

**Characterization of candidate metastasis founder cells in
breast cancer using combined genome and limited
proteome analysis**



Dissertation
zur Erlangung des Doktorgrades
der Biomedizinischen Wissenschaften
(Dr. rer. physiol.)

der
Fakultät für Medizin
der Universität Regensburg

vorgelegt von
Atieh Alyasin

aus
Arak, Iran

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FREISEITE

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Dekan: Prof. Dr. Dirk Hellwig

Betreuer/in: Prof. Dr. Christoph Klein

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List of abbreviations

Abbreviation	Meaning
ATP	Adenosine triphosphate
BC	Breast cancer
bp	Base pair
BSA	Bovine serum albumin
CITE-seq	Cellular indexing of transcriptomes and epitopes by sequencing
CNV	Copy number variation
CTC	Circulating tumor cell
DCC	Disseminated cancer cell
DCCD	DCC density; number of DCCs per million mononuclear cells
DCIS	Ductal carcinoma in situ
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DOP-PCR	Degenerate oligonucleotide-primed PCR
EDTA	Ethylenediaminetetraacetic acid
EMT	mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FISH	Fluorescent <i>in situ</i> hybridization
G&P	Genome and proteome
GII	Genomic integrity index
H ₂ O	Water
IDC	Invasive ductal carcinoma
IF	Immunofluorescence staining
IgG	Immunoglobulin G
kb	Kilobase
LASIC	Light Activated Site-Specific Conjugation
LCIS	Lobular Carcinoma In Situ
LMD	Laser microdissection
mAb	Monoclonal antibody
MFI	Mean fluorescence intensities
mL	Milliliter
MMI	Molecular Machines & Industries
NA	Not available
NBT/BCIP	Nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolylphosphate p-tuluidine salt
NGS	Next generation sequencing
OPA	One Phor All buffer
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEA/STA	proximity extension assay with specific RNA target amplification
PFA	Paraformaldehyde
PLAYR	proximity ligation assay for RNA
QC	Quality control
REAP-seq	RNA expression and protein sequencing assay
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SCOMP	Single-cell comparative genomic hybridization
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.
ssDNA	single-stranded DNA
TAE	Tris-Acetate-EDTA buffer
TBE	Tris/Borate/EDTA buffer
TNBC	Triple negative breast cancer
TNM	Tumor-Node-Metastasis staging system
WGA	Whole genome amplification

Summary

Die metastatische Ausbreitung einzelner Tumorzellen aus dem Primärtumor erfolgt oft schon in einem frühen Stadium der Brustkrebsentwicklung. Das Vorhandensein von disseminierten Krebszellen (DCCs) im Knochenmark bei Brustkrebspatientinnen ist ein starker Indikator für ein erhöhtes Risiko, Knochen- und viszerale Metastasen zu entwickeln. Aufgrund der Seltenheit von DCCs werden Objektträger aus Knochenmarkproben oft mit einer hohen Zelldichte präpariert, um die Chancen auf einen Nachweis zu erhöhen. In dieser Arbeit wurde zunächst eine Methode zur Identifizierung und Isolierung dieser sehr seltenen Zellen aus diesen Knochenmarkproben entwickelt. Darüber hinaus wurde ein neuartiger Arbeitsablauf für die Genom- und Proteomanalyse (G&P) entwickelt, der eine umfassende Analyse der Einzelzell-DNA und die Erstellung von limitierten Proteomprofilen in einzelnen Zellen ermöglicht. Das optimierte Protokoll zur Isolierung von DCCs aus Knochenmark umfasste eine Immunfluoreszenzfärbung zur Identifizierung von Tumorzellen in Kombination mit einer AP-Polymerfärbung, um laserinduzierten Schäden entgegenzuwirken. Darauf folgte eine lasergestützte Ablation der umgebenden Zellen, eine manuelle Mikromanipulation der Zielzellen, eine umfassende Genom-Amplifikation, eine Qualitätskontrolle und eine Low-Coverage-Sequenzierung zur Analyse von Kopienzahlveränderungen. Das Protokoll wurde an präparierten Zytospins validiert, die aus MCF-7-Zellen und mononukleären Blutzellen generiert wurden, bevor es auf archivierte DCCs aus dem Knochenmark von DCIS-Patienten angewendet wurde. Anschließend wurden vier gefrorene archivierte Knochenmark-Zytospins von DCIS-Patientinnen untersucht, die an der Universitätsklinik Tübingen behandelt wurden. Bei einem von vier DCIS-Patientinnen konnten wir erfolgreich DCCs aus archivierten Knochenmarkzytospins isolieren, was eine anschließende CNV-Analyse ermöglichte. Diese zeigte das Vorliegen multipler Veränderungen bei DCCs und ein ausgewogenes genomisches Profil bei normalen Zellen, welches die Zuverlässigkeit der Methode weiter bestätigte.

Darüber hinaus wurde der G&P-Arbeitsablauf entwickelt. Dabei wurden Antikörper verwendet, die mit spezifischen DNA-Oligonukleotiden konjugiert sind, sodass Einzelzellgenome amplifiziert werden können. Dies ermöglichte eine parallele Erstellung von Genom- und Proteomprofilen durch quantitative PCR. Die Leistungsfähigkeit der Methode wurde durch die Quantifizierung der Kopienzahl des

ERBB2-Gens sowie der Proteinexpression von HER2 und Phospho-HER2 in Einzelzellen nachgewiesen. Weiterhin wurde diese validiert, indem die zellulären und molekularen Reaktionen auf eine Lapatinib-Behandlung auf Einzelzellebene untersucht und die Aktivierung des HER2-Signalwegs überwacht wurde. Darüber hinaus wurde die Methode angewandt, um kritische Signalwege zu analysieren, die an der Progression von Brustkrebs beteiligt sind. Insbesondere HER2 und wichtige nachgeschaltete Proteine wie phospho-HER2, phospho-ERK1/2, phospho-AKT und phospho-STAT3 standen im Fokus der Untersuchung. Die Analyse der Signalwege wurde zunächst anhand von Zelllinien (MDA-MB-231, MDA-MB-468 und BT474) validiert, wobei die Heterogenität der verschiedenen Brustkrebs-Subtypen und das Zusammenspiel zwischen vorhandenen molekularen Veränderungen und aktivierten Signalwegen deutlich wurde. Schließlich wurde die Anwendung der Methode in einem klinischen Kontext geprüft, indem Knochenmarkproben einer HER2-positiven Brustkrebspatientin analysiert und damit das Potenzial zur Untersuchung der Heterogenität von DCCs und der Mechanismen der Medikamentenresistenz weiter unter Beweis gestellt wurden. Zusammenfassend lässt sich sagen, dass in dieser Arbeit eine Methode zur Isolierung von DCCs aus dem Knochenmark aus Zytospins mit hoher Zelldichte entwickelt wurde, die zusammen mit G&P-Arbeitsablauf eine hochzuverlässige Isolierung und Charakterisierung ermöglicht. Zusammen werden die Methoden bislang nicht durchgeführte Multiomics-Analysen von DCCs sogar aus archivierten Knochenmarkproben ermöglichen.

Introduction

1. Introduction

1.1. Breast cancer

Breast cancer is the most prevalent cancer among women and the leading cause of cancer-related deaths globally [1]. In 2022, it accounted for approximately 2.3 million new cases, representing 11.7% of all cancer diagnoses worldwide. Furthermore, it was the primary cause of cancer-related deaths among women, contributing to approximately 15.5% of all cancer fatalities [2]. The incidence of breast cancer in males is very low and accounts for <1% of all breast cancers and <1% of all diagnosed cancers in males [3]. Only female breast cancer has been addressed in this dissertation.

1.1.1. Breast cancer classification

Breast cancer is a highly heterogeneous neoplasm with distinct subtypes. Histological classifications differentiate between pre-invasive and invasive types based on cellular behavior and tissue spread. Pre-invasive types include Ductal Carcinoma In Situ (DCIS) and Lobular Carcinoma In Situ (LCIS) [4]. DCIS is a non-invasive form of breast cancer where abnormal cells are confined to the ducts and have not invaded surrounding breast tissue. It accounts for about 20% of all breast cancers diagnosed and can be further classified based on its nuclear grade (low, intermediate, and high) and architectural patterns (e.g., comedocarcinoma, solid, cribriform) [5]. LCIS, originating in lobules, is less likely to progress but signifies a heightened cancer risk [6]. Similarly, invasive carcinoma includes both ductal and lobular types. Invasive ductal carcinoma (IDC) is the most common type of breast cancer, accounting for approximately 75% of all breast cancer cases. It originates in the milk ducts and invades surrounding breast tissue [7]. Invasive lobular carcinoma (ILC) represents approximately 10-15% of breast cancers and arises from the lobules (milk-producing glands) of the breast. The cancer cells in ILC typically invade in a single-file pattern, leading to a more insidious infiltration of surrounding tissues [8].

Immunohistochemical classification further assesses breast cancer based on the expression of estrogen (ER), progesterone (PR) and human epidermal growth factor 2 (HER2) receptors. 1) ER, PR positive and HER2 negative cancers account for

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around 70% of all breast cancers; 2) HER2 enriched cancers, account for 15-20% of tumors; 3) Around 15% tumors do not express any of these cell surface receptors and are thus called triple-negative breast cancers (TNBC) [9].

Clinically, the American Joint Committee on Cancer (AJCC) provides a widely accepted staging system (TNM classification) which basically characterizes breast cancer on the basis of three major parameters, i) T denotes the features of the primary tumor, mostly size and local invasion and varies from T1-T4 with ascending order of severity; ii) N denotes the presence of disseminated cancer cells (DCCs) or micro-metastases in the lymph nodes, whereby N0 implies no involvement of lymph nodes, while N1-N3 indicate spread in increasing order; iii) M indicates the absence or presence of distant metastasis, which could be either M0 or M1, respectively [10]. The combination of the above parameters defines the stage of the disease. Stage 0 is assigned to non-invasive cancers like DCIS, stage I-III are considered early breast cancer without distant metastasis and stage IV refers to metastatic breast cancer [7].

Gene expression studies have led to the molecular subtype classification of breast cancer, identifying four clinically relevant subtypes: Luminal A, Luminal B, HER2-enriched, and basal-like. Each of these subtypes exhibits distinct prognostic outcomes, treatment strategies, and responses to therapies [11]. Hormone receptor (HR)-positive cancers, often referred to as luminal-like cancers, are further divided into Luminal A and Luminal B subtypes. Both subtypes demonstrate expression patterns similar to luminal cells in the breast epithelium, with Luminal B cancers exhibiting higher expression of cell proliferation markers and lower expression of hormone receptors [12]. Ki67, a well-known cell proliferation marker, is used to clinically identify Luminal B cancers through immunohistochemistry [12]. In contrast, basal-like tumors have a gene expression pattern similar to basal/myoepithelial cells of the breast epithelium, with approximately 80% of triple-negative breast cancers (TNBCs) classified as basal-like [13]. Another major subtype within the TNBC group includes claudin-low tumors, which exhibit enhanced mesenchymal and stem-like properties [14].

1.1.2. Breast cancer treatment

Introduction

Primary breast cancer is typically treated through loco-regional approaches, which include surgical resection of the tumor and the removal of adjacent lymph nodes, often referred to as axillary lymph node dissection. This surgical intervention is frequently followed by adjuvant therapies, such as postoperative radiation therapy, to eliminate residual cancer cells and reduce the risk of local recurrence [15]. Recent advancements in breast cancer treatment have significantly improved patient outcomes, particularly through the introduction of targeted therapies and immunotherapy. Notably, CDK4/6 inhibitors like palbociclib, ribociclib, and abemaciclib are increasingly used in HR-positive breast cancers, often in combination with hormone therapies [16]. Additionally, PI3K inhibitors such as alpelisib target PIK3CA mutations and enhance treatment efficacy [17]. Immunotherapies, including checkpoint inhibitors like pembrolizumab and atezolizumab, are showing promise in treating triple-negative breast cancer (TNBC), especially when combined with chemotherapy [18]. Newer agents, such as elacestrant, represent a novel class of selective estrogen receptor degraders (SERDs) for HR-positive cancers [19]. Furthermore, precision medicine approaches, including genomic profiling with tests like Oncotype DX, help tailor treatment strategies to individual patient tumor biology [20]. Ongoing clinical trials continue to explore innovative drug combinations and novel agents, further expanding the therapeutic landscape for breast cancer [20].

