiratory gas is promoted within the artificial interstitium.

Keeping piled and/or paved stem/progenitor cells at the interface of an artificial polyester interstitium the influence of newly developed morphogenic drugs, innovative drug delivery systems in combination with advanced biodegradable fleece materials can be tested.

The challenge for the future is to implant stem/progenitor cells seeded in a concentrated form within an artificial interstitium and to find a secure protocol for guiding the process of regeneration within a diseased kidney.

**[search: kidney stem cell microvasculature]**

**Self-study: Read about microvaculature after implantation of stem/progenitor cells**

## 40. Selecting polyester fleeces for implantation

Since several years the implantation of stem/progenitor cells for the regeneration of renal parenchyma in acute and chronic renal failure is under actual biomedical investigation. Independently if stem/progenitor cells are applied by infusion over blood vessels, by accidental injection into diseased parenchyma or by a subcapsular implantation, in all of the cases they have to migrate through the interstitial space into diseased areas of the organ. At this site stem/progenitor cells have to terminate the process of degradation and turn it in a process of regeneration. One of the presuppositions is that an optimal microenvironment has to be created suppressing inflammation but promoting cell proliferation with subsequent differentiation so that functional tubules for the process of restoration can arise.

Provider	Walraf	Positech			
Identification	I-7	Posi-4	Posi-5	Posi-6	Posi-7
Thickness [mm]	0.59	0.13	0.17	0.18	0.18
Water porosity [L/m <sup>2</sup> /S]	-	1 480	1 200	1 100	700
Micron rating [μm]	-	40	30	25	15

Figure 163: Physical parameters of polyester fleeces used for the generation of renal tubules within an artificial interstitium. Except date for the I-7 fleece, all other data are given by the provider.

Following the strategy that stem/progenitor cells are implanted into the subcapsular space of the kidney, one can imagine that the binding of them within a fleece covers surgical and cell biological needs. Due to the rigidity of the material this method allows first an optimal transfer of stem/progenitor cells to the site of implantation by forceps and appears then to be able to produce an optimal surrounding during the initial phase of implantation as proved by multiple culture experiments.

For example, previously performed experiments have demonstrated that primary steps and basic mechanisms of tubule development can be optimally investigated under innovative perfusion culture conditions, when stem/progenitor cells are developing within a fleece. Further it could be demonstrated that the formation of numerous tubules was optimally achieved by the application of an artificial interstitium made by an I-7 polyester fleece.

However, an I-7 polyester fleece layer is measuring 0.59 mm in thickness (Walraf, Grevenbroich, Germany) (Fig. 164). In consequence, this thickness is rather high in relation to the little amount of contained stem/progenitor cells. Problematic is further that biophysical parameters such as water porosity and micron rating were not given by the provider.

The implantation of stem/progenitor cells only can be successful, when the necessary amount of potent cells is available at the site of regeneration. However, when stem/progenitor cells are mounted within a thick fleece such as an I-7 specimen and when several layers are piled, the concentration of contained cells will not be high enough to promote a process of regeneration. Thus, the thickness of the I-7 fleece appears as the most limiting factor to concentrate renal stem/progenitor cells. For that reason other kinds especially more thin polyester fleeces had to be taken under consideration.

**[search: stem cell polyester fleece]**

**Self-study: Get informed about polyester fleeces**

## **40.1. Finding the correct type of fleece**

When later a pad is used for implantation, the number of cells within piled fleece layers must be increased by avoiding at the same time to increase its total thickness. In consequence, the previously used I-7 polyester fleece is regarded to be too thick so that it has to be

replaced by much thinner polyester fleeces. Promising candidates are Posi fleeces such as Posi-4, Posi-5, Posi-6 and Posi-7 (Positech, Hallwil, Suisse) (Fig. 163).

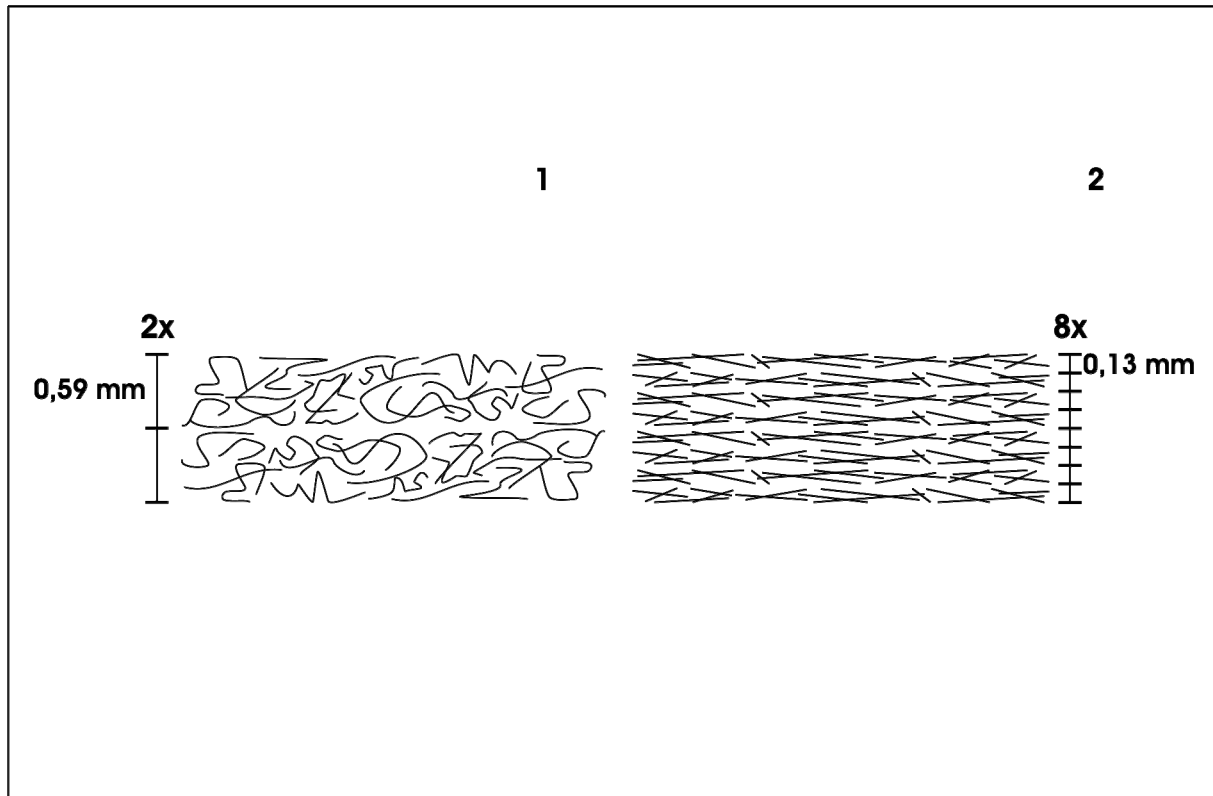


Figure 164: Schematic illustration of stacked I-7 fleeces (1) and stacked Posi-4 fleeces (2). As compared to two layers of I-7 fleece (1) eight layers of Posi-4 fleece (2) can be applied until the same height is reached. Only one row of stem/progenitor cells can be placed between two I-7 fleeces, while seven rows can be mounted between eight Posi-4 fleeces. Thus, the sevenfold amount of stem/progenitor cells can be cultured in specimens within Posi-4 fleeces.

The selected fleece material must harbour on the one hand stem/progenitor cells and on the other hand the resulting fleece pad must be implanted between the capsule and the outer cortex of the renal parenchyma. In consequence, the specific site of implantation requires a minimal thickness of the fleece layer containing adherent stem/progenitor cells so that it can be easily shoved in the narrow cleft between the renal capsule and the superficial nephrons.

It is obvious that the selected material must further exhibit certain stiffness so that a secure transfer by forceps can be made. Further the fleece mechanically has to stabilize adherent

stem/progenitor cells. Then the material must be biocompatible with both surrounding renal parenchyma and implanted stem/progenitor cells. Finally, the implant has to stand inflammatory processes and must promote the mechanism of regeneration including vascularization.

It was demonstrated that Posi (Fig. 164.2) fleeces reveal a decreased thickness as compared to an I-7 fleece (Fig. 164.1). In consequence, Posi fleeces were taken into experimental consideration for the adherence of stem/progenitor cells. All of them consist of pure polyester. They exhibit a thickness between 0.13 to 0.18 mm as compared to 0.59 mm of earlier used I-7 fleece (Fig. 163). When piling of the fleece layers is made, it is obvious that eight layers of Posi-4 fleeces can be used beside two layers of I-7 specimen to reach the same thickness (Fig. 164). However, only one row of stem/progenitor cells fits between two I-7 fleeces, while seven rows can be placed between eight Posi-4 fleeces to reach the same height. In conclusion, a sevenfold increase of stem/progenitor cells can be obtained by piling of Posi-4 fleeces.