1.1.3. Breast cancer prognosis and survival

The prognosis for breast cancer patients is primarily influenced by several key factors, including histological type, grade, stage and molecular subtypes. IDC generally has a good prognosis when detected early, with a high 5-year survival rate of 90-95%. However, the prognosis worsens in cases with larger tumors, lymph node involvement, or HER2 positivity [15]. ILC presents a more indolent course but is associated with a higher risk of late recurrence, especially in the bones and gastrointestinal tract [21]. The breast cancer-specific mortality for DCIS is approximately 2%, while it is 4.1% for small invasive cancers (node-negative) [22]. Histological grade plays a crucial role in predicting the prognosis of breast cancer, as it has been shown to significantly impact survival outcomes. Higher-grade tumors, which are more aggressive and poorly differentiated, generally correspond to a poorer prognosis compared to lower-grade tumors, even when patients have similar TNM. Additionally, the stage at

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diagnosis is another critical factor influencing survival, with more advanced stages correlating with reduced survival. For instance, patients diagnosed with early-stage breast cancer (Stage 0-I) have a ten-year survival rate of approximately 90-95%, while those diagnosed at later stages such as Stage II-III and Stage IV experience survival rates of 60-75% and 10%, respectively [23]. Molecular subtype classification has further refined prognosis; Luminal A tumors typically have the best outcomes, while HER2-enriched and basal-like subtypes, which include many TNBCs, are linked to worse survival rates [24]. The survival outcomes for patients with metastatic breast cancer are highly dependent on the subtype; for instance, patients with triple-negative breast cancer (TNBC) have a median survival of about one year, while those with hormone receptor-positive (HR+) and HER2-positive tumors can expect a median survival of around five years [15].

1.1.4. Metastasis

Metastasis is defined as dynamic and multistep process by which cancer cells spread from the primary (original) tumor to distant organs and tissues. This process involves cellular changes in which the cancer cell leaves their primary site, circulate in the bloodstream, by various mechanisms like collective migration, undergoing epithelial to mesenchymal transition (EMT) or by passive shedding. Next the circulating tumor cells (CTCs) encounter numerous challenges, including the shear stress of blood flow and the immune response, which can lead to their elimination. Upon arrival at their destination, CTCs can either successfully extravasate, crossing the vascular endothelium to infiltrate surrounding tissues, or become trapped in narrow capillaries, which hinders their ability to disseminate. Extravasated CTC may attracted to and captured by adhesion molecules on the surface of endothelial cells in the bone marrow [25]. The interaction between tumor cells and the bone marrow microenvironment is facilitated by direct cell-to-cell contact and signaling molecules like transforming growth factor β (TGF β), fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), Jagged, parathyroid hormone-related protein (PTHrP), and receptor activator of nuclear factor kappa-B ligand (RANKL) [26]. Through these molecular signals, CTCs can invade the bone marrow and become disseminated cancer cells (DCCs). Some DCCs may survive in a dormant state in the bone marrow for extended periods, but under a mesenchymal-to-epithelial transition

Introduction

(MET) begin to proliferate and form metastases. This transition facilitates their colonization of new tissues and is crucial for the development of overt metastases. Metastatic breast cancer often targets organs such as the lungs, liver, bones, and brain [27]. This targeting can be influenced by various factors including genetic alterations, the tumor microenvironment, and the specific subtype of breast cancer. According to meta-analysis DCCs in the bone marrow could be detected in 4,703 stage I-III breast cancer patients [28].

1.1.5. Models of metastasis

There are two main models that explain tumorigenesis and systemic cancer progression: linear progression and parallel progression. The linear progression model of cancer suggests that metastasis founder cells (MFCs) evolve primarily within the primary tumor, gradually acquiring mutations until they become highly advanced clones that spread later in tumor development [29]. Therefore, dissemination of cancer cells should be a late event, and bone marrow DCCs should contain most of the mutations present in the primary tumor. However, evidence from clinical data and animal studies increasingly supports the idea of parallel evolution, where primary tumors and metastatic sites evolve simultaneously and independently, challenging the linear model (Figure 1) [30].

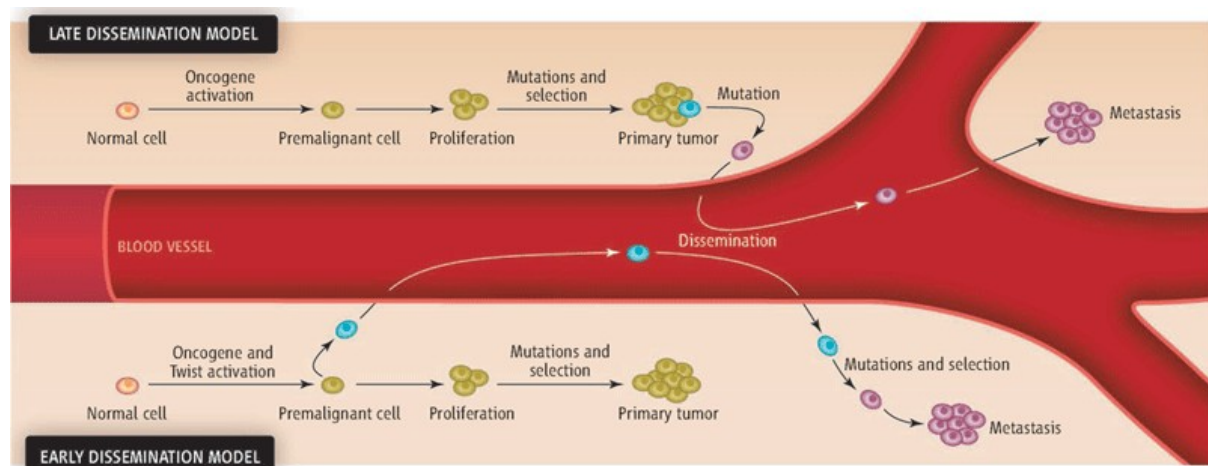


Figure 1. Linear vs. parallel progression. Top: The late dissemination model suggests that DCCs primarily evolve within the primary tumor, accumulating somatic mutations over time and resembling the most advanced clone within the tumor. In this model, the genomic profile of DCCs is expected to align closely with the primary tumor, meaning treatments targeting the primary tumor should effectively prevent relapse. Bottom: The early dissemination model proposes that DCCs leave the primary tumor at an early, genomically immature stage and evolve independently at distant sites by acquiring

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additional somatic mutations. In this case, metastases may not respond to therapies designed based on the primary tumor's characteristics. Adapted from [30].

Since the 1950s, research has questioned the late-dissemination model of MFCs due to discrepancies in metastatic growth rates [31]. Though single cell analysis has played an important role in enhancing our understanding on cancer progression models. The first single-cell analysis on bone marrow DCCs conducted demonstrated that these cells shared chromosomal aberrations with distant metastases and showed loss of tumor suppressor genes like APC and CDH1 [32]. Later studies built on this, found that DCCs from breast cancer patients without metastasis had fewer chromosomal abnormalities compared to those from patients with metastasis, suggesting early dissemination with limited evolution in a distinct microenvironment [33]. A study further confirmed that cytokeratin (CK) positive cells with normal chromosomal profiles from the same patient group showed higher loss of heterogeneity compared to normal cells, supporting their origin from primary breast tissue and indicating very early dissemination [34]. Further analysis of mammographic data revealed that if cancer cells spread only in late tumor stages, metastasis would require up to 12 years to manifest. However, data shows that median metastasis-free survival for small tumors (<2 cm) is about 35 months, suggesting parallel development of primary tumors and metastases [30]. Furthermore, studies have detected DCCs in the bone marrow of patients with DCIS, suggesting that metastasis may begin even before the invasive stage, complicating the traditional view of metastasis as a purely late-stage phenomenon [35-37]. The phenomenon of metastasis originating from pre-malignant tumors has been also demonstrated in various mouse models of breast cancer, revealing that a significant proportion of metastases can arise prior to the development of invasive cancer. For example, studies have shown that early dissemination of tumor cells can occur from non-invasive lesions, leading to distant metastasis without prior invasive growth [38, 39]. Similar findings have been observed in other cancer types, such as melanoma and pancreatic cancer [40, 41]. As a result, single-cell analysis challenges traditional models by demonstrating early dissemination of tumor cells and genetic divergence from the primary tumor. These studies also underscore the complexity of DCC heterogeneity, highlighting the need to understand the mechanisms of parallel evolution through the analysis of residual disease (DCCs) to improve therapeutic strategies.

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1.2. Analysis of disseminated cancer cells (DCCs)

1.2.1 Detection of DCCs

Specific detection of carcinoma cells in bone marrow, lymph nodes, and blood can be achieved through immunocytochemical staining for epithelial markers, as these tissues are typically of mesenchymal origin and free of epithelial cells. Over the years, antibodies specific to cytokeratin (CK) have proven highly effective in identifying disseminated cancer cells (DCCs) [42, 43]. Epithelial cell adhesion molecule (EpCAM) is another widely used marker used for DCC detection [44, 45]. Although EpCAM is not as specific as CK, being a cell surface marker, it allows isolation of live cells for transcriptomic analysis [46, 47]. Detection of CK positive cells in bone marrow or EpCAM positive cells in lymph nodes of non-metastatic cancer patients is strongly associated with a poor prognosis [48-53].

1.2.2 Genetic analysis of DCCs

Single-cell analysis enables detailed examination of limited DNA material, uncovering the complex biology within diverse cell populations. Over the last 25 years substantial efforts have been invested to develop and improve methods allowing amplification of the entire human genome [32, 54-62]. These approaches, collectively termed whole genome amplification (WGA), were designed to provide a comprehensive and unbiased representation of the entire genome from minimal DNA samples. Among these, two primary techniques, ligation-mediated PCR (LM-PCR) and degenerate oligonucleotide-primed PCR (DOP-PCR), are widely applied in research on bone marrow DCCs [63-65]. Copy number variation (CNV) is indeed a critical genomic alteration often observed in tumorigenesis [66]. CNV is defined as an increased or decreased number of DNA segments that is equal to or larger than 1kb while single-nucleotide polymorphisms are defined as genetic alterations in one single base [67, 68]. To study CNVs in bone marrow DCCs, various high-resolution methods have been employed. Chromosome Comparative Genomic Hybridization (cCGH) detects large-scale alterations but is limited in resolution [32, 69], while Array CGH (aCGH) improves this by using microarrays [70]. When CNVs are not detected, Loss of Heterozygosity (LOH) analysis, often through microsatellite analysis or PCR-RFLP, helps to assess malignancy [71]. A single-nucleotide polymorphism (SNP) array

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provide even higher resolution by measuring DNA signal intensities with numerous probes [72]. Finally, Next-Generation Sequencing (NGS) enables comprehensive, high-throughput detection of genomic alterations at the single-cell level [73]. Many studies revealed genomic heterogeneity and malignant characteristics in DCCs. Fluorescence in-situ hybridization (FISH) experiments on CK positive cells from bone marrow have confirmed their malignant origin by detecting tumor-specific chromosomal aberrations [74] [75]. Early studies using FISH and CGH have demonstrated high heterogeneity among DCCs across various cancers, such as breast, prostate, and esophageal cancers [33, 34, 76]. Notably, in patients without overt metastases (i.e. in stage M0), DCC genomes tend to have fewer chromosomal aberrations and often lack the specific mutations or alterations found in primary tumors or metastasized (M1) DCCs [43, 77]. This suggests that M0 DCCs may represent a more “immature” genetic state, with limited but potentially latent metastatic potential. In contrast, DCCs in patients with metastasis exhibit genomic profiles more similar to primary tumors, often sharing key mutations, such as HER2 amplification or PIK3CA mutations, which drive cancer progression [78, 79]. The genomic differences between DCCs isolated from M0-stage and M1-stage patients raise questions about the evolution of systemic cancer. Are there intrinsic cellular properties that could drive the genomically immature M0 DCCs to evolve into cells with M1-like genomic characteristics? Thus, to identify MFCs in breast cancer patients, STR based phylogenetic analysis was applied.

1.2.3 Phylogenetic analysis of DCCs

In our lab, we previously established a protocol allowing phylogenetic reconstruction of healthy and tumor cells [80]. Recently, we employed this technology to reconstruct the phylogenetic relationship between the primary tumor and the corresponding DCCs and CTCs obtained from a breast cancer patient. This analysis revealed that DCCs and primary tumors of breast cancer patients showed a clear separation of primary tumor cells from metastatic cells (DCCs and CTCs) and control cells (hematopoietic). Interestingly, primary tumor cells formed a cluster, which was more differentiated from the normal cells than CTCs and DCCs, suggesting that primary tumor cells represent the most advanced stage of genetic progression in the studied patient. CTCs and DCCs, on the other hand, represented an intermediated stage of genetic progression

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between the primary tumor and control cells. Surprisingly, DCCs appeared to be heterogeneously with individual DCCs clustering with either CTCs or normal cells (Figure 2). This may suggest that the analyzed DCCs represented different stages of genetic progression and that some of these cells may stem from cancer clones representing early stages of the disease. While genetic analysis of DCCs provides valuable insights into their potential for metastasis, integrating phenotypic analysis is essential to fully understand tumor behavior.

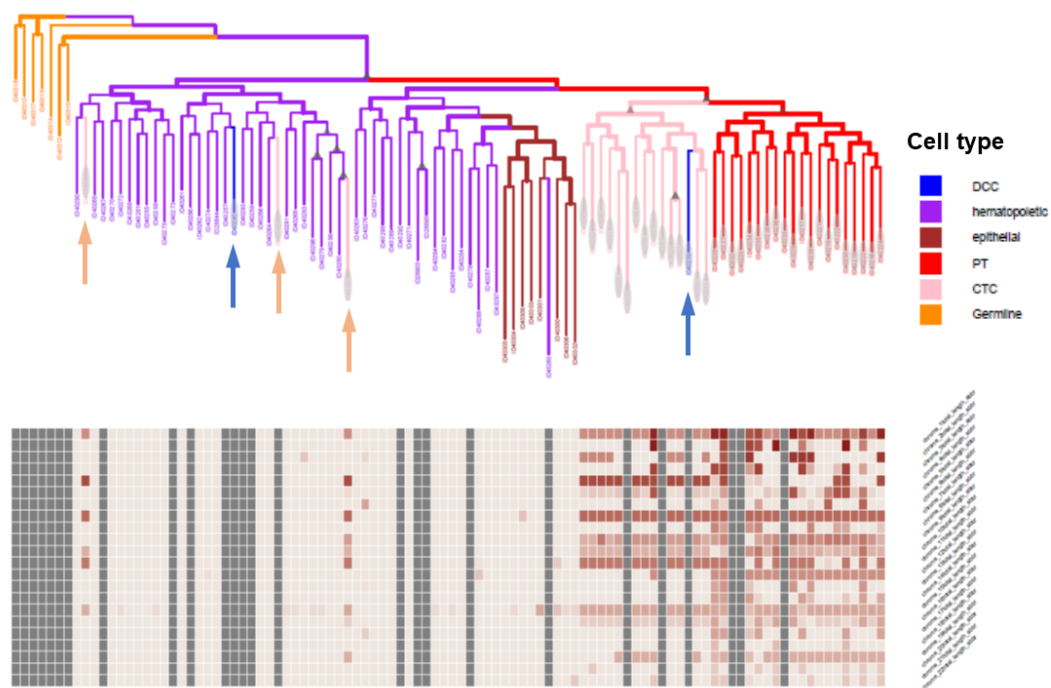


Figure 2. STR based lineage tree associated with CNV analysis. Presented phylogenetic analysis (upper panel) revealed a clear separation of primary tumor cells (red) from metastatic cell (CTCs and DCCs – pink and blue, respectively) and hematopoietic cells (violet). Oral epithelial cells (brown) form a separate cluster distinct from the hematopoietic cells and all cancer cells. Interestingly, primary tumor cells represent the most advanced stage of genetic progression, which is visualized in the lineage analysis by the highest distance from the germline samples (cells derived from PBMCs of the same patient, orange). Blue arrows indicate clustering of DCCs. Note that one DCC clustered with hematopoietic and the second DCC clustered with CTCs. Similar clustering pattern was observed for three CTCs, with individual CTCs clustering with hematopoietic cells (pink arrows). The highlighted cells likely represent early stages of systemic progression. Lower panel depicts results of the complimentary CNV analysis, with row depicting aberrations in individual chromosomes. Brown color shading is proportional to the size of detected aberrations. Note that genomic aberrations (dark brown) were detected only in cancer cells. Gray boxes indicate samples, where CNV analysis failed or could not be performed.

Introduction

1.3. Challenges and innovations in multi-omics profiling of single cells

Single-cell technologies designed for comprehensively analyzing individual types of biomolecules, often termed mono-omics or unimodal approaches, provided better understanding of cellular heterogeneity and functional states than bulk sample analysis due to the genotypic and phenotypic heterogeneity of cancer cells. Despite the power of these mono-omics approaches, they provide only a partial view of cellular complexity [81, 82]. For a more complete understanding of the molecular landscape from the genome to the phenome within individual cells, there is a growing need for multi-omics methodologies that can simultaneously capture multiple biomolecular layers at the single cell level. Multi-omics approaches enabled investigation of intermolecular interactions linking gene regulatory mechanisms in the epigenome and gene expression patterns in the transcriptome and proteome within the same individual cells, offering a more integrated and comprehensive view of cellular dynamics [83]. Therefore, more recently multi-omics workflow was established allowing parallel analysis of two or at most three types of biomolecules in a single cell (i.e. parallel analysis of genome and transcriptome [46, 84, 85], methylome and transcriptome [86, 87] or genome, methylome and transcriptome [88, 89]). Several advanced techniques have been developed to measure both the transcriptome and proteome within individual cells. Key methods include the proximity extension assay with specific RNA target amplification (PEA/STA), the proximity ligation assay for RNA (PLAYR), cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), and RNA expression and protein sequencing assay (REAP-seq) (Figure 3) [90, 91]. In particular, CITE-seq and REAP-seq are similar in that they both use DNA-tagged antibodies for protein detection and RNA sequencing for mRNA profiling, with quantification based on DNA tag/barcode frequency. In contrast, PEA/STA relies on DNA oligonucleotide hybridization for protein detection and PCR amplification for readout, while PLAYR uses isotope-labeled probes and mass cytometry to measure both proteins and mRNAs, employing rolling circle amplification for mRNA quantification [91]. In contrast to CITE-seq and REAP-seq, which focus on cell surface proteins, the single-cell RNA and immunodetection (RAID) method allows the simultaneous detection of intracellular or phosphorylated proteins alongside mRNAs (Figure 3).

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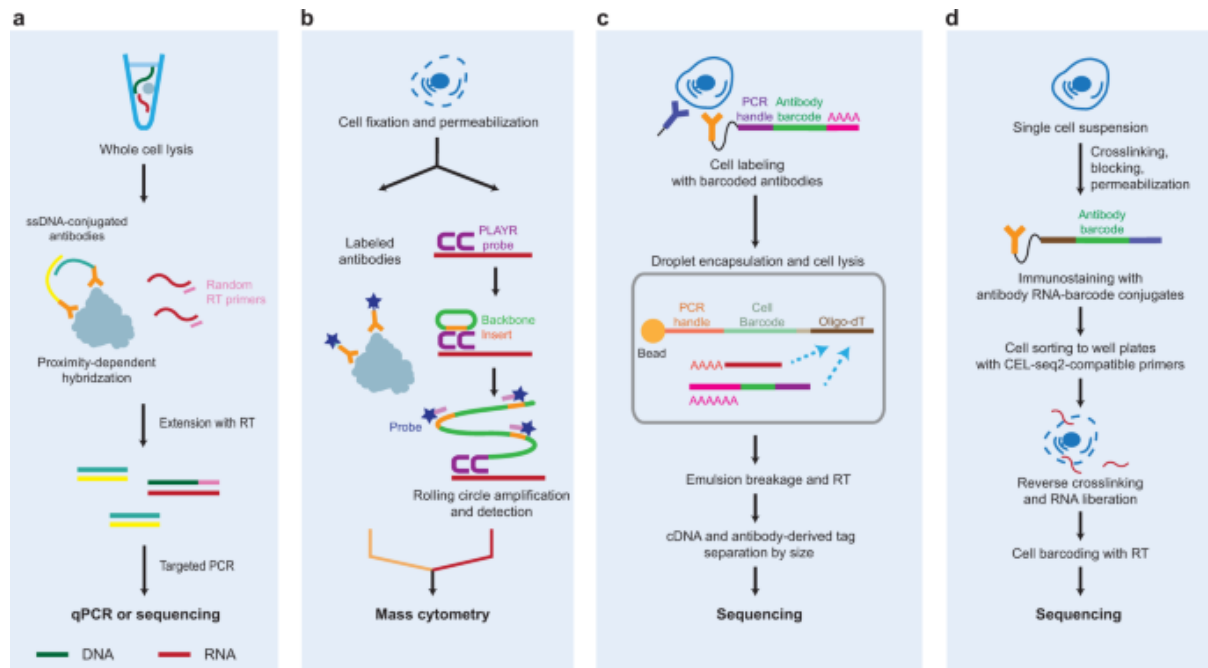


Figure 3. Four multiomics methods integrating transcriptome and proteome analyses. (a) PEA/STA, (b) PLAYR, (c) CITE-seq, and (d) RAID. Key elements are represented as follows: green-blue lines denote single-stranded DNA (ssDNA) oligos attached to antibodies, rotated U shapes signify PLAYR probes, green-orange circles indicate backbone-insert oligos, and DNA fragments with stars represent isotope-labeled probes [92].

While transcriptomic and phenotypic measurements reveal insights into gene expression and cellular activity, they do not capture genetic variations that lie outside the expressed regions, such as mutations in non-coding regions, regulatory elements, or structural variations, all of which play significant roles in gene regulation and disease mechanisms. Therefore, transcriptomic and proteomic analyses cannot alone provide a full understanding of how genetic variants affect biological processes [93]. While many technologies have emphasized the importance of high-throughput single-cell genotype-phenotype measurements, there are fewer studies focused on genome and proteome analysis at the single-cell level. In this context, Mission Bio's Tapestry Platform utilizes high-throughput single-cell proteogenomic profiling (DAb-seq), which uses DNA sequencing and flow cytometry to assess genotype and immunophenotype in separate assays (Figure 4).

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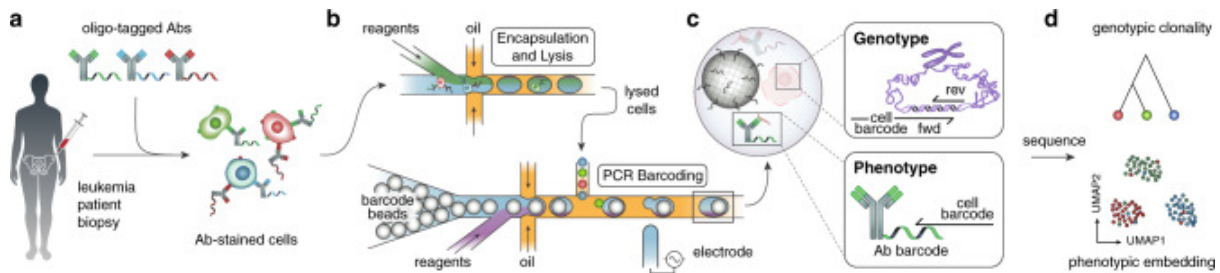


Figure 4. The DAb-seq workflow. (a) Bone marrow aspirates from acute myeloid leukemia patients consist of both healthy and malignant cells, displaying diverse genotypes and immunophenotypes. These cells are stained using antibodies conjugated with DNA tags. (b) The stained cells are paired with barcode beads and encapsulated in droplets using the Mission Bio Tapestry instrument. (c) Within each droplet, PCR is performed to label antibody tags and genomic DNA targets simultaneously with a unique cell index. (d) Sequencing of the barcoded amplicons and antibody tags provides integrated single-cell immunophenotype and genotype data for thousands of cells [93].

However, like many other multi-omics methodologies, this platform is also optimized for superficial analysis of large cell populations and is less suited for profiling rare cells (DCCs and CTCs), as it relies on emulsion or droplet-based methods [93].

Introduction

1.4. Aim

The first aim of this thesis focused on developing a reliable method for isolating bone marrow-derived DCCs from archived cytopins which contain densely packed cells that cannot be isolated using conventional micromanipulation. To improve upon previously established isolation techniques, we introduced two key advancements: First, we employed the Microdissection Micromanipulation Imaging (MMI) system, a high-tech tool that enabled more precise isolation of DCCs from cytopins with a high density of bone marrow cells. Second, we incorporated immunofluorescence (IF) staining to enhance the identification of cancer cells, allowing for more accurate detection of DCCs. The second aim of this thesis was to establish a combined genomic and proteomic (G&P) profiling approach at the single-cell level. Building on the genetic data obtained from DCCs, our goal was to perform phenotypic characterization to identify specific signatures that could predict the metastatic potential of these cells.

Materials

2. Materials

2.1 Patients sample

Bone marrow derived archived frozen cytopspins from four patients with DCIS were used in this project to test the application of LMD. Additionally, bone marrow derived archived frozen adhesion slides from HER2+ patients were utilized for the application of the G&P assay. Additionally, as control and reference samples, single mononuclear cells were obtained from peripheral blood of six healthy donors.

2.2 Cell lines

The breast cancer cell lines including BT474, MDA-MB-453 and MDA- 231, MDA-MB-468 and MCF7 were acquired from the American Type Culture Collection (ATCC). All cell lines have been maintained in the conditions recommended by the distributor. Identity of all cell lines was confirmed using a PCR-based fingerprinting.