Before any surgical implantation is performed the selected support material for the adherence of stem/progenitor cells has to be critically tested by perfusion culture experiments.

**[search: artificial interstitium polyester]**

**Self-study: Read about fleeces for implantation**

## **40.2. Testing tubule formation on promising polyester fleeces**

To investigate the development of tubules on I-7 polyester fleece (Fig. 165.1) in comparison to thin polyester fleeces such as Posi-4 (Fig. 165.2) renal stem/progenitor cells were placed

in a Minusheet® tissue carrier within a perfusion culture container with horizontal flow characteristics. As described earlier the interface between the fleece layers produces an artificial interstitium promoting the spatial development of tubules during the entire culture period. The spatial area for tubule formation was 5 mm in diameter and up to 250 µm in height. During a culture period of 13 days always fresh medium was transported with 1.25 ml/h by an IPC N8 peristaltic pump (Ismatec, Wertheim, Germany). To maintain a constant temperature of 37°C the perfusion culture container was placed on a thermo plate (Medax-Nagel, Kiel, Germany) and covered with a transparent lid.

For the generation of renal tubules chemically defined IMDM (Iscove's Modified Dulbecco's Medium including phenol red, GIBCO/Invitrogen, Karlsruhe, Germany) was used as described earlier. To sustain a constant pH of 7.4 under atmospheric air containing 0.3% CO<sub>2</sub>, 50 mmol/l HEPES (GIBCO) was contained in the medium. To induce tubulogenic development aldosterone ( $1 \times 10^{-7}$  M, Fluka, Taufkirchen, Germany) was added to the culture medium. An antibiotic-antimycotic cocktail (1%, GIBCO) was used in all culture media to prevent infections.

In the present series of experiments whole mount label with SBA was performed to obtain information about the spatial growth pattern of renal tubules generated with polyester fleeces I-7 (Fig. 165.1) in comparison with Posi-4 (Fig. 165.2). Whole mount fluorescence microscopy demonstrated that in all of the analyzed specimens numerous labeled tubules are detected. They exhibit a basal lamina, lining epithelial cells and a visible lumen. However, the pattern of growth differs between I-7 fleeces (Fig. 165.1) and Posi-4 fleeces (Fig. 165.2). Surprisingly, when an I-7 fleece is used, fibers of the fleece between the tubules cannot be observed (Fig. 165.1). In contrast, samples generated with Posi-4 (Fig. 165.2) illustrate that numerous fibers are detected between generated tubules.



To obtain more information about the growth pattern differing between I-7 and Posi-4 fleeces histochemistry on cryosections of generated tubules was performed (not shown). The presented series of experiments revealed that the expression of typical proteins in both series is the same. In contrast, the spatial growth of tubules in Posi-4 fleeces (Fig. 165.2) is different in comparison to previously used I-7 polyester fleeces (Fig. 165.1).

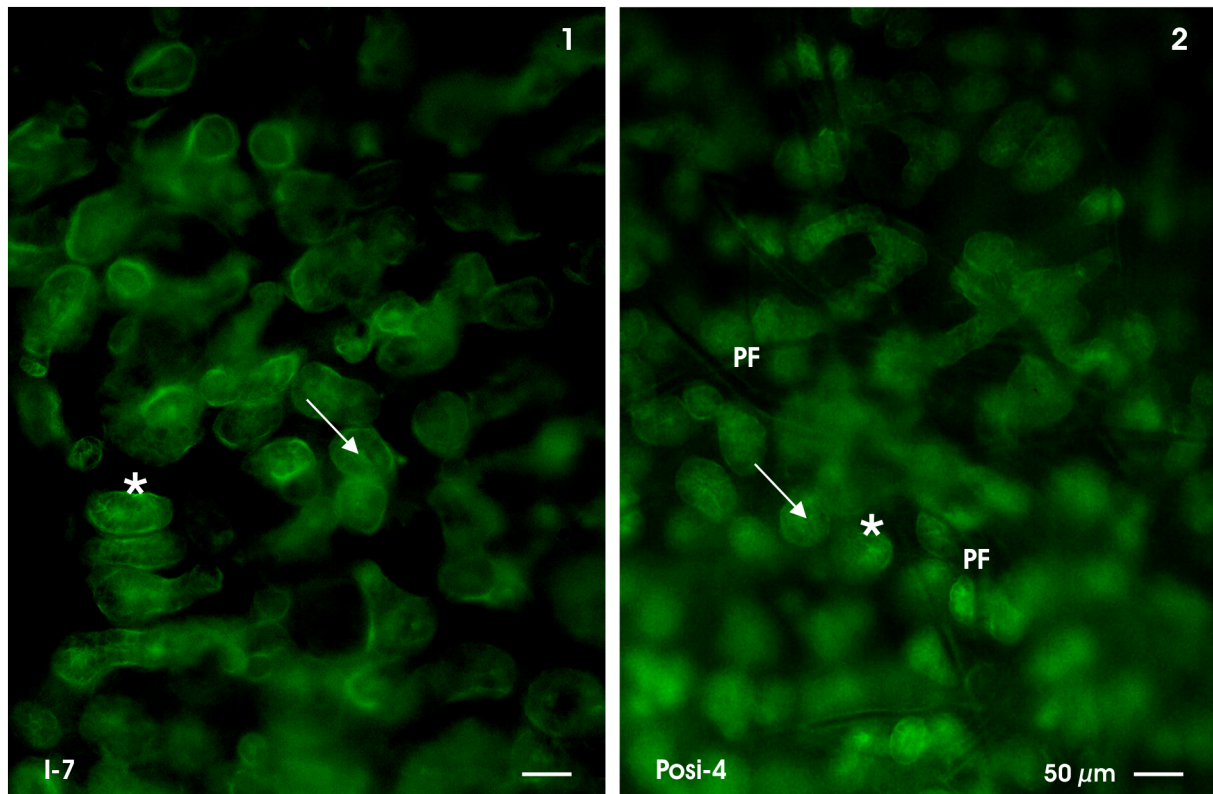


Figure 165: Fluorescence microscopy on whole mount specimens labeled with SBA. Tubules were generated within 13 days at the interface of an artificial interstitium made of I-7 (1) and Posi-4 (2) polyester fleeces. SBA-label shows that polyester fibers are lacking between generated tubules using an I-7 fleece (1). In contrast, specimens generated on Posi-4 (2) fleeces depict numerous polyester fibers (PF) between generated tubules. All of the tubules exhibit a lumen (arrow) and a basal lamina (asterisk).

In series with I-7 tubules are exclusively observed at the artificial interstitium produced between the layers of fleece (Fig. 166.1). Thus, applying I-7 development of tubules between the fibers of the fleece is not observed.

In contrast, numerous fibers between tubules are detected, when Posi-4 fleeces were used (Fig. 166.2). The experiments clearly demonstrate that growth of tubules is not only promoted at the interface between two fleece layers but can also occur between the fibers of tested Posi-4. Since all of the fleeces consist of polyester, the differing spatial distribution of fibers can be given as the only reason for the different growth of tubules.