2.3 Reagents

2.3.1. Antibodies and dyes

Table 1. List of antibodies and dyes

Antibody	Manufacturer	Catalog No.
A45B/B3 biotinylated	in house biotinylated	N.A.
Alexa Fluor 350 goat anti-rabbit IgG1	Invitrogen	2128964
Alexa Fluor 488 goat anti-mouse IgG1	Invitrogen	2179204
Alexa Fluor 488 goat anti-Rabbit IgG1	Invitrogen	2506099
Alexa Fluor 555 goat anti-mouse IgG1	Invitrogen	XK359306
AP-polymer anti-mouse	Zytomed Systems	ZUC077-100
AP-polymer anti-rabbit	Zytomed Systems	ZUC031-006
HEA125-PE	Miltenyi Biotec	5231100201
IgG1, Kappa from murine myeloma, Clone MOPC 21	Sigma Aldrich	M9269
Monoclonal Mouse anti-ERK1/2 P44/42 MAPK	Cell Signaling	4696S
Monoclonal Mouse anti-ERK1/2 Phospho (Thr202/Tyr204),	Biolegend	B292934
Monoclonal Mouse anti-human CD340 (erbB2/HER-2)	Biolegend	B266137
Monoclonal Mouse anti-human CD45	Biolegend	B283767
Monoclonal Mouse anti-Human Pan cytokeratin, Clone A45B/B3	Micromet, Germany	N.A.
Monoclonal Mouse anti-phospho-ErbB2 (HER-2, Tyr1248)	Invitrogen	WA3179601
Monoclonal Mouse anti-STAT3 Phospho (Ser727)	Biolegend	B331149
Monoclonal Rabbit anti- AKT Phospho (Ser473)	Cell Signaling	31957SF
Monoclonal Rabbit anti-CD3, T Cell antibody	Sigma Aldrich	C7930
Monoclonal Rabbit anti-phospho-HER2/ErbB2 (Tyr1221/1222)	Cell Signaling	2243S
MOPC21 biotinylated	in house biotinylated	N.A.
Rabbit IgG isotype control	Southern Biotech	0111-01

Materials

2.3.2. Tag oligonucleotides sequences

Table 2. List of DNA tag sequences used for antibody conjugation

Name	Sequence (5' → 3')	Length (bp)
Cytokeratin	AGTGGGATTCTGCTGTCAGTTCGTCGGCAGCGTCAGATGTGTATAAGA GACAGGTTGAGGCCGGGAGAAATGGGGGCNNNNNNNCTGTCTCTTATACA CATCTCCGAGCCACGAGACAC TGACAGCAGGAATCCCACT	138
HER2	AGTGGGATTCTGCTGTCAGTTCGTCGGCAGCGTCAGATGTGTATAAGA GACAGCGGGGAGAGTTCCGTAGCGATCNNNNNNNCTGTCTCTTATACA CATCTCCGAGCCACGAGACACGACAGCAGGAATCCCACT	138
pHER2	AGTGGGATTCTGCTGTCAGTTCGTCGGCAGCGTCAGATGTGTATAAGA GACAGCAGGCCTAACCGAGGCGCTGCCANNNNNNCTGTCTCTTATACA CATCTCCGAGCCACGAGACACTGACAGCAGGAATCCCACT	138
pERK1/2	AGTGGGATTCTGCTGTCAGTTCGTCGGCAGCGTCAGATGTGTATAAGA GACAGGCGAGGGGAGGAACCTCCGCGCGNNNNNNNNNNCTGTCTCTT ATACACATCTCCGAGCCACGAGACACTGACAGCAGGAATCCCACT	142
pAKT	AGTGGGATTCTGCTGTCAGTTCGTCGGCAGCGTCAGATGTGTATAAGA GACAGCGCAACACGCAGTTTACCGCGTCNNNNNNNNNNCTGTCTCTTA TACACATCTCCGAGCCACGAGACACTGACAGCAGGAATCCCACT	142
*pSTAT3	AGTGGGATTCTGCTGTCAGTTCGTCGGCAGCGTCAGATGTGTATAAGA GACAGCGCATGCGGGCAGGTTATGCAGNNNNNNNNNNCTGTCTCTTA TACACATCTCCGAGCCACGAGACACTGACAGCAGGAATCCCACT	142
Spike-in	AGTGGGATTCTGCTGTCAGTTCGTCGGCAGCGTCAGATGTGTATAAGA GACAGTGGACGACTAGCCATCCCTCTGGNNNNNNNCTGTCTCTTATACAC ATCTCCGAGCCACGAGACACTGACAGCAGGAATCCCACT	138

*This sequence was initially used to conjugate with the CD45 antibody. However, since that conjugation was unsuccessful, we used the same sequence for pSTAT3 conjugation instead.

2.3.3. Primers

Table 3. List of primers

Name	Sequence (5' → 3')	Length (bp)	Amplicon size (bp)
Index #2	AATGATACGGCGACCAACCGAGATCTACACTAGATCGCTCGTCGG CAGCGTC	51	165
Index #1	CAAGCAGAAGACGGCATAACGAGATCGCCTTAGTCTCGTGGGCT CGG	46	

2.3.4. TaqMan probes

Table 4. List of TaqMan probes used in qPCR

Name	5' Modification	Sequence (5' → 3')	3' Modification
Cytokeratin	BHQ-2	GTTGAGGCCGGGAGAAATGGGGGC	LC610
HER2	BHQ-3	CGGGGGAGAGTTCCGTAGCGATC	Cy5

Materials

pHER2	BHQ-1	CAGGCCTAACCGAGGCGCTGCCA	HEX
pERK1/2	BHQ-3	GCGAGGGGAGGAACCTCCGCGCG	Cy5
pAKT	BHQ-1	CGCAACACGCAGTTTACCGCGTC	HEX
pSTAT3	BHQ-1	CGCATGCGGGCAGGTTATGCAGG	HEX
Spike-in	BHQ-1	TGGACGACTAGCCATCCCTCTGG	FAM

2.3.5. Chemical and commercial solutions

Table 5. List of chemical and commercial solutions

Name	Manufacturer	Catalogue number
1 kb Plus DNA Ladder + Dye	New England Biolabs	N3200L
AB serum, human	Bio Rad	805135
Acetic acid (100%)	Merck	1000631000
Acetone	VWR Chemicals	MFCD00008765
Agarose LE	Anprotec	AC-GN-00009
Agencourt AMPure XP	Beckman Coulter	A63881
BCIP/NBT detection system	Bio-Rad	1706432
Bovine serum albumin (BSA) (for picking and basal medium)	Sigma Aldrich	B8667-5ml
Bovine serum albumin (BSA) (for PCRs)	Roche Diagnostics	10711454001
dNTP Set; 100 mM each A, C, G, T; 4x 24 µM	GE Healthcare/ Roche Diagnostics	28-4065-51/ 11814362001
DMEM	Anprotec	AC-LM-0011
DMSO	Sigma Aldrich	D4540-100ml
Ethanol absolute Mol. Bio. Grade 250 ml	VWR Chemicals	437443T
Ethanol absolut ≥99.8%	VWR Chemicals	20821.330
Ethidium Bromide Solution (10 mg/ml)	Sigma-Aldrich	E1510-10ML
Ethylenediaminetetraacetic acid (EDTA)	J.T. Baker	B-1073.1000
Expand Long Template Buffer 1	Roche Diagnostics	11751
FastStart DNA Polymerase	Roche Diagnostics	4738420001
FastStart dNTP mix	Roche Diagnostics	4738420001
FastStart PCR buffer with MgCl ₂	Roche Diagnostics	4738420001
Fetal bovine serum (FBS) sera Plus	PAN Biotech	P30-3702
Fetal calf serum (FCS) sera Plus	PAN Biotech	P30-3702
FluoroFix™ Buffer	Biolegend	422101
Formaldehyde, 37%	Merck	104003
Gel loading dye 6x, purple, no SDS	New England Biolabs	B7025S
Hank's balanced salt solution (HBSS) 10x	Biochrom	L2045
Hematoxylin Solution, Mayer's	Sigma Aldrich GmbH	MHS16-500ml
Igepal CA-630 viscous liquid	Sigma Aldrich	I3021-20ml
Intracellular Staining Permeabilization Wash Buffer (10x)	Biolegend	421002
MseI	New England Biolabs	R0525M
Percoll™	GE Healthcare	17089101
Pick PBS	Gibco	10-010-023
RPMI 1640	Anprotech	AC-LM-0060
Tris EDTA 1x pH 8.0 low EDTA for mol. Biology	AppliChem	A8569,0500
Trypan blue	Sigma Aldrich	T8154-20ml
Trypsin/ EDTA (10x)	PAN Biotech	P10-024100
TWEEN 20	Sigma-Aldrich	P9416-50ml
Water for chromatography, LiChrosolv®, LC-MS grade (PCR-water)	Merck	1.15333.1000
Water, aqua ad injectabilia (NGS-water)	Braun	2351744

2.3.6. List of custom-made buffers

Table 6. List of custom-made buffers

Materials

Name	Composition
AB serum 10 %	5 ml AB serum human, 45 ml 1X (PBS) pH 7.4
FACS buffer	10 ml FCS2 ml, 0.5 M EDTA, 0.5 ml 10% NaN ₃ , PBS ad 500 ml
Igepal 10%	2 ml Igepal CA-630, 18 ml DEPC-water
Loading dye	0.3% Orange G, 25% Ficoll, TBE
NBT/BCIP-solution	100 µl AP development buffer*, 25 µl AP reagent 1*, 25 µl AP reagent 2* 25 µl Levamisol, Distilled water ad 2.5 ml * From AP-conjugate substrate kit, Bio Rad
One Phor All (OPA) buffer 10X	5 ml 1 M Tris acetate, 5 ml 1 M Magnesium acetate ,1 ml 5 M Potassium acetate, PCR-Water (final volume 1L)
PBS pH 7.4 10X	450 g Sodium chloride, 71.65 g Disodium phosphate (Na ₂ HPO ₄), 13.35 g Monopotassium phosphate (KH ₂ PO ₄), Milli-Q water (final volume 5L)
Percoll 60 %	60 ml Percoll 100 % ,40 ml NaCl (0.9% Braun)
PFA 0.5%	1.35 formaldehyde solution, Milli-Q water (final volume 100 ml)
Tris/Borate/EDTA (TBE) buffer 10x	539 g Tris, 275 g Boric acid, 37 g EDTA, Milli-Q water (final volume 5l)
TBS	8 g of Sodium chloride, 0.2 g of potassium Chloride, 12.1 g of Tris (final volume 1L)
Tween 10 %	2 ml 100 % TWEEN. 20 in 18 ml PCR water

2.3.7. Commercial kits

Table 7. List of commercial kits

Kit	Manufacturer	Catalog Number
Ampli1™ LowPass Kit (SETA+Set B)	Menarini Silicon Biosystems	WGLPAB
BCIP/NBT (AP conjugate substrate Kit)	Bio-rad	170-6432
Expand Long Template PCR System	Roche Diagnostics	11759060001 (Sigma Aldrich)
FastStart™ Taq DNA Polymerase, dNTPack	Roche Diagnostics	4738420001 (Sigma Aldrich)
MiSeq Reagent Nano Kit v2 (300-cycles)	Illumina	MS-103-1001
LightCycler® 480 Probes Master (5 x 1 ml)	Roche	4707494001

2.4. Cell culture media

Table 8. List of cell culture media

Name	Components	Cell lines
RPMI-1640	500 ml RPMI 50 ml FCS 5 ml 200 mM L-Glutamine 5 ml Penicillin/ Streptomycin	BT474, MCF7
DMEM	500 ml DMEM 50 ml FCS 5 ml 200 mM L-Glutamine 5 ml Penicillin/ Streptomycin	MDA-MB-231, MDA-MB-453, MDA-MB-468

2.5. Consumables

Table 9. List of consumables

Product Name	Company	Catalog Number
AdhesiveCap 200 clear	Carl Zeiss	415190-9191-000
Adhesive sealing sheets	Thermo Fisher Scientific	AB0558
CellSave Preservative Tubes	Menarini Silicon Biosystems	7900005
Cellstar. serological pipette 2 ml	Greiner Bio-one	710180
Cellstar. serological pipette 5 ml	Greiner Bio-one	606180
Cellstar. serological pipette 10 ml	Greiner Bio-one	607180
Cellstar. serological pipette 25 ml	Greiner Bio-one	760180
Centrifuge tube 15 ml	Greiner Bio-One	188271
Centrifuge tube 50 ml	Greiner Bio-One	227261

Materials

Diagnostic adhesion slides	Thermo Fisher Scientific	ER-203B-CE24
Eppendorf twin.tec®-PCR-Platte 96 LoBind, skrited	Eppendorf	0030129512
Eppendorf. protein LoBind tube 0.5ml	Eppendorf	0030 108.094
Eppendorf. protein LoBind tube 1,5ml	Eppendorf	0030108.116
Eppendorf. protein LoBind tube 2ml	Eppendorf	0030 108.132
Eppendorf. protein LoBind tube 5ml	Eppendorf	0030 108.302
LabTek Chamber Slides, glass, 8 fields	Nunc	11367764
MAXYMum Recovery™ 0.2 ml	Axygen Scientific	11370145
Micro-hematocrit capillary	Brand	749321
Microcentrifuge tube 1.5 ml	Greiner Bio-One	616201
Microcentrifuge tube 2 ml	Greiner Bio-One	623201
Multichannel Reagent reservoirs	Integra	4331
PCR tube 0.2 ml	4titude Deutschland	4ti-0795
PCR SingleCap 8er-SoftStrips 0.2 ml	Biozym	710970
SafeSeal Surphob filertips 1250µl	Biozym	VT0270
SafeSeal Surphob filertips 200 µl	Biozym	VT0240
SafeSeal Surphob filertips 20 µl	Biozym	VT0220
SafeSeal Surphob filertips 10 µl	Biozym	770010
Superfrost® Plus slides	Thermo Fisher Scientific	10149870
Transparent 96-well PCR plate	Biozym	710884

2.6. Devices

Table 10. List of devices

Name	Manufacturer
AlphaThera LEDPX2 Device	AlphaThera
Autoclave 3150 EL	Systec
Bioanalyzer 2100	Agilent Technologies
Cell culture incubator	Heraeus, Hanau
Cell culture laminar flow	Heraeus, Hanau
CellTram Pump	Eppendorf
Centrifuge 5424	Eppendorf, Hamburg
Centrifuge 5810R	Eppendorf, Hamburg
Centrifuge Rotina 380R	Hettich
Centrifuge Rotofix 32A	Hettich
Digital dispenser	Tecan
DMZ Universal Puller	Zeitz
DNA Engine Peltier Thermal Cycler	Bio Rad
DNA Engine Tetrad2 Peltier Thermal Cycler	Bio Rad
Gel electrophoresis chambe	Biostep, Jahnsdorf
Gel electrophoresis power supply	MRC Lab
GeneTouch Thermal Cycler, Block 96 or Block 48/48	Biozym
Incubator Heraeus BB15	Thermo Fisher Scientific
Incucyte® Live-Cell Analysis System	Essen BioScience
Laminar flow bench Her Safe KS18	Thermo Fisher Scientific
Manual pipettes (2µl, 10µl, 20µl, 200µl, 1000µl)	Gilson
Microscope Axiovert 200M (fluorescence)	Zeiss
Microscope IB inverted (cell culture)	Optech
Microscope IX81 (fluorescence)	Olympus
Microvawe	Micromaxx
MiSeq	Illumina
Molecular Machines & Industries (MMI)	MMI
Multipette Stream	Eppendorf
NanoDrop 2000c	Thermo Fisher Scientific
Neubauer Counting Chamber	Marienfeld
P-H Waterbath WB 20	P-D Industriegesellschaft mbH
Patchman NP2 micromanipulator	Illumina
PCR bench UVT-S-AR	Thermo Fisher Scientific
pH-meter PB-11	Sartorius
Pipet-Lite™ XLS+ manual 8-channel pipette, 20-200 µL	RAININ
Power Supply MP-250N	Kisker Biotech
Research Pro 8-channel pipette	Eppendorf
Roller Mixer SRT1	Stuart Scientific

Materials

Scale AVW220D
Scale PLS 510-3
UV hand lamps
UV Illuminator
Ventana BenchMark Ultra
Vortexer

Shimadzu
Kern
Avantor
Intas
Roche
VELP Scientifica

2.7. Softwares and databases

Table 11. List of softwares and databases

Name	Provider/ URL	Application
CellSens Dimension 1.9	Olympus	Microscope
GraphPad Prism v9	https://www.graphpad.com/scientificsoftware/prism/	Plotting of data
FlowJo V10	https://www.flowjo.com/solutions/flowjo/	Flow cytometry
IncuCyte ZOOM® 2018A	Essen BioScience	Killing assay
Microsoft Office 2016	Microsoft	Data management
PubMed	https://www.ncbi.nlm.nih.gov/pubmed	Literature search

Methods

3. Methods

3.1. Patient samples and collaborations

3.1.1. External collaborations

Table 12. List of collaborations

Name	Institution at time of collaboration start	Project part
Prof. Dr. med. Andreas Hartkopf	Department of Gynecology, University of Tübingen	Sample acquisition
Prof. Dr. med. Stephan Seitz	Department of Gynecology and Obstetrics	Sample acquisition
Prof. Dr. med. Christian Schürch	University Hospital and Comprehensive Cancer Center Tübingen, Germany	Antibody-DNA conjugation assay
Dr. Jens Warfsmann; Dr. Adithi Ravikumar Varadarajan	Fraunhofer ITEM-R, Regensburg	CNA analyses

3.1.2. Internal collaborations

LowPass sequencing and CNA analysis were guided and supported by Dr. Zbigniew Czyż, whereas bioinformatic analyses were performed by Dr. Jens Warfsmann and Dr. Huiqin Koerkel-Qu.

3.1.3. Sample acquisition

Patient samples were received between 2021-2023 in cooperation with the Department of Gynecology, University of Tübingen, owing to their huge archive of BM slides in collaboration with Prof. Dr. med. Andreas Hartkopf. Additionally, patient samples were provided through the collaboration with Prof. Dr. med. Stephan Seitz.

3.1.4. Ethics

The study was approved by the local ethics committee of University of Regensburg (ethics vote numbers 07-079, 18-948-1) and University of Tübingen (ethics vote number 535/2016BO2).

Methods

3.2. Preparation of slides for single cell isolation

3.2.1. Standard cell culture

For thawing frozen cell lines, the cryo vial was put into a water bath (37°C) for around 2 minutes. If only a small piece of ice was visible, the cryo vial was wiped with 70% ethanol and the cell suspension was transferred into a new 50 ml tube. Then, 10 ml of pre-warmed standard cell culture medium (Table 8) was added dropwise. After centrifuging (500g, 5 min), the supernatant containing cell-toxic DMSO was removed and cells were resuspended in culture medium having a density of 10^6 cell/ml and seeded in T 25 flask. On the next day, cells were washed with PBS and received fresh medium. After several days, when reaching a confluency of 80-90%, cells were transferred into a T75 flask. For this, old medium was removed and cells were washed with 1x PBS. Then, 1 ml 0.05% trypsin/ ETDA solution was added and incubated at 37 °C. After 3 minutes, trypsin was inactivated by adding 10 ml of fresh pre-warmed medium. After centrifugation, cells were resuspended in 15 ml of fresh medium transferred into a T75 flask. Cells were passaged having a confluency of around 90%. After washing with PBS, cells were trypsinized using 2 ml 0.05% trypsin/ ETDA solution as already described. After centrifugation, cells were resuspended in 5 ml of fresh medium and counted. Depending on the experiments $1-2 \times 10^6$ cells were resuspended in 25 ml of fresh medium and seeded into a new T175 flask. For preparation of cell suspension, after washing with PBS, cells were trypsinized and after centrifugation, cells were resuspended in 1 ml of PBS and counted. The cell suspension was then either used for direct staining or for the preparation of adhesion slides. All cells were cultured at 37°C and 5% CO₂. In order to freeze cells in cryo vials, 10^6 cells were frozen in 70% FCS/ 20% culture medium/ 10% DMSO. Cryo vials were put into a Cryo 1°C Freezing Container and stored at -80°C overnight. Cells were either kept at -80°C or transferred to liquid nitrogen tanks.

3.2.2. Preparation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood was collected in EDTA vacutainers. PBMCs were initially washed with Hank's solution (200g, 10 min, 4°C). Resulting cell pellet was diluted in 15 ml Hank's solution followed by centrifugation at 1000g for 20 min at 4 °C on a 60% Percoll density gradient (9 ml of mixture + 6 ml of 60 % Percoll in 15 ml falcon tube). The interphase containing PBMCs was collected and washed with PBS at 200 g for 10 min

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at 4 °C. PBS was discarded, and the cell pellet was resuspended in fresh PBS and counted in a Neubauer counting chamber. The cell suspension was then either used for direct staining or for the preparation of adhesion slides.

3.2.3. Cell counting

To determine the cell number, 10 µl of a cell suspension is taken, transferred to a well of a 96 well plate and diluted 1:10 with Trypan Blue (The dilution factor adjusted depending on the expected cell count). The cover glass of the Neubauer counting chamber is moistened and placed on the chamber. Then 10 µl of the 1:10 dilution transferred into the counting chamber. Cells in 4 large corner squares were counted and the mean value calculated. The cell counts per milliliter calculated by multiplying the mean value of the counted corner squares by the volume of a corner square (0.1 mm^2) $\times 10^4 \times$ dilution factor.

3.2.4. Preparation of adhesion slide

Diagnostic slides were prepared from cell line suspension or PBMCs. After cell counting, cells were transferred onto diagnostic slides at a density of 0.5×10^6 cells/slide (0.17×10^6 cells/spot). Following sedimentation of the cells after one hour at RT, PBS was discarded and slides were stored on the next day at -20°C after having air-dried overnight at RT.

3.2.5. Preparation of cytopsin

Cytopsins were prepared from cell line suspension. After cell counting, cells were resuspended in 1x PBS to achieve a concentration of 1.5×10^6 cells/ml. To generate the cytopsins, 1 ml of the cell suspension was centrifuged onto glass slides at 1600 rpm for 2 minutes using a cytocentrifuge (Hettich, Tuttlingen, Germany). The slides were then left to air-dry overnight at RT and subsequently stored at -20°C.

3.3. Identification of cells of interest for single cell isolation

3.3.1. Double Alkaline Phosphatase (AP)-Polymer staining against CD3 and Cytokeratin

After thawing of cytopsin for 30 min at RT, unspecific binding was blocked using PBS/10% AB-serum for 30 min at RT. Next, primary antibody incubation was done using 2 µg/ml rabbit anti-CD3 and 2 µg/ml mouse anti-human pan cytokeratin A45-

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B/B3 (CK) antibody in PBS/10% AB-serum for 60 min at RT. Each spot of the adhesion slide was covered with 160 μ l antibody dilution. Following this, slides were washed 3 times with 1x PBS and were incubated for 30 minutes with secondary antibody including anti-mouse AP-Polymer and anti-Rabbit AP-Polymer solution followed by washing 3 times with 1x PBS. For development, BCIP/NBT solution (AP Conjugate Substrate Kit; Bio-Rad Laboratories) was prepared according to manufacturer's instructions with 0.5% levamisole followed by incubating the slides for 12 min. After development the slides were washed again for 3 times with 1x PBS. Fixation with PFA was not performed for slides undergoing LMD. Throughout all staining procedures performed in this study, isotype controls were included to ensure specificity of the staining. For mouse IgG1 antibodies, MOPC 21 was used as the isotype control. For Rabbit IgG1 antibodies, the corresponding Rabbit IgG1 isotype control antibody was employed.

3.3.2. Triple Immuno-polymer staining against CK, EpCAM and CD3

Cytospins were defrosted for 20 minutes before being blocked using PBS/10% AB-serum for 30 min at RT. Following this, the slides were incubated with primary antibodies (2 μ g/ml rabbit anti-CD3 and 2 μ g/ml mouse anti-human CK) for 1 hour. After incubation, the slides were washed with PBS 3 times for 3 minutes each. Secondary antibodies (2 μ g/ml goat anti-mouse IgG-Alexa fluor 488 and 2 μ g/ml goat anti-rabbit IgG-Alexa Fluor 350) incubation were applied for 1 hour. Slides were washed 3 times with PBS and this followed by a 20-minutes incubation with 0.25 μ g/ml HEA125-PE (EpCAM) antibody. The slides were washed again three times for 3 minutes each. Slides scanned and screened using MMI and the coordinates of the target cells exported as a file. The slides removed from microscope and underwent a second incubation with primary antibodies (2 μ g/ml rabbit anti-CD3 and 2 μ g/ml mouse anti-human CK) overnight. On the next day, the slides were washed three times for 3 minutes. Polymer staining was conducted using AP-Polymer anti-rabbit and AP-Polymer anti-mouse for 1 hour, followed by another series of three washes for 3 minutes. The slides were then incubated with BCIP/NBT solution for 12 minutes and washed three times for 3 minutes. Counterstaining was performed using Mayer's hematoxylin for 2 minutes, followed by a 2-minute wash with PBS. Dehydration was conducted to prepare slide for LMD (See 3.4.2.1). After inserting the slides on microscope, coordinates of the cells imported in the software and targeted cells

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underwent LMD. This protocol was initially optimized using MCF7 cells spiked into PBMCs and later applied on frozen bone marrow derived cytopins of DCIS patients.