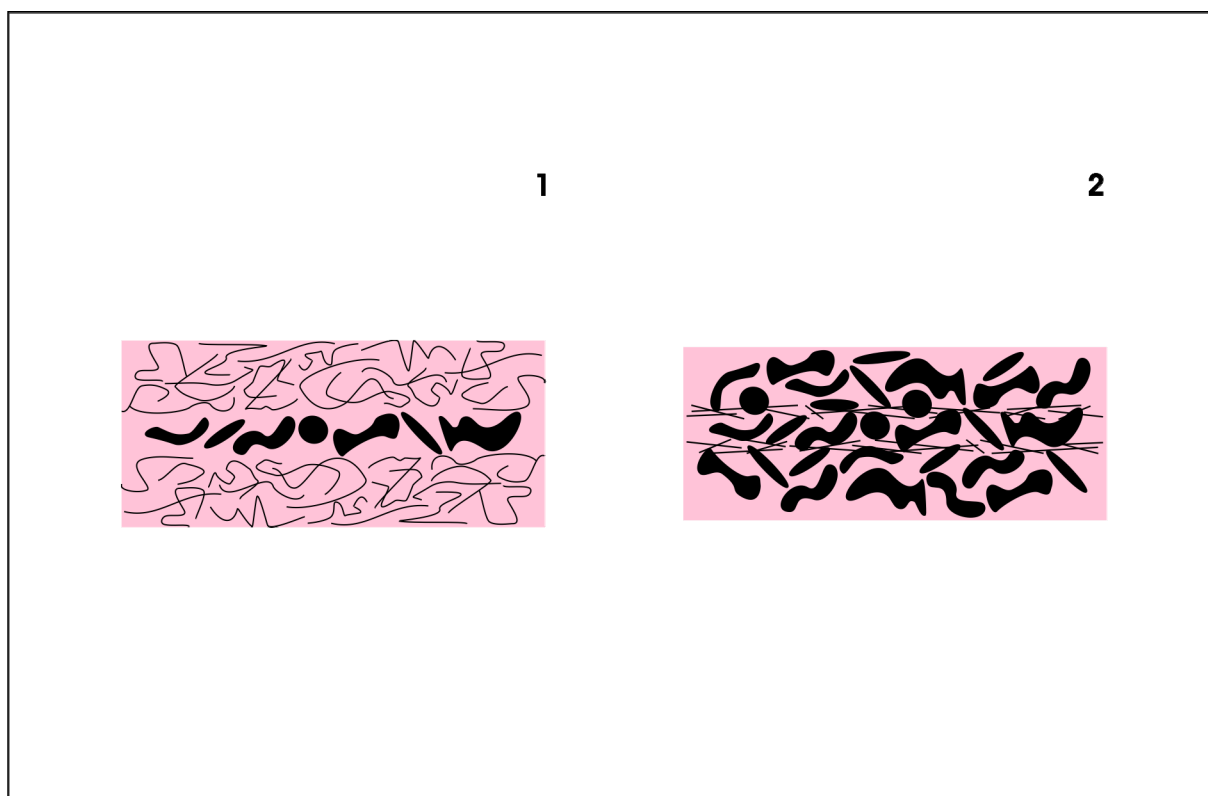


Figure 166: Schematic illustration of the growth pattern of tubules generated on an artificial interstitium made by I-7 (1) and Posi-4 (2) polyester fleeces. Applying I-7 growth of tubules is only observed at the interface between the layers and not between the fibers of the fleece (1). On Posi-4 fleeces formation of tubules is detected as well at the interface between the layers as between and in the neighbourhood of fibers (2).

As revealed by whole mount label all of the Posi fleeces exhibit excellent features for the spatial development of tubules. Most important, toxic effects cannot be observed. Immunohistochemistry of specimens growing in Posi-4, Posi-5, Posi-6 and Posi-7 fleeces (not shown) further demonstrate that the generated tubules exhibit the same pattern of cell biological differentiation profile as demonstrated with an I-7 fleece.

However, application of an I-7 fleece also exhibits that generation of tubules occurs only at the interface between two layers (Fig. 167.1). In contrast, in Posi-4 fleeces development of tubules occurs not only in the space between the fibers, but is also found in a sprouting manner in the wider neighbourhood (Fig. 167.2). Further the thickness of the tubule layer could be increased by piling Posi-4 fleeces. In this case the over all thickness of the explant would be the same as obtained with two I-7 fleece layers (Fig. 167.3).

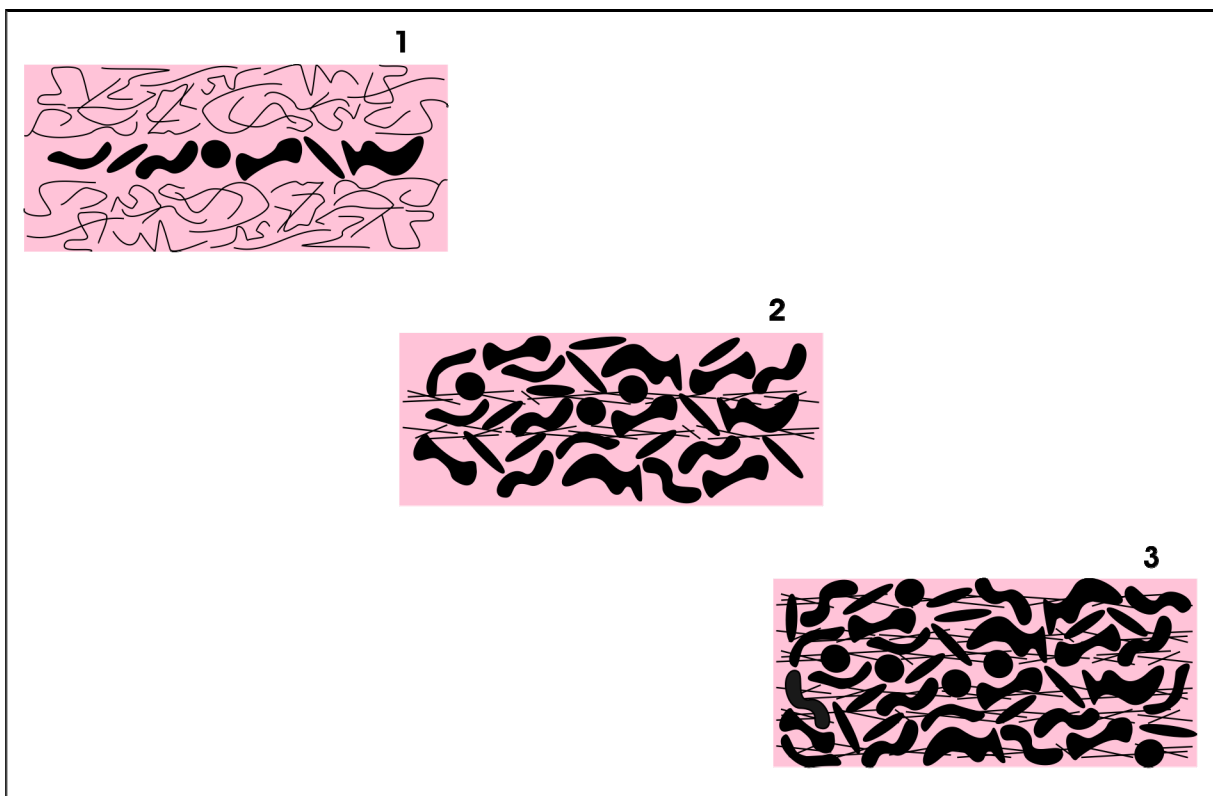


Figure 167: Schematic illustration of the growth pattern of tubules generated on an artificial interstitium made by I-7 (1) and Posi-4 (2,3) polyester fleeces. When an I-7 fleece is used, growth of tubules is only observed at the interface between the two layers (1). In contrast, application of Posi-4 fleeces stimulates formation of tubules in a spatial manner (2). When eight layers of Posi-4 fleeces are piled the same thickness of two I-7 fleece layers is reached. In parallel the amount of tubules can be strongly increased (3).

This result contrasts experiments with Posi-5, Posi-6 and Posi-7 fleeces. Here the growth of tubules is restricted to the close vicinity of polyester fibers. An explanation for the differing pattern of growth cannot be given, since the fleeces consist of the same material.

Although the development of tubules in the wider neighbourhood is only found in series with Posi-4 fleeces data cannot be correlated with physical properties such as thickness, water porosity and micron rating (Fig. 163). Besides thickness, water porosity and micron rating Posi-4, Posi-5, Posi-6 and Posi-7 fleeces differ in diameter of the fibers and in crossing points of fibers.

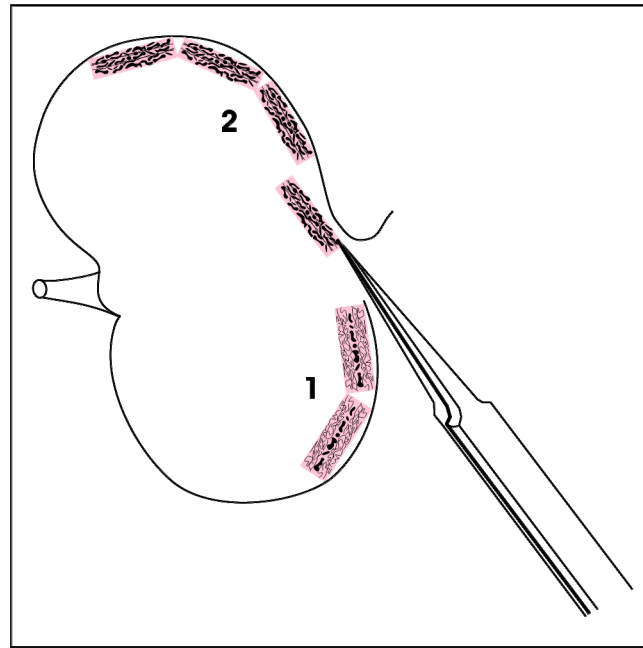


Figure 168: Schematic illustration depicts implantation of stem/progenitor cells generated on an artificial interstitium made by I-7 (1) and Posi-4 (2) polyester fleeces. When an I-7 fleece is applied, regeneration of tubules is only observed at the interface between the two layers (1). In contrast, application of Posi-4 fleeces stimulates formation of tubules in a spatial manner (2). When for example eight layers of Posi-4 fleeces are piled the same thickness of two I-7 layers is reached. In parallel the amount of tubules can be strongly increased.