3.3.3. IF staining against HER2, pHER2 (Tyr1248) and (Tyr1221/1222)

To establish multiplex staining, single staining was optimized for each marker. Washing steps and antibody preparations were conducted using TBS for phosphorylated markers and PBS for HER2. Adhesion slides of BT474 cells spiked into PBMCs were thawed, and non-specific binding was blocked by incubating the slides with PBS or TBS/10% AB-serum for 20 minutes at RT. After blocking, the slides were stained with primary antibodies, including 2 µg/ml mouse anti-human CD340 (erbB2/HER2), 2 µg/ml mouse anti-phospho-ErbB2 (HER2, Tyr1248) (pHER2, Tyr1248), and 2 µg/ml rabbit anti-phospho-HER2/ErbB2 (Tyr1221/1222) (pHER2 tyr1221/1222). Each antibody was diluted in PBS or TBS containing 10% AB serum, and 160 µL of antibody solution was applied per spot on the slide. Isotype controls using mouse IgG and rabbit IgG at the same concentration (2 µg/ml) were included on separate adhesion slides. For isotype controls, mouse IgG and rabbit IgG were used at the same concentration (2 µg/ml), with the controls performed on separate adhesion slides. After primary antibody incubation, the slides were washed three times with PBS or TBS, 3 minutes each. Secondary antibody incubation followed for 1 hour at RT, using 2 µg/ml goat anti-mouse IgG-Alexa Fluor 555 for the first two markers and 2 µg/ml goat anti-rabbit IgG-Alexa Fluor 488 for the third marker. After incubation, the slides were washed three times for 3 minutes each and then screened using an Olympus microscope.

Optimization of protocol:

- I. Increased primary antibody concentration: To evaluate the effect of using a higher concentration of primary antibodies, the protocol was repeated using double the concentration of each primary antibody (4 µg/ml for each antibody). All other aspects of the protocol remained unchanged
- II. Fixation with Paraformaldehyde (PFA): To determine if fixation improves staining quality, a 5-minute fixation step with 1.37% PFA was introduced immediately after thawing the cells. After fixation, the slides were washed three times for 3 minutes each. The staining process then continued as described above.

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III. Combination of increased antibody concentration and fixation: To enhance the staining protocol, a combination of two previously tested modifications was applied simultaneously (See 4.2.3.1)

3.3.4. G&P staining with dextran sulfate to minimize unspecific binding of DNA-tagged antibody

Optimization of G&P staining initially was done using HER2 antibody on BT474 cell line spiked into PBMCs and MDA-MB-231 adhesion slides. After thawing the adhesion slide at RT, the cells were fixed using 1.37% PFA for 5 minutes and washed 3 times with PBS or TBS. The non-specific binding was blocked by incubating with PBS or TBS/5% BSA/0.5% dextran sulfate (DS) for 20 minutes at RT. After blocking, the slides were stained with 2 µg/ml of mouse anti-HER2 DNA-tagged mAb diluted in the same solution as used for blocking for 1 hour. The slides washed 3 times each 3 minutes with PBS or TBS and incubated with 2 µg/ml goat anti-mouse IgG-Alexa Fluor 555 for another 1 hour. After 3 washing each 3 minutes, the slides screened using Olympus. The optimized G&P staining protocol was subsequently applied to other markers.

3.3.5. Staining for flow cytometry and FACS

Flow cytometric analysis was performed for detecting cell surface or intracellular markers.

Surface markers: Following cell lines harvesting and centrifugation, cells resuspended in 1ml of FACS buffer, counted and transferred into 5ml FACS tube. (500K cells/tube for stained samples and 100K cells/tube for unstained samples). Following centrifugation, the supernatant was discarded and Fc receptors were blocked for 10 minutes using 2.5 µl of AB serum at RT. Following blocking, 50 µl of primary antibody against marker of interest was added to the cell. After mixing cell suspensions, cells incubated for 30 minutes at RT in the dark. Following 2 washing (centrifugation of 300g, 5 min), supernatant discarded and 50 µl of secondary antibody was added to the cells and incubated for another 30 minutes. Cells were washed twice and fixed by adding 200 µl of FluoroFix™ Buffer and incubated for 20 minutes at RT in the dark. Following another centrifugation step, cells were washed with FACS buffer. lastly the cells were resuspended in 150 µL of FACS buffer and kept at 4°C in the dark until measurement.

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Intracellular markers: after collecting cells in FACS tube and centrifugation, the cells were washed twice with Intracellular Staining Permeabilization Wash Buffer (Perm Wash), which had been previously diluted 1:10 in ddH₂O. The remainder of the protocol was followed as described for surface marker staining, with the key difference being that all washing steps and antibody incubations were performed using Perm Wash instead of FACS buffer. For measurements, the cells were resuspended in 150 µL of FACS buffer and kept at 4°C in the dark until measurement.

3.3.6. Staining against pERK, pAKT and pSTAT3

Adhesion slides of cell lines (See 4.2.11.1) were thawed and immediately fixed using 1.37% PFA for 5 minutes. After fixing, the slides were washed three times for three minutes each with TBS. The slides were then incubated with ice-cold methanol in a cuvette at 4°C for 10 minutes. Following this, the slides underwent another three washes with TBS, each lasting three minutes. To block non-specific binding, the slides were incubated with TBS/5% BSA for 20 minutes at RT. After blocking, the slides were incubated with the primary antibodies for 120 minutes at RT, applying 160 µl of the antibody solution per spot on the slide. Isotype controls were performed using mouse IgG and rabbit IgG (2 µg/ml) on separate adhesion slides. After the primary antibody incubation, the slides were washed three times with TBS, each wash lasting three minutes. Secondary antibody incubation followed for one hour at room temperature (the working concentration of each antibody is provided). Finally, the slides were washed again three times with TBS, each wash lasting three minutes, and screened using Olympus. List of antibodies and dyes used with this protocol summarized in the table 13.

Table 13. List of antibodies and concentrations used to establish phospho-protein staining

Primary antibody	Stock concentration	Working concentration	Secondary antibody
Anti-ERK1/2 P44/42 MAPK, mIgG1 mAb	84 µg/ml	1 µg/ml	Goat anti-Rabbit Alexa 488, 2 µg/ml
Anti-pAKT (Ser473) Rabbit IgG mAb	1 mg/ml	1 µg/ml	Goat anti-Rabbit Alexa 488, 2 µg/ml
Anti-pERK1/2 (Thr202/Tyr204) mIgG2b mAb	0.5 mg/ml	2 µg/ml	Goat anti-mouse Alexa 555, 2 µg/ml
Anti-pSTAT3 (Ser727), mIgG1 mAb	0.5 mg/ml	2 µg/ml	Goat anti-mouse Alexa 555, 2 µg/ml

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Mouse IgG Isotype	0.5 mg/ml	2 µg/ml	Goat anti-mouse Alexa 555, 2 µg/ml
Rabbit IgG Isotype	0.5 mg/ml	2 µg /ml	Goat anti-Rabbit Alexa 488, 2 µg/ml

3.3.7. Multiplex G&P staining; identification and characterization

The protocol initiated by defrosting frozen adhesion slides for 20 minutes. The cells were first fixed with 1.37% PFA for 5 minutes, followed by three times washing with TBS (3 minutes each). Permeabilization was performed using ice-cold methanol (100%) for 10 minutes, and the cells were washed again three times. The samples were then blocked with TBS/5% BSA/1% DS for 20 minutes. Primary antibody incubation was carried out using biotinylated CK and a cocktail of DNA-tagged monoclonal antibody including HER2, pHER2, pERK1/2, pAKT and pSTAT3 in TBS/BSA 5%/DS 1% for 120 minutes. After another round of three washes, secondary antibody incubation was performed for 1 hour using Streptavidin-Alexa Fluor 488 and EpCAM-PE in TBS. The staining process was completed with three washes of 3 minutes each. Screening of positive cells was performed using Olympus microscope. This protocol initially established using frozen adhesion slides of BT474, MDA-MB-231 and MDA-MB468 and PBMCs and later applied on bone marrow derived archive adhesion slides of HER2+ patient samples.

Table 14. List of antibodies and concentrations used in the multiplex G&P staining

Primary antibody	Stock concentration	Working concentration	Secondary antibody
Anti-HER2 mlgG1 mAb	0.5 mg/ml	2 µg/ml	-
Anti-pAKT (Ser473) Rabbit IgG mAb	0.5 mg/ml	1 µg/ml	-
Anti-pERK1/2 (Thr202/Tyr204) mlgG2b mAb	0.333 mg/ml	2 µg/ml	-
Anti-pHER2 (Tyr1248) mlgG1 mAb	0.1667 mg/ml	2 µg/ml	-
Anti-pSTAT3 (Ser727), mlgG1 mAb	0.333 mg/ml	2 µg/ml	-
Biotinylated CK mlgG1 mAb	0.2 mg/ml	2 µg/ml	Streptavidin-Alexa Fluor 488 goat-anti mouse, 2 µg/ml
EpCAM-PE	0.05 mg/ml	0.25 ug/ml	-

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3.4. Single cell isolation

3.4.1. Standard Micromanipulation

Single cells were either isolated from diagnostic slides or from cell suspension using an inverted fluorescence microscope (Olympus or Zeiss) equipped with a micromanipulator and a pump. First, a picking field of an 8-chamber slide was prepared. After coating with BSA, 200 μ l of PBS was filled in the picking field. Stained cell suspensions having a maximum density of 10^6 cells per 200 μ l PBS were transferred on a free BSA-coated field of a chamber slide. If using diagnostic slides, 200 μ l of PBS was put on the spots of the slide. First, 1 μ l of cell-free PBS from the picking field was taken and served as a negative control. Positively stained cells or cells of interest were identified, sucked in the FCS-coated glass capillary, which was attached to the micromanipulator, and transferred into the picking field. By using a small pipette, the cell was transferred in 1 μ l into a 0.2 ml Axygen™ PCR tube containing 2 μ l of proteinase K digestion mix. In addition to single cells, also a pool of cells was isolated by transferring 1 μ l of cell suspension to the prepared tubes.

3.4.2. Laser Microdissection (LMD)

3.4.2.1. Sample preparation for LMD

The entire slide undergoing LMD was stained in addition to the immunohistochemical staining of cells of interest. For counterstaining, the slides were incubated with Mayer's hematoxylin for 2 minutes followed by washing 2 times with 1X PBS (pH 7.4) for 2 minutes. The cuvette containing the slides was left in 4 °C for at least 30 min for bluing. Next, the slides were dehydrated by dipping into pre-chilled (-20 °C) solutions of 70%, 90%, 100% ethanol and acetone for 5 seconds each (dehydration at RT should be avoided). Slides were used for LMD immediately after dehydration.

3.4.2.2. LMD adjustment

The MMI system allows for the precise fine adjustment of all key dissection parameters, such as laser intensity, focus, and cutting speed. In this context, the laser power adjusted to ensure clean cutting of the cells, avoid causing excessive heat

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damage. Proper laser focus was optimized to maintain sharp precision, enabling the system to target specific regions. Additionally, balancing laser speed and pulse duration adjustment was done for efficient ablation of the surrounding cells, allowing the isolation of the target cell without compromising DNA quality. Two main features of microscope used for LMD described below:

1- CellScan:

 The area of the scan was first defined using the “Select area” tool allowing the selection of the region of interest in the overview.

 Once set, the scan was initiated by pressing the “Start scan” button, with the option to interrupt the process at any time using the “Stop Scan button” or the Esc key.

 To scan all slides in the stage insert configuration, the “Scan all” button was used to sequentially capture each slide (The icon provided from software version 6.1)

2- CellCut:

The cutting velocity was controlled using a velocity slider, which determined how quickly the stage moved during the cutting process, measured in micrometers per second ($\mu\text{m/s}$). This setting did not influence any other stage movements. The laser focus was adjusted with a focus slider, controlling the z-axis focus position of the laser beam within the tissue sample. Cutting performance was highly sensitive to this setting, with different focus values required for varying tissue types. The focus position was measured in micrometers (μm) or millimeters (mm), with its range dependent on the objective in use. The laser power was set using a power slider, which defined the laser’s output based on the sample’s thickness, ensuring clean cutting. Power settings displaying as a percentage referred to the average laser power before it passed through the optical components of the MMI CellCut system and the microscope. LMD can be done using any types of drawing tools and these shapes can be separated and handled in different groups (Figure 5).