The demonstrated results exhibit that a realistic chance exists for the surgical application of polyester fleeces in combination with stem/progenitor cells. For example, the minimal thickness of 0.13 mm in a Posi-4 in contrast to an I-7 fleece makes piling of stem/progenitor cells possible and increases efficiently the amount of implanted cells (Fig. 168).

Further the presented experiments show the feasibility to regenerate renal tubules not only with one but in combination with Posi-4, Posi-5, Posi-6 and Posi-7 fleeces. This result in turn

is an important presupposition to concentrate stem/progenitor cells within more suitable fleeces to investigate mechanisms steering development of regenerating tubules.

**[search: potential of stem/progenitor cells within polyester fleeces]**

**Self-study: Get informed about advantages and disadvantages of fleeces**

### **40.3. Pathologists need to look more closely**

The implantation of stem/progenitor cells into diseased parenchyma appears as an attractive option to treat acute and chronic renal failure in future. However, such a therapy needs time and careful evaluation for realization, since patients are faced by several kinds of risks.

One of the problems is that after an implantation stem/progenitor cells are exposed to harmful interstitial fluid and altered extracellular matrix. This harsh transition results in an only minimal cell survival. A second specific problem is that an environment of inflammation may cause beside typical development also arise of atypical cell features. Although such abnormal cell development was commonly ignored, it has been accidentally discovered.

For example, to investigate basic parameters for the repair of parenchyma, renal stem/progenitor cells were isolated and mounted in a polyester interstitium during perfusion culture in chemically defined Leibovitz's L-15 Medium. Then generated tubules are fixed by conventional glutaraldehyde (GA) solution or by GA solution containing tannic acid to analyze by morphological methods how far development of typical characteristics is paralleled by the arise of abnormal cell features.



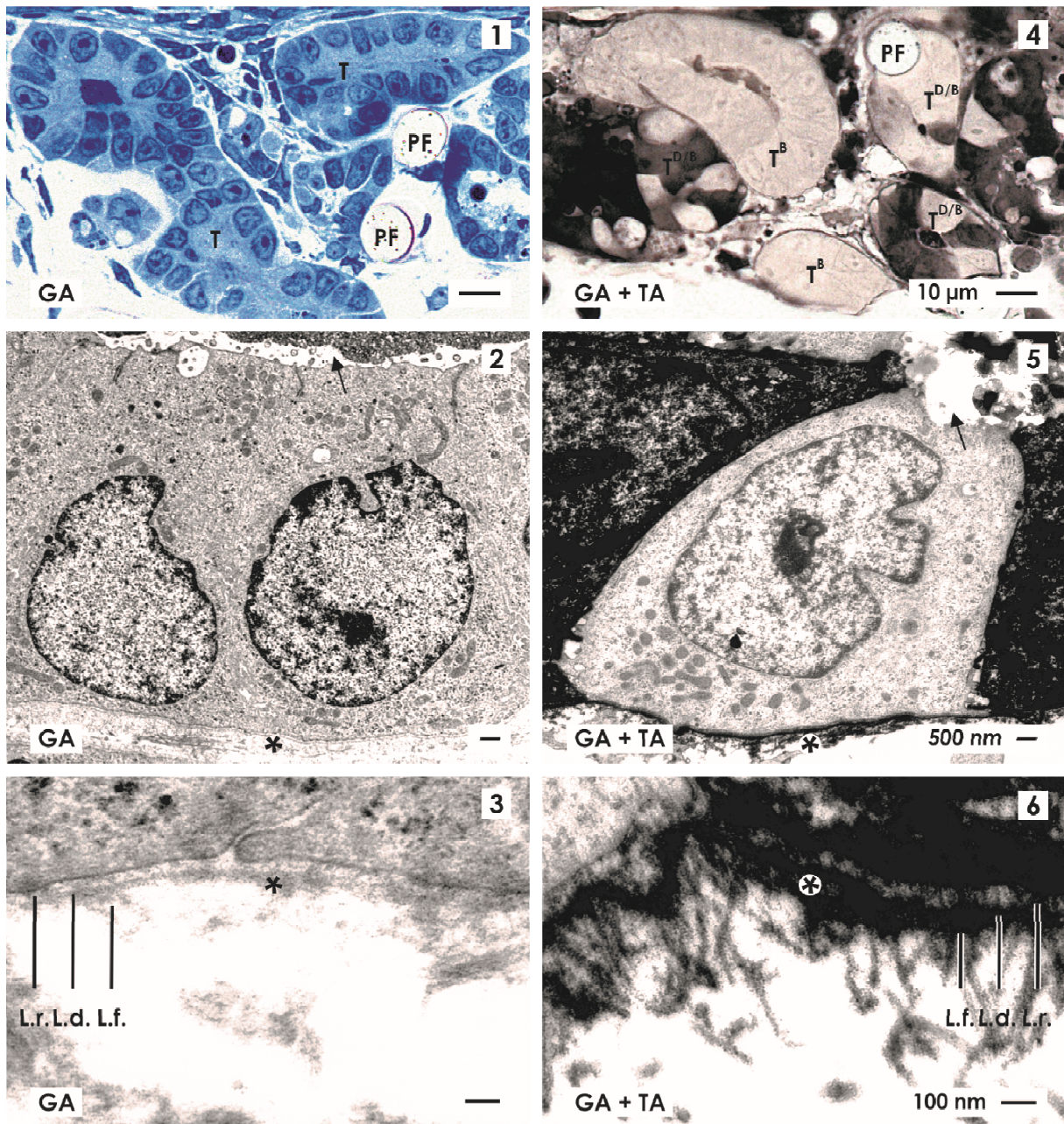


Figure 169: Detection of abnormal cell features and extracellular matrix by tannic acid label. Renal tubules (T) were generated in a polyester interstitium by Leibowitz's L-15 Medium in perfusion culture during 13 days. Then specimens were fixed by conventional glutaraldehyde (GA) solution (1,2,3) or GA solution containing tannic acid (TA) (3,4,5). Samples fixed by GA solution show on a semithin section (1) tubules containing a single cell type. In transmission electron microscopy can be seen that the polarized epithelium contains bright cells (2) and is based on a typical basal lamina (3) including a lamina rara, densa and fibroreticularis. In contrast, specimens fixed by GA solution including tannic acid exhibit on a semithin section (4) tubules with bright ( $T^B$ ) and abnormal dark ( $T^D$ ) cells. In electron microscopy can be seen that the tubule epithelium contains bright and abnormal dark cells (5). Further the tannic acid label unveils on the basal lamina an abnormal lamina fibroreticularis (6). PF – polyester fibers; arrow – lumen; asterisk – basal lamina

Specimens fixed by GA solution illustrate on a semithin section that the epithelium of generated tubules consists of a single cell type (Fig. 169.1). Transmission electron microscopy elucidates that the tubule epithelium is polarized and bordered by a lumen and a basal lamina. In each of analyzed cases only bright cells are present (Fig. 169.2). Higher magnification illustrates that a typical basal lamina is developed at the basal aspect of the epithelium consisting of a lamina rara, densa and fibroreticularis (Fig. 169.3).

In contrast, generated tubules fixed by GA solution containing tannic acid demonstrate on a semithin section that different kinds of tubules exist. The one type of tubules contains only bright epithelial cells. The other type of tubules contains a heterogeneously composed epithelium consisting of bright and abnormal dark cells (Fig. 169.4). Transmission electron microscopy demonstrates that tubules developed a polarized epithelium containing both bright and abnormal dark cells (Fig. 169.5). Higher magnification in electron microscopy reveals that as well bright as dark cells are underlined by a basal lamina containing an abnormal lamina fibroreticularis (Fig. 169.6).

Summing up, conventional fixation for transmission electron microscopy does not, but advanced fixation of specimens by GA solution containing tannic acid detects arise of abnormal cell features and severe alterations of the basal lamina in generated tubules derived from stem/progenitor cells. A comparable cell type was neither found in the embryonic or adult kidney.

**[search: renal stem/progenitor cells abnormal development]**

**Self-study: inform yourself about pathology and abnormal cell development**

## 41. Challenging aspects for the next future

In the last decades many experiments have demonstrated that the expansion of isolated cells does not pose great difficulties in classical dishes, while adherent cells often show severe morphological, physiological and biochemical changes caused by dedifferentiation. As demonstrated further in the text, numerous investigations have shown that the quality of cultured cells or bioengineered tissues highly depends on suitable scaffolds, optimal adhesion sites, intense cell attachment, close intercellular communication and excellent environmental culture conditions. All of these factors have to complement one another to promote functional development, while at the same time arise of atypical features must be prevented.

Characteristic examples derived from bad culture conditions are the expression of atypical collagen in tissue engineered cartilage and bone, calcification of artificial heart valves or loss of endothelial growth on vessel implants. A down-regulation of specific cellular functions was further observed in liver, pancreas and kidney cell cultures used for physiological and toxicological assays.

Many years of experiments have further shown that it is not possible to generate fully functional tissues within the static environment of a culture dish. This can be accounted by the fact, that cell respectively tissue development in vivo is triggered by a variety of extracellular influences which cannot simply be replaced by optimizing a culture medium or by offering a single growth factor. In consequence, the need for an optimal culture system for adherent cells and for the generation of specialized tissues must be the simulation of very individual environments to achieve a high degree of differentiation and functionality.



### **41.1. Offering an interstitium promoting typical development**

To surmount present limitations of a culture dish, different kinds of matrices, tissue carriers and microreactors respectively perfusion culture containers were constructed. However, most of the known microreactors or perfusion culture containers show a significant dead space around the developing cells that is taken up by culture medium and an artificial interstitium (Fig. 170). This fluid filled space can cause substantial hydraulic effects and passes pressure differences directly on to the cultured tissue.

The dead volume is also a preferred site for gas bubbles to accumulate. The aggregation of gas bubbles in turn is problematic, because the supply of culture medium is shortened regionally so that cells can be damaged by surface tensions. In consequence, the dead space can be reduced by decreasing the measures of the container or by the creation of an artificial interstitium. Such an artificial interstitium consists of highly porous biocompatible material that equally decreases and distributes fluid pressure across the cell layer by capillary effects. The use of an artificial interstitium will further provide mechanical protection to the contained tissue and minimizes the dead space within the culture container.

The selected material of an artificial interstitium can be in direct contact with the developing cells or tissues. Further the interface between cells and fleece fibers can be engineered individually to modulate spatial extension and differentiation. For example, a positive surface interaction can lead to directional growth of the developing tissue (Fig. 170.1), while a negative surface interaction of the artificial interstitium can inhibit spatial extension (Fig. 170.2). Consequently, the interface between the growing cells or tissues and the surrounding artificial interstitium can be experimentally defined.

To induce a guided growth and spatial development of parenchyma structures, the artificial interstitium can be locally coated with defined extracellular matrix proteins. An additional option is to link morphogens, growth factors or hormones to the artificial interstitium. When

biodegradable materials as an artificial interstitium are used, these substances will be released in a fashion like matricellular proteins into immediate vicinity of the developing cells and tissues contributing to its functional maturation. By releasing linked growth factors and by providing extracellular guiding structures the artificial interstitium will promote spatial outgrowth of cells into the surrounding leading finally to an increase in size of the tissue. It is also thinkable that such morphogenic factors are not any more added to the culture medium but rather exclusively to the artificial interstitium to increase the local bioavailability.

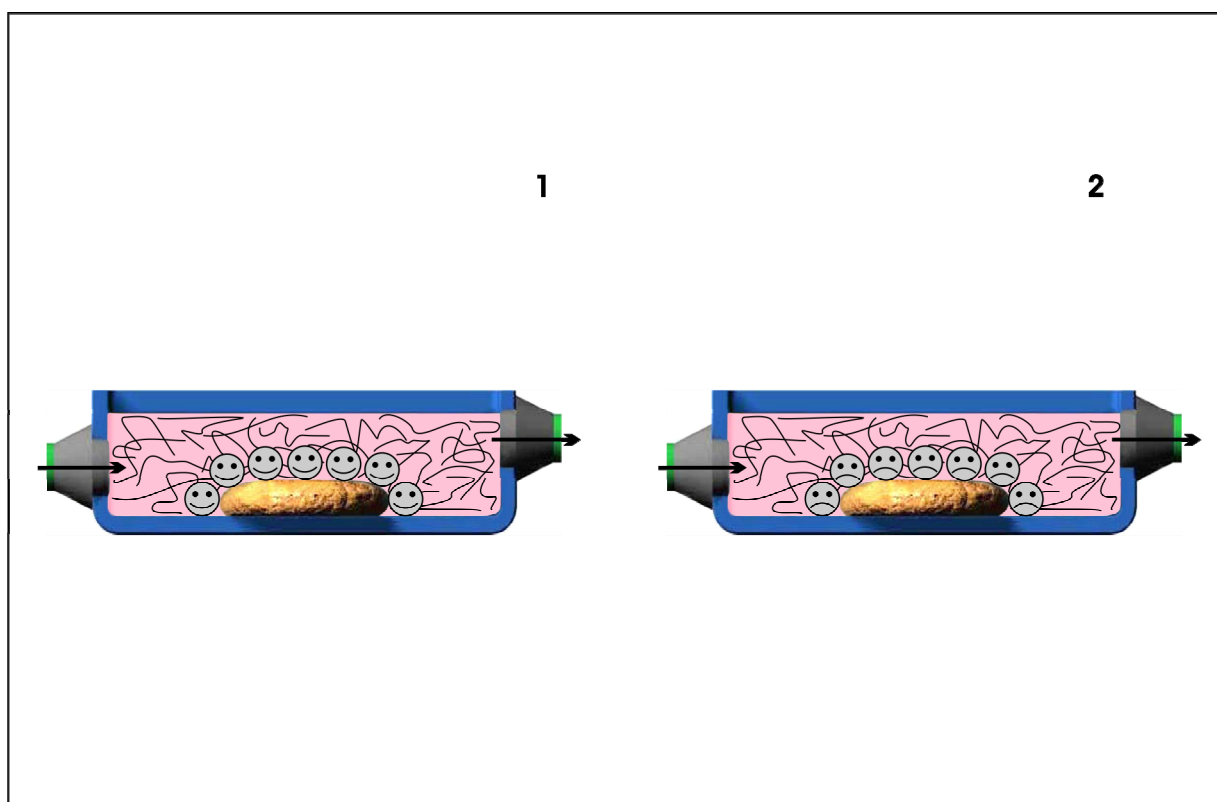


Figure 170: Schematic illustration to demonstrate the dead space around a developing tissue by an artificial interstitium. A positive surface interaction would lead to a guided growth (1), while a negative surface interaction will inhibit spatial extension (2).

As an artificial interstitium natural or chemically defined extracellular matrix proteins can be used. Possible materials are fleeces made from cellulose, fiber glass, wool or silk. Also synthetic sponges made from biopolymers such as polyester, polyurethane or cell-free extracellular matrices are thinkable. A possible source of such artificial interstitial matrices

may be recombinant matrix proteins. In analogy to the amino acid sequence of known structural proteins, artificial collagenous or non-collagenous monomers can be created. These proteins in turn can be polymerized into three-dimensional networks of defined mesh sizes to attract cell ingrowth. In contrast to natural collagens an artificial interstitium can be optimized regarding its functional qualities.

For example, assembly of recombinant matrix proteins or other motifs can be added to the naturally occurring sequences. Such motifs are of particular importance for cell adhesion urgently needed to improve anchorage via integrins, cadherins, and selectins. It is further thinkable to incorporate defined signals for cell attachment, cell migration, mitosis, spreading and differentiation. Thus, the surrounding artificial interstitium will continuously guide cells during migration and spatial development of tissue constructs.