Methods

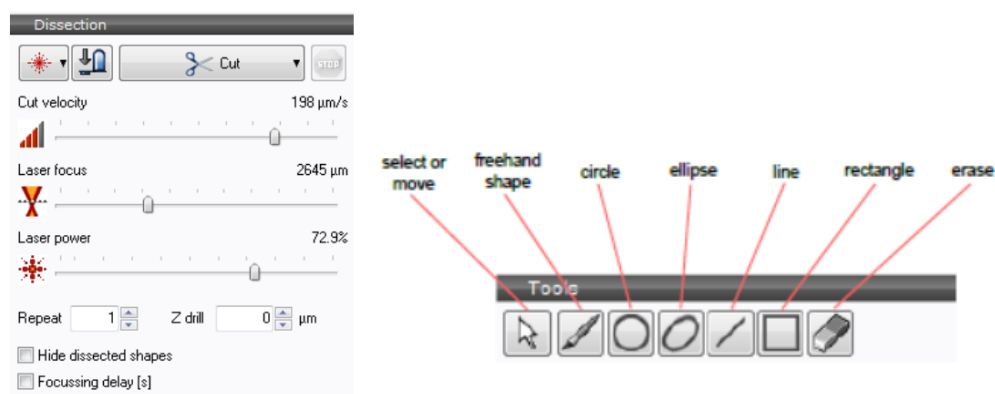


Figure 5. Dissection panel with laser parameters and drawing tool.

The laser cutting process was divided into two main parts:

I: cutting near the cell which are precise cuts made close to the target cell without touching it, creating a boundary around the cell

II: ablation around target cell which are broader cuts to clear the surrounding area, ensuring the target cell can be isolated without contamination (Figure 6).

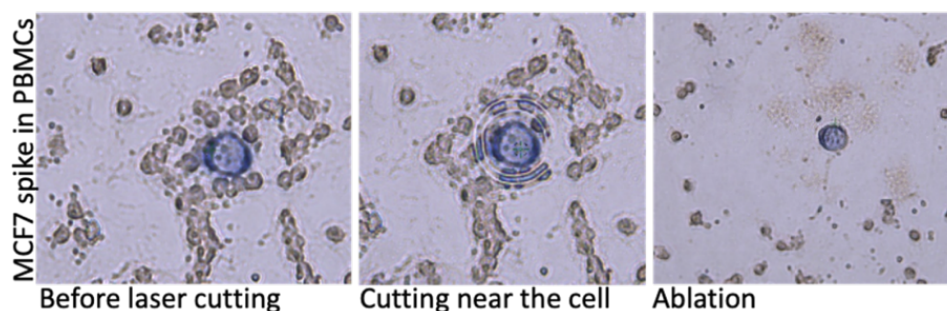


Figure 6. MCF7 cells spiked into PBMCs illustrating two distinct types of laser cutting.

3.5. Antibody-oligo conjugation assays

For antibody-DNA conjugation, we utilized the commercial service provided by BioLegend and developed two additional conjugation methodologies: the Light Activated Site-Specific Conjugation (LASIS) method and a conjugation assay based on the CODEX method. Details for each method are provided below.

3.5.1. LACIS Method

To develop this method we utilized proprietary technology, called oYo-Link® Conjugation provide by AlphaThera, that can be used to label the Fc region of antibody with oligonucleotides in a site-specific manner. We provided AlphaThera our desired oligonucleotide sequence, which used to generate a custom oYo-Link oligo reagent

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comprising a DNA oligonucleotide with the pre-defined sequence. Following receiving oYo-Link following steps were done:

1- oYo-Link oligo reconstitution: The tube containing the lyophilized oligo was spun down at 1000g for one minute. We then added 25 μ L of nuclease-free H₂O to the tube, which contained 25 μ g of the custom single-stranded DNA (ssDNA). The mixture was vortexed briefly and spun down. The tube was placed on a thermal shaker at 400 rpm for five minutes at room temperature (22 °C), followed by additional vortexing and spinning. Finally, it was placed back on the thermal shaker at the same settings for another five minutes, then vortexed, spun down, and stored. The reconstituted product was stored at 4 °C for short-term use (less than one week) or between -20 °C and -80 °C for long-term storage.

2- Antibody-oligo mixture preparation: Following reconstitution, each tube was used to label 25 μ g of antibody. The antibody and oligo were mixed in a 1.5 mL Eppendorf tube according to Table 15. Details of the antibodies conjugated using this method, including their concentrations before and after conjugation are provided in the table.

Table 15. List of antibodies conjugated using LASIC method

Primary antibody	Stock concentration	The volumes of antibody and oligo required to prepare 25 μ g of DNA-conjugated antibody	Final conjugate conjugation
Monoclonal mouse IgG1 anti-CK	0.2 mg/ml	125 μ l Ab+25 μ l of oligo	166.6 μ g/ml
Monoclonal mouse IgG1 anti-pHER2	0.2 mg/ml	125 μ l Ab+25 μ l of oligo	166.6 μ g/ml
Monoclonal Mouse IgG2b anti-pERK1/2	0.5 mg/ml	50 μ l Ab+25 μ l of oligo	333.3 μ g/ml
Monoclonal Rabbit IgG anti-pAKT	1 mg/ml	25 μ l Ab+25 μ l of oligo	500 μ g/ml
Anti-pSTAT3 (Ser727), mIgG1 mAb	0.54 mg/ml	46.3 μ l Ab+25 μ l of oligo	350 μ g/ml

3- Photo-crosslinking: the antibody-oligo mixture was exposed to non-damaging UV light (λ = 365 nm) for 2 hours, enabling the oYo-Link to form a covalent bond with the antibody. For UV exposure, we used two LED devices: a handheld LED device and AlphaThera LEDPX2 device (Figure 7). The handheld LED device, equipped with two 6 W bulbs emitting non-damaging UV light (365 nm). This device required adjusting

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the ice height in the bucket so the sample was positioned within 3 to 10 cm of the light source. The capped sample tube was placed horizontally on the ice for optimal exposure for approximately 2 hours. AlphaThera LEDPX2 device, also containing two LED bulbs, provided uniform UV light exposure (365 nm). For this setup, the acrylic sample container was filled with ice until two-thirds full, and the sample tube was placed on its side on top of the ice. The device ran for 1 hour, after which the ice was replaced, and the process continued for another hour.

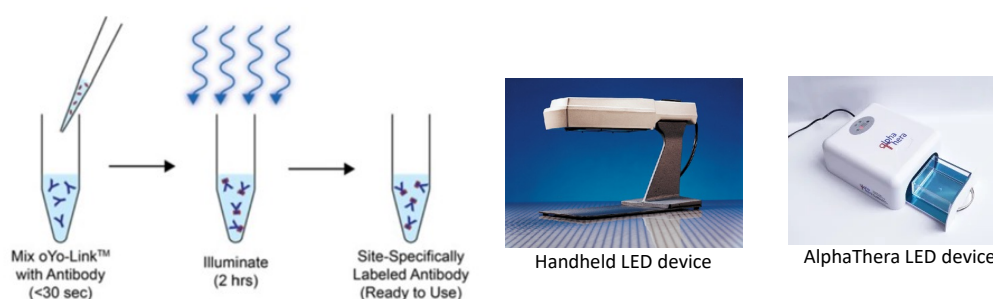


Figure 7. Principle of LACIS method. The graph designed by AlphaThera.

Validation of antibody-oligo conjugation using SDS-PAGE gel

Following the conjugation, the products were run on SDS-PAGE gel to assess the result of the conjugation. For this samples were reduced with 2% β -mercaptoethanol and run on 10% Bis-Tris denaturing gel.

SDS-PAGE gel preparation

To prepare a 10% separation gel (1 mm, 2 gels), we first mixed 4.6 mL of H_2O , 2.6 mL of 1M Tris-HCl (pH 8.8), 3.7 mL of 30% acrylamide, 50 μ L of 20% SDS, 50 μ L of 10% APS, and 10 μ L of TEMED, with TEMED and APS added last to initiate radical polymerization. The gel mixture was then poured between the plates using a serological pipette, filling to about 2 cm below the top of the front plate. The gel was then covered with 80% ethanol to create a smooth surface and left to polymerize, which typically took about 45 minutes. The remaining gel in the falcon tube was used to monitor solidification. For the preparation of a 4% concentration gel (1 mm, 3 gels), we first mixed 3.9 mL of H_2O , 630 μ L of 1M Tris-HCl (pH 8.8), 825 μ L of 30% acrylamide, 25.5 μ L of 20% SDS, 49.5 μ L of 10% APS, and 7.5 μ L of TEMED, adding TEMED and APS last to initiate polymerization. Once the separation gel had polymerized, the ethanol was poured off and any remaining liquid was dried off with a

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cloth or paper towel. The concentration gel mixture was then poured between the glass plates up to the upper edge using a pipette, ensuring no air bubbles formed. The comb was inserted into the gel, which was allowed to polymerize. The remaining gel in the Falcon tube was monitored to check when it had solidified. To load samples onto the gel, we first prepared ice, a protein ladder, dye, and PBS. We mixed 1 μg of conjugated antibody with 3.75 μL of dye and added PBS to achieve a final volume of 15 μL (the volumes of antibody and PBS varied depending on the antibody concentration), followed by a quick centrifugation. The samples were then denatured by heating for 5 minutes at 95°C. Next, the glass plates and gel were placed into the holder. When using only one gel, a fake glass plate was inserted on the opposite side to balance the holder. The gels were placed into the gel chamber in the back slot of the tank. Running buffer was added, filling up to the upper edge between the gels and reaching the corresponding mark (based on whether 2 or 4 gels were used) in the outer chamber. The chamber was placed under a fume hood, and the wells were carefully loaded with samples. The gel was run at 70V for approximately 1 hour, ensuring the ladder separated well. The voltage was then increased to 100V until the protein bands reached the desired separation, compared with the bands in the protein standard. Finally, the gels were carefully removed from the chamber, and the glass plates were separated using the green plastic wedge.

Ethidium bromide and Coomassie blue staining

For Ethidium bromide staining, the gel was first washed twice with water for 5 minutes each. It was then submerged in a solution of 100 mL of water, 2.5 μL of Ethidium bromide and 10 μL of Syber Gold, and placed on a shaker for 25 minutes. After staining, the gel was removed, placed on a plastic bag, and visualized using a UV illuminator. Following ethidium bromide staining, the gel was washed twice with water (each 5 minutes) to eliminate excess stain. For Coomassie blue staining, the gel was stained with 20 mL of SimplyBlue SafeStain for 1 hour, then destained in 100 mL of water for another hour. An additional hour of washing was performed to achieve a clearer gel. The gel was subsequently scanned using a standard scanner.

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3.5.2. Maleimide conjugation method

Antibody conjugation using this method consists of partially reducing disulfide bonds in the IgG antibody with tris [2-carboxyethyl] phosphine (TCEP), incubating with a unique maleimide-modified DNA oligonucleotide and purifying the DNA-conjugated antibody. Implementation of CODEX involves staining a tissue section with a unique DNA-conjugated antibody, adding the corresponding fluorescent oligonucleotide, hybridizing this fluorophore with the conjugated antibody and visualizing it with light microscopy (Figure 8). The details of the conjugation steps provided below. List of oligos used for conjugation of CD45 antibody and detection provided in the table 16.

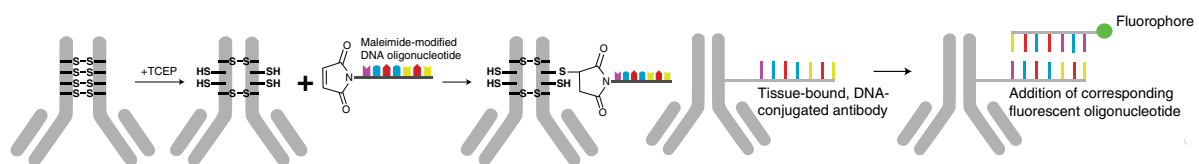


Figure 8. Principle of CODEX method. Modified graph from codex paper [94].

Antibody preparation: Initially, the concentration of monoclonal mouse IgG CD45 antibody was measured using nanodrop, ensuring 100 ug was available for reaction. The ratio of oligo to antibody was adjusted to 2:1. Non-specific absorption was blocked in a 50k MWCO spin column by adding PBS-Tween solution, which was then spun at 12,000g for 2 minutes. After removing the liquid, the antibody was added to the pre-blocked column, spun again at 12,000g for 8 minutes, and the flow-through was discarded.

Antibody reduction: a mixture of 1 mL PBS, 5 μ L of 500 mM tris (2-carboxyethyl) phosphine (TCEP) (pH 7.0), and 5 μ L of 500 mM EDTA (pH 8.0) was prepared. This solution was added to the top of the MWCO filter unit, and the lid was closed before briefly vortexing the tube. The mixture was incubated at RT for 30 minutes, ensuring that no additional incubation time was taken to avoid destabilizing the antibody structure. During this incubation, the oligo aliquot was removed from the freezer and placed in the fridge, while a box for liquid nitrogen was prepared in a -80°C freezer. After the incubation, the solution was spun at 12,000g for 8 minutes, and the flow-through was discarded. Then, 400 μ L of Buffer C (1 ml of 1M Tris (pH 7.0), 1 ml 1 M Tris (pH 7.5), 30 ml of 5 M NaCl, 2 ml 0.5 M EDTA (pH 8.0) and 966 ml MilliQ-H₂O)

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was added to the column, followed by another spin at 12,000g for 8 minutes to remove excess TCEP.