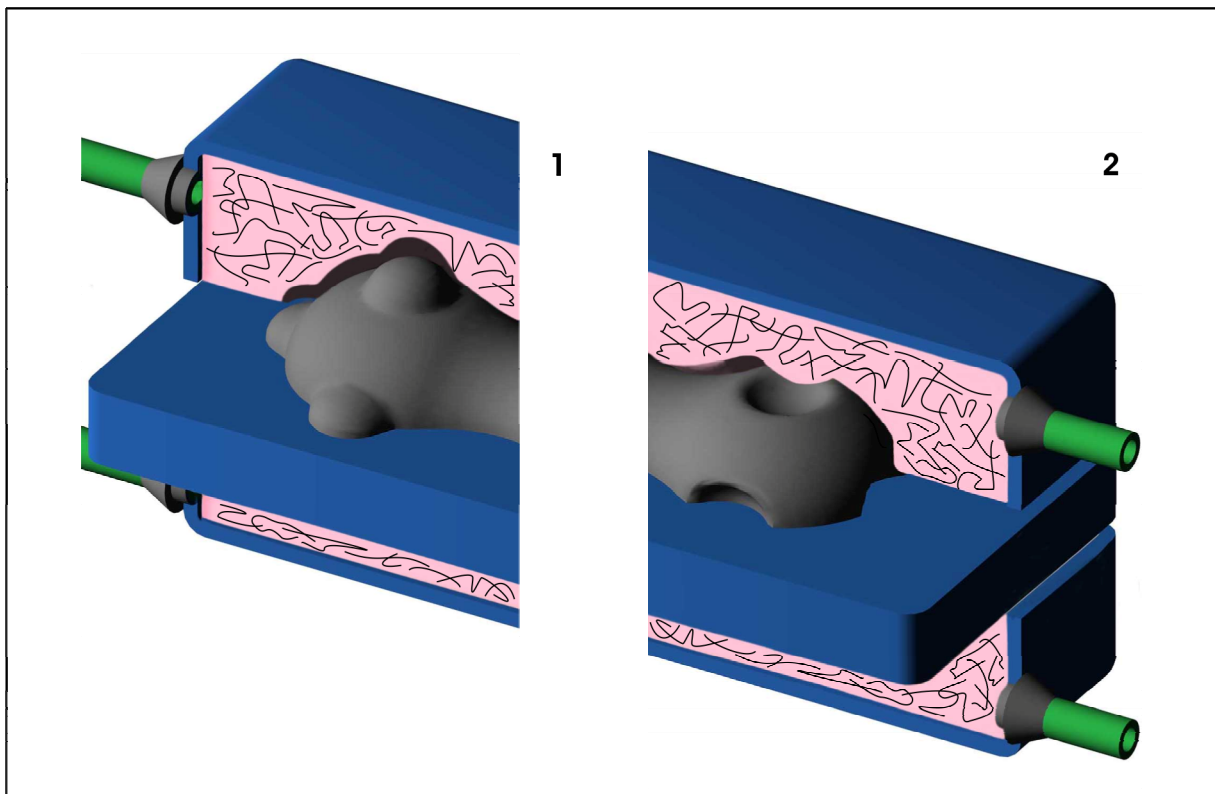


Figure 171: Schematic illustration shows a gradient container with connective tissue resting on a carrier. The dead space is filled by an artificial interstitium. The contour of the artificial interstitium can show projections (1) or recesses (2) to guide the final shape of the tissue construct.

Further the contour of an artificial interstitium can influence the outer surface of the tissue construct. This feature can be used to trigger not only the shape but also spatial characteristics of the surface.

Using an artificial interstitium alternately dotted with promoting or inhibiting signal motifs will result in projections (Fig. 171.1) respectively recesses (Fig. 171.2) influencing thereby the surface structure of the tissue construct. In consequence, the surface of generated tissues is not smooth but exhibits a more or less intense roughness in form of projections (Fig. 171.1) or recesses (Fig. 171.2). Such constructs can be used in tissue engineering implants accelerating perfect integration of the implant for dental, bone or cartilage repair.

Further prospective materials for parenchyma cells are collagen type III and gelatine sponges coated with growth promoting nanomaterials. Such materials can be easily shaped so that they can be used as powerful artificial interstitium.

**[Search: cell culture artificial interstitium]**

**Self-study: Read about possibilities to create an artificial interstitium for cultured cells**

## **41.2. Shaping constructs with suitable tissue carriers**

The housing for developing tissue must cover on the one hand an artificial interstitium and must hold on the other hand carriers supporting the growth of specialized tissues. Since specialized tissues exhibit very individual features, not a single type of tissue carrier but various differently composed ones are needed to fulfill the specific demands (Fig. 172).

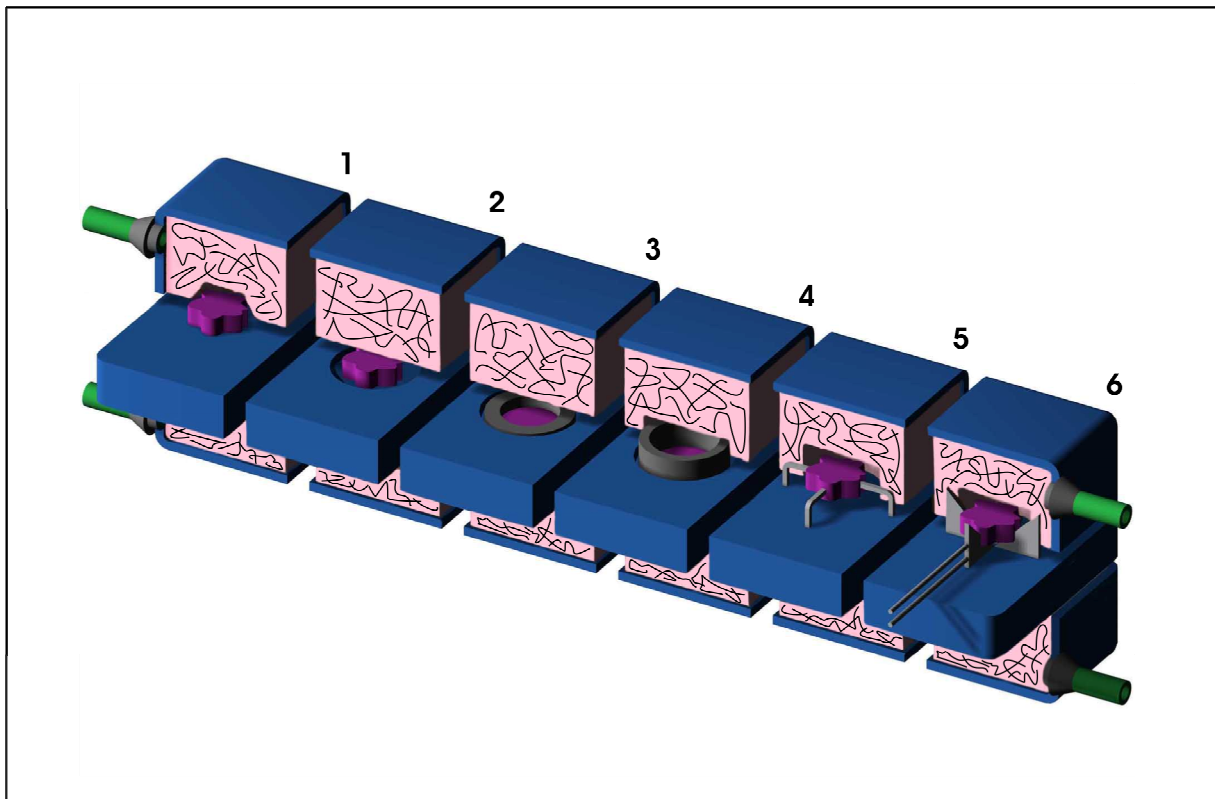


Figure 172: Schematic illustration of a perfusion culture container filled with an artificial interstitium and various tissue carriers. When bone (1) or cartilage (2) are tissue engineered, the scaffold included in a carrier will determine the form of the arising tissue. When a monolayered epithelium is cultured on a filter, the tissue carrier can exhibit a flat form (3). In contrast, when a multilayered epithelium such as epidermis is cultured on a relatively thick basal lamina substitute, the tissue carrier must have a certain height for holding both (4). When neurons are cultured within a spider net like substrate, it is of interest to register synaptic activity by electrodes (5.). In this case the tissue carrier must hold beside neurons also the spider net like substrate, electrodes and wires. When muscle cells are cultured within an elastic matrix, the carrier must be accessible to measure activity of contractile cells, to register stiffness of extracellular matrix, to preload drugs for cardiac contractility assays, to determine toxicity and to elaborate signal transduction cascades (6).

For example, when pieces of bone (Fig. 172.1) or cartilage (Fig. 172.2) are tissue engineered, the individual scaffold included in a carrier will influence the arising spatial form of the tissue construct. However, when a monolayered epithelium is cultured on a filter, the tissue carrier can exhibit an extremely flat form (Fig. 172.3). When a multilayered epithelium such as epidermis is cultured on a relatively thick basal lamina substitute, the tissue carrier must increase in height to hold the construct (Fig. 172.4). When neurons are cultured within

a spider net like substrate, it is of interest to register their biologic activity by electrodes (Fig. 172.5). In this case the carrier has to hold beside neurons also the spider net like substrate, electrodes and lining wires. Finally, when muscle cells are cultured within an elastic matrix, the tissue carrier must have quite other characteristics (Fig. 172.6). During culture the generated muscular tissue must be accessible to investigate activity of contractile cells, to register stiffness of extracellular matrix, to preload drugs for cardiac contractility assays, to determine toxicity and to elaborate signal transduction cascades. Finally, the contractile tissue must be accessible to be investigated under the fluorescence microscope or has to be available for example to a Palpator<sup>®</sup> tool integrated in an automated system.

**[Search: cell culture automated assay system]**

**Self-study: Read about technical possibilities to create an innovative tissue carrier**

### **41.3. Providing developing tissues within a controlled environment**

One important limitation in cell and tissue culture is the lack of exact knowledge about factors influencing cell proliferation, spreading and differentiation in specialized tissues. Although numerous parameters such as extracellular matrix, cell number, electrolyte environment, nutrition, hormones, growth factors and serum can influence development to a certain degree, the maintenance of tissue specific differentiation over long periods of time remains up to date obscure.

Consequently, a main task in future is to measure metabolic changes over time in the used culture medium. For example, studies have demonstrated that different types of spectrophotometric assays including Raman and Near-InfraRed (NIR) techniques are capable to detect specific changes of the secretome. These experiments have also shown

that the data correlate well with tissue development. Applying these techniques minimal changes in the presently used culture medium can be recognized so that necessary corrections of environmental parameters can be performed within shortest time.

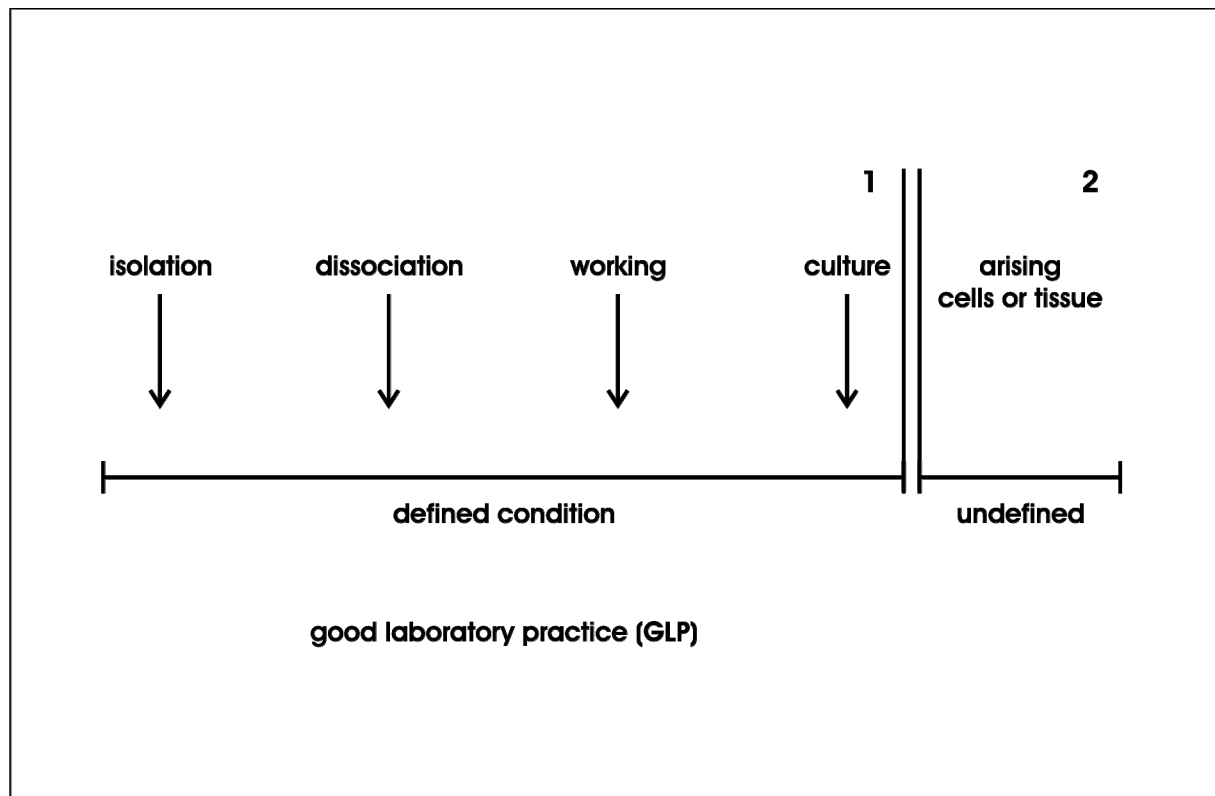


Figure 173: Schematic illustration of defined and undefined areas of Good Laboratory Practice (GLP). While isolation, dissociation, treatment of cells and culture conditions of cells are defined (1), the quality of harvested cells remains obscure (2)

Further the quality assessment is becoming more and more important. Good Laboratory Practice (GLP) and Good Manufacturing Practice (GMP) are established methods. However, one has to consider that all of these standards only define the performance of the experiments (Fig. 173.1) and the technical run over time but they do not say anything about the quality of harvested cells and tissues (Fig. 173.2).

A black box in this coherence is the actual composition of the individually used culture medium. Information on the formulation by the companies is often limited and unknown

supplements are contained in a medium so that they cannot be regarded as completely defined. There is only one alternative. The experimental solution is to use chemically defined culture media and to adapt them to the needs of differentiating cells and specialized tissues.

**[Search: cell culture good laboratory practice]**

**Self-study: Get informed about quality assessment in cell culture**

#### **41.4. Urgent need of powerful culture media**

A fertilized egg is developing from a morula stage over the embryoblast into an embryo and then into a foetus. Everyone knows that the development of specialized tissues is not terminated at the day of birth but proceeds over many years. In all of these very different developmental stages the cells are exposed to very specific and stage dependent environmental conditions.

However, regarding recently published literature concerning experiments with stem/progenitor cells most of the experiments are performed in a single culture medium. It is not surprising that under these fluid conditions only a minority of cells survive and expected differentiation does not arise. Thus, the challenge in future culture experiments is to offer stem/progenitor cells a fluid environment adapted to the individual needs and actual stages of development. According to the cell cycle and to the potential development at least three different culture media have to be offered (Fig. 174).

The first culture medium has to support proliferation of cells. It stimulates mitosis so that always the necessary amount of cells is available. At the same time the proliferation medium is able to suppress development into specific cell lineages (Fig. 174.1).



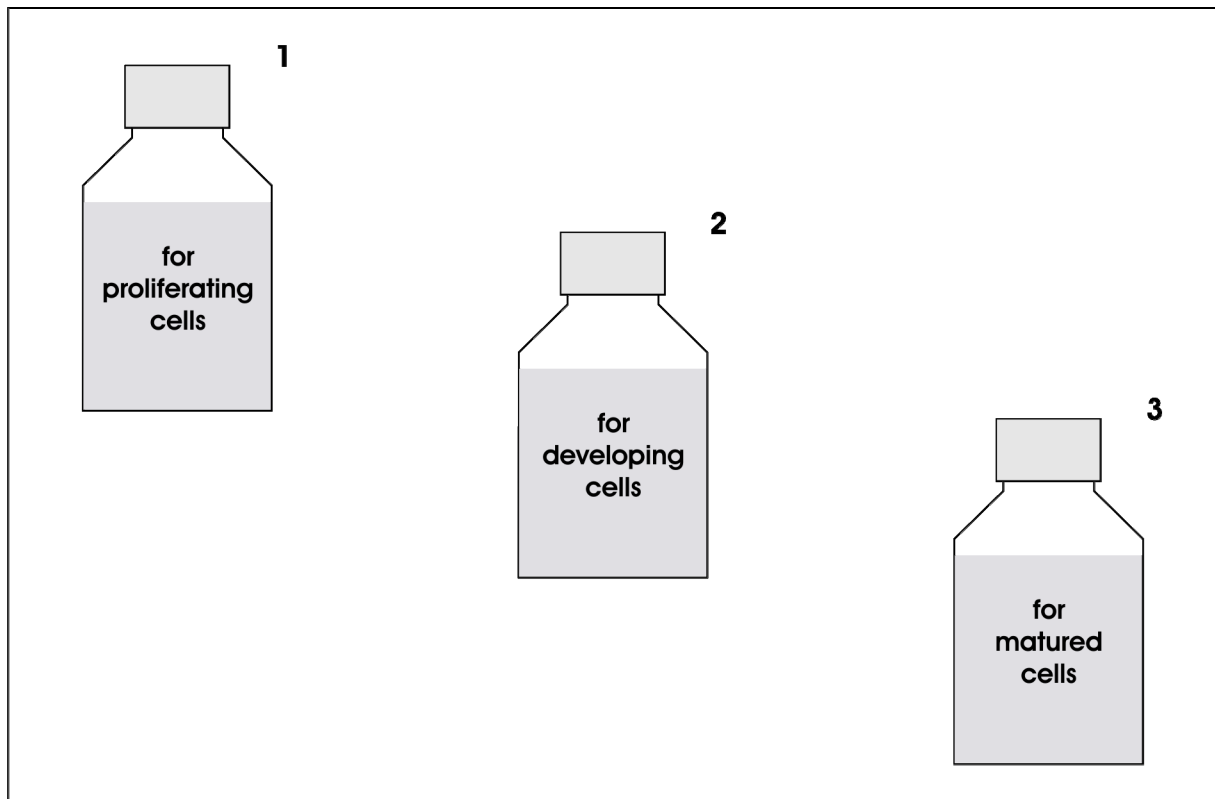


Figure 174: Proposal for a three bottle concept to culture stem/progenitor cells in a competent, maturing and matured stage. A first culture medium has to support proliferation of cells (1). The second one determines features in future cell lineage (2). A third one down-regulates proliferation so that functional differentiation takes place (3).

The second culture medium has to down-regulate mitosis so that cells are able to enter over extended periods of time the postmitotic phase. Treatment with this medium determines features in future cell lineage so that first functional characteristics in blast cells arise (Fig. 174.2).

The third culture medium is suitable to keep proliferation at a low level but is able to promote functional differentiation so that mature cells will arise (Fig. 174.3). For example, such a medium will stimulate neuroblasts, immature epithelial cells, chondroblast, osteoblasts and myoblasts to develop into related functional cells found within the specialized tissues.

If always three culture media have to be applied, has to be elaborated in future. However, without a sound concept essential progress in this area will not be made.

**[Search: cell culture medium]**

**Self-study: Read about possibilities to adapt a culture medium to specialized tissues**

## 42. Biologic security

Beside perfect technical equipment in the cell culture laboratory, one must always keep the biological risks in sight, which are present during manipulation, static and dynamic culture with adherent cells and maturing tissues. One has to accept that all the experiments have to be very exactly carried out according the general and specific instructions for use (Fig. 175). In all of other cases one has to consider that static and dynamic cultures may exhibit biological hazards.

Biological hazard	Kind of culture
Residual risk	Cultures of non-infected animals with a limited period of life. Single and excellently characterized cell lines
Minor risk	Many frequently used cell lines Barely characterized cell lines
High risk	Cells from human and primates Human cell lines infected with pathogens

Figure 175: Potential of risks during experimentation with adherent cells.

**Performing cell and tissue culture experiments three different levels of potential risk have to be considered (Fig. 175). Further in the different countries very individual instructions have to be noted performing experiments with adherent cells. These rules of protocol must be strictly considered.**

Thus, in any case danger by infection has to be prevented. Biological hazard can arise by the transfer of cells by a pipette, by manipulation on cells adherent on a biomaterial, during run

of a perfusion culture experiment or during cell biological analysis. Furthermore, dangerous are infection by a conscious or unconscious contact with cells or tissues. Finally waste must be sterilized in each case so that infections cannot arise.

It is further important to note that danger can arise not only from cells and tissues but also from culture media and additives such as serum and tissue extracts frequently used in culture experiments. Dangerous molecules are further drugs with unknown pharmacological and immuno-modulatory effects. Additional risks arise during fusion of cells and transformation of cells with recombinant DNA. Not to underestimate is the risk, when in cells virus is reactivated or when oncogens are expressed.

Due to biological hazard and ethical reasons cells or tissues of the own body are not used for culture experiments. Further genetic modifications and in-vitro transformation of cells of the own body can lead to malignant disease, when infection takes place. An ethical principle must be during experimental work with human cells that they do not have any personal relation to relatives.

Since in single countries very individual laws and rules exist regulating the experimental work with cells and tissues, the local administration is the personal contact for further questions. Information to this subject in European countries is found in the internet under: [www.hse.gov.uk](http://www.hse.gov.uk). For example, there are found instructions of the Advisory Committee on Dangerous Pathogens (ACDP), categorisation of biological agents according to hazard and categories of containment. For other countries the local administration has to be contacted.

**[Search: cell culture risk]**

**Self-study: Get informed about risks performing culture experiments**

## 43. Publications of authors related to cultures

- W.W. Minuth** (1978) Mesodermalization of Amphibian Gastrula Ectoderm in Transfilter Experiments. *Medical Biol.* 56: 349-354
- W.W. Minuth**, M. Minuth, H. Tiedemann (1979) Amino Acid Transport and Protein Synthesis in Induced Ectoderm of Amphibian Embryos. *Wilhelm Roux's Arch* 187: 211-217
- W.W. Minuth** and W. Kriz (1980) Newly Synthesized Proteins in Rat Glomerular Fractions. *Renal Physiol.* 3: 88-96
- W.W. Minuth**, M. Minuth, H. Tiedemann (1981) Changes in the Electrophoretic Pattern of Nuclear Proteins from Noninduced and Induced Embryonic Ectoderm. *IRCS Medical Science* 9: 36
- W.W. Minuth** and W. Kriz (1982) Culturing of renal collecting duct epithelium as globular bodies. *Cell Tissue Res.* 224: 335-348
- W.W. Minuth** and W. Kriz (1982) Renal Collecting Duct Cells Cultured as Globular Bodies and as Monolayers. *J Tissue Cult Meth* 7: 93-96
- W.W. Minuth** (1982) Cell Associated Glycoproteins Synthesized by Cultured Renal Tubular Cells. *Histochem.* 76: 89-106
- W.W. Minuth** (1993) Die Kultur von Epithelzellen unter organtypischen Bedingungen. Alternativen zu Tierversuchen, Springer Verlag Wien - New York: pp 238 - 245.
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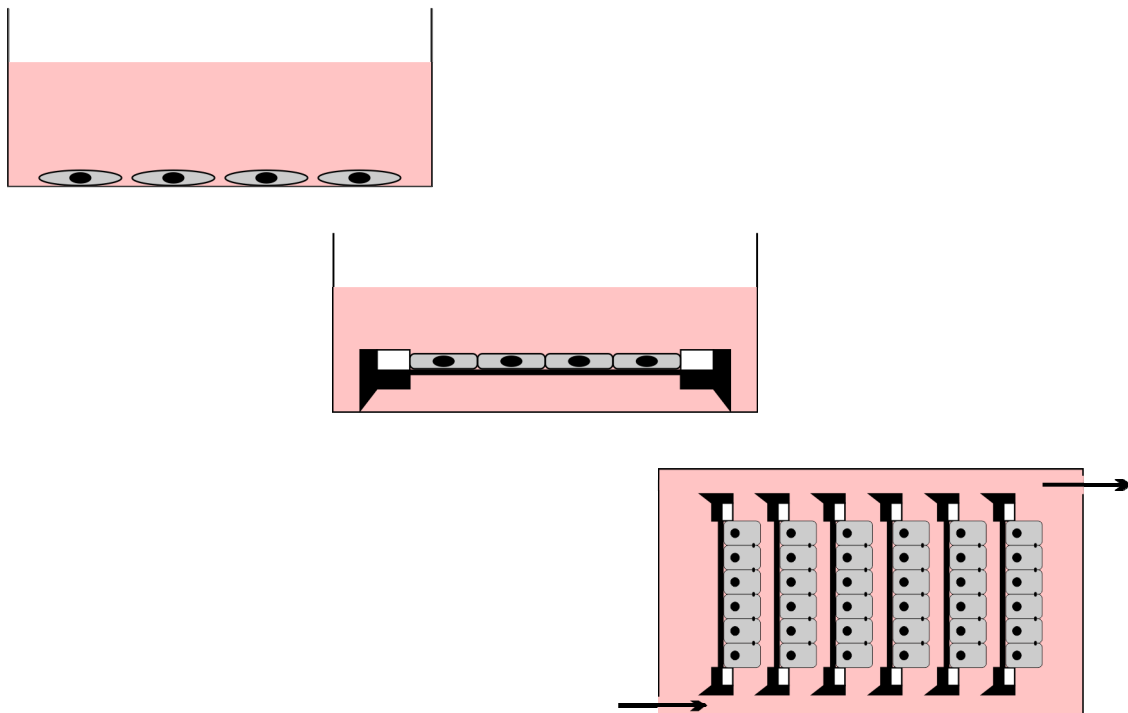
## 44. Authors



**Professor Dr. Will W. Minuth** studied at the University of Cologne (1968-1974), worked as a PhD student in the Institute of Molecular Biology and Biochemistry at the Free University of Berlin (1974-1978) and was Assistant Professor at the Department of Anatomy at the University of Heidelberg. Since 1989 he is a Professor of Anatomy at the University of Regensburg/Germany teaching medical students in Microscopic Anatomy and Neuroanatomy. The research interest is focused to the barrier function in epithelia including the tubulogenic action of aldosterone. A driving force in present experiments is the application of stem/progenitor cells to treat acute and chronic renal failure. Sophisticated perfusion culture technique including an artificial interstitium plays an essential role in this hot spot of research.



**Lucia Denk** is pharmaceutical technician at the Department of Molecular and Cellular Anatomy/University of Regensburg. She is working together with Will Minuth since more than 10 years. In the laboratory she is performing all of the micro-surgical tissue isolation, perfusion culture experiments and cell biological analysis to investigate the development of stem/progenitor cells. Here special interest is arise of specific functions in epithelia, survival of stem/progenitor cells within a diseased environment, creation of an optimal artificial interstitium for parenchyma regeneration. Together with Will Minuth she is a co-author in numerous papers. For the actual book she performed all of the histochemical label experiments, documented the numerous results and prepared all of the actual illustrations.



When you are happy with the results of your culture experiments dealing with adherent cells, do not further look into this book. However, when you feel that your cells might have a higher degree of differentiation, we invite you to look inside and to read about advanced culture set ups and innovative techniques.