Oligo preparation: the oligo solution was prepared in an inert gas environment by dissolving each oligo in 150 μ L of 1x PBS. This solution was transferred to a 1 mL Eppendorf tube containing 250 μ L of 1X PBS. The entire 400 μ L solution was then added to the top of the column containing the reduced antibody solution and incubated at room temperature for two hours. After incubation, the column was spun at 12,000g for 8 minutes, and the flow-through was discarded. The column was washed three times with 450 μ L of high salt PBS, each time spinning at 12,000g for 8 minutes and discarding the flow-through.

Stabilization of the antibody-oligo: 50 μ L of CODEX antibody stabilizer solution was added, rinsing it over the membrane four times. The column was inverted into a new collection tube, which was capped to prevent evaporation. The solution was spun down at 3,000g for 2 minutes, and the collected solution was stored at 4°C.

Table 16: list of oligos used in maleimide conjugation and validation

Oligos used in maleimide conjugation method	Sequence (5'→ 3')	Length (bp)
Maleimide oligo	5'-Maleimide- AGTGGGATTCTGCTGTCAGTTCGTCGGCAGCGTCAGATGTGTATAAGA GACAGCGCATGCGGGCAGGTTATGCAGGNNNNNNNNNNCTGTCTCTTA TACACATCTCCGAGCCCACGAGACACTGACAGCAGGAATCCCACT-3'	142
Bottom oligo	5'-Cy5-CCTGCATAACCTGCCCGCATGCG-3'	23

3.5.2.1. Validation of antibody-oligo conjugation using FACS

FACS tubes were labeled as negative controls (compensation beads), test conjugates (antibody-DNA conjugated), and positive controls (CD45 antibody-APC). One drop of compensation beads was added to each tube, followed by 200 μ L of 1x PBS. Next, 1 μ L of the antibody conjugate was added to the test conjugate tube, and 1 μ L of the positive control antibody was added to the positive control tube. The tubes were incubated in the dark at room temperature for 10 minutes. After incubation, 3 mL of 1x PBS was added to each tube, and the samples were spun at 500g for 5 minutes. The supernatant was carefully decanted to avoid losing the pellet. Then, 1 μ L of the corresponding bottom oligo (Reverse complementary sequence) was added to the negative control and conjugated test tube (but not to the positive control tube), and the

Methods

tubes were incubated for another 5 minutes in the dark. Another 3 mL of 1x PBS was added, and the tubes were spun again at 500g for 5 minutes. The supernatant was decanted, and the pellets were resuspended in 200 μ L of 1x PBS. The samples were kept at RT until flow cytometry analysis was performed.

3.6. Primary whole genome amplification (WGA)

All single cells and cell pools were amplified using the SCOMP WGA technology (References) which is based on ligation mediated PCR, wherein genomic sequence is deterministically fragmented with MseI endonuclease prior to ligation and amplification steps (Figure 9). Single cells or cell pools were collected in a 1.0 μ L of PBS and added to 2 μ L of the proteinase K digestion buffer (Table 17), which had been previously dispensed into 0.2 mL reaction tube. The digestion was conducted in a PCR cyclor with heated lid at 42°C for 10 hours. Subsequently proteinase K was heat-inactivated at 80°C for 10 min. Next, the sampled DNA was digested with MseI restriction endonuclease by adding 0.2 μ L 10x OPA buffer, 10 U of MseI and water to a total volume of 5 μ L. The restriction digestion was performed for 3 hours at 37°C and thereafter enzyme was heat-inactivated at 65°C for five minutes. Simultaneously, to the MseI digestion, PCR adaptors were prepared for the subsequent ligation mediated PCR. The PCR adaptors consisted of two oligonucleotides, either LIB1 (5'-AGT GGG ATT CCT GCT GTC AGT-3') and ddMse11 (5'-TAA CTG ACA GCdd-3') which were mixed in equimolar concentrations (0.5 μ L of 100 μ M stock solution, each) with 0.5 μ L of 10x OPA Buffer and 1.5 μ L of H₂O. The annealing of the oligonucleotides forming the PCR adaptors occurred during an incubation step, in which the temperature was initially raised to 65°C and then decreased with a constant ramp of 1°C/minute to 15°C. Next, 1 μ L of ATP (10 mM) and 1 μ L T4-DNA-Ligase (5 units) were added to the pre-annealed PCR adaptors, which were then ligated over night at 15°C with the genomic DNA previously processed with MseI. The resulting genomic representation was amplified in a subsequent PCR reaction consisting of reagents outlined in Table 17. For this procedure, the MJ thermocycler was programmed using the settings listed in Table 18.

Methods

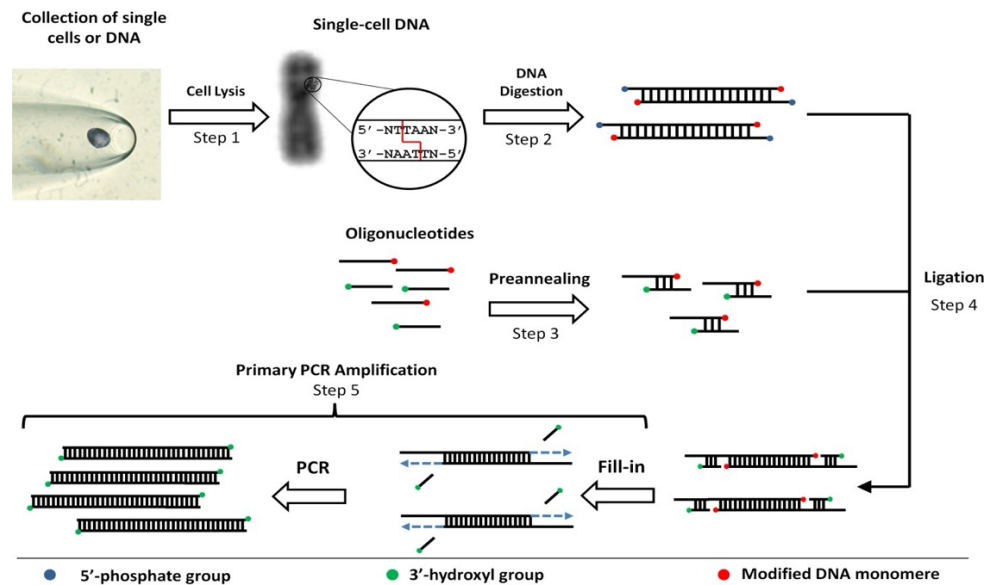


Figure 9. Overview of the processes underlying WGA. SCOMP WGA consists of five consecutive steps: (1) cell lysis, (2) digestion of template, (3) preannealing of PCR adaptors, (4) ligation and (5) PCR-based amplification. Utilization of restriction endonuclease (MseI) at the second step of the procedure assures deterministic and reproducible pattern of DNA fragmentation and priming. Moreover, the use of dedicated design of oligonucleotide comprising PCR adaptors prevents their self-ligation, thereby increasing the efficiency of PCR.

Table 17. Composition of master-mix prepared for WGA.

Master Mix	Constituents	*Volume per sample (μl)	**Volume per sample (μl)
Proteinase-K digestion	OPA 10x	0.2	0.5
	Tween 10 %	0.13	0.13
	Igepal 10 %	0.13	0.13
	Proteinase K (10mg/ml)	0.26	0.26
	PCR-water	1.28	3.48
	***Spike-in oligo	0.1	
MseI digestion	OPA 10x	0.2	--
	MseI 50,000 U/μl	0.2	0.25
	PCR-water	1.6	0.25
Pre-annealing	OPA 10x	0.5	0.5
	Lib1 100 μM	0.5	0.5
	ddMse11 100 μM	0.5	0.5
	PCR-water	1.5	1.5
Primary PCR	Buffer 1	3	3
	dNTPs 10 mM	2	2
	DNA Pol Mix	1	1
	PCR-water	34	34

* Indicates amounts for standard WGA

** Indicates amounts for WGA of cells isolated by LMD

*** Spike-in oligo introduced to the PK digestion step for the sample going through G&P assay

Table 18. WGA Primary PCR program

Step	Temperature	Duration	Cycles
Step 1	68.0 °C	3 min	
Step 2	94.0 °C	40 s	
Step 3	57.0 °C	30 s	
Step 4	68.0 °C	1:30 min	extend 1s/cycle
Step 5			GOTO 2, 14 times

Methods

Step 6	94.0 °C	40 s	
Step 7	57.0 °C	30 s	Increase 1.0°C/cycle
Step 8	68.0 °C	1:45 min	extend 1s/cycle
Step 9			GOTO 6, 8 times
Step 10	94.0 °C	40 s	
Step 11	65.0 °C	30 s	
Step 12	68.0 °C	1:53 min	extend 1s/cycle
Step 13			GOTO 10, 22 times
Step 14	68.0 °C	3:40 min	
Step 15	4.0 °C	infinite	

3.7. WGA quality control (WGA-QC)

Quality of WGA samples were assessed by a multiplex endpoint PCR consisting of primers (KRAS, CK 19, TP53 Exon2/3, D5S2117) to amplify four genomic regions corresponding to different MseI fragments, as previously described (reference) and commercially available as *Ampli*TM QC kit. Primer sequences, amplicon lengths and corresponding MseI fragment length is provided in the table 19. 1 µl of the templates (primary WGA product) were mixed with 1 µl of the 10x FastStart PCR Buffer, 0.2 µl of PCR Grade dNTP Mix (10 mM each), 1 µl of primer mix (4 µM per primer), 0.1 µl FastStart Taq Polymerase (5 U/µl), 0.2 µl BSA (20 mg/ml) and 6.5 µl HPLC Grade H₂O to a final volume of 10 µl (Table 20). As negative control 1 µl of PCR water was used instead of template DNA, and a previously assessed good quality sample was used as a positive control. The PCR was run using the following program: initial denaturation of 4 min at 95°C was followed by 40 cycles of 30 s at 95°C, 30 s at 58°C, 1:30 min at 68°C and a final elongation step for 7 min at 72°C (Table 21). The products of the PCR reaction were visualized by agarose gel electrophoresis. The number of bands displayed on the gel corresponded to the genome integrity index (GII) of the sample. Samples with $GII > 2$ were considered to be of good quality. Since KRAS corresponds to a small MseI fragment, it is usually amplified in the QC PCR if a cell was isolated, irrespective of its DNA quality. Absence of all the bands $GII = 0$, thus means that the starting material might not have contained any cell.

Table 19. QC-Multiplex PCR Primers and amplicon description

Primer	Chromosomal Location	Sequence	MseI fragment length (bp)	Amplicon length (bp)
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Methods

KRAS_91bp_5'	12p	ATAAGGCCTGCTGAAAATGAC	192	91
KRAS_91bp_3'		CTGAATTAGCTGTATCGTCAAG G		
*D5S2117_5'	5q	CCAGGTGAGAACCTAGTCAG	1376	140
D5S2117_3'		ACTGTGTCCTCCAACCATGG		
CK19 5'	6q	GAAGATCCGCGACTGGTAC	1146	621
CK19 3'		TTCATGCTCAGCTGTGACTG		
TP53 Exon2/3 5'	17p	GAAGCGTCTCATGCTGGATC	1374	301
TP53 Exon2/3 3'		CAGCCCAAC CTTGTCCTTA		

*D5S2117 is a microsatellite marker, homozygous samples show one band, heterozygous samples show two bands but with a minor difference in length, 108-166 bp. These two bands would be counted as one band for calculating GII.

Table 20. Master mix composition for QC-Multiplex PCR

Reagents	Volume per sample
10x FastStart PCR Buffer (with 20mM MgCl ₂)	1.0 µl
Primer mix (4 µM per primer)	1.0 µl
dNTPs (10 µM)	0.2 µl
BSA (20 mg/ml)	0.2 µl
FastStart Taq Pol (5 U/µl)	0.1 µl
PCR-Wasser	6.5 µl
Template	1.0 µl

Table 21. QC-Multiplex PCR program

Step	Temperature	Duration	Cycles
Step 1	95.0 °C	4 min	
Step 2	95.0 °C	30 s	
Step 3	58.0 °C	30 s	
Step 4	68.0 °C	1:30 min	
Step 5			GOTO 2, 32 times
Step 6	72.0 °C	7 min	
Step 7	4.0 °C	forever	

3.8. Endpoint PCR for DNA tag detection

Endpoint PCR was conducted to confirm the presence of DNA tag in WGA products. 1 µl of the templates (primary WGA product) were mixed with 1 µl of the 10x FastStart PCR Buffer, 0.2 µl of PCR Grade dNTP Mix (10 mM each), 0.5 µl of of Nextera i7 and 1.5 µl of Nextera i5 oligo (10 µM), 0.1 µl FastStart Taq Pol and 6.7 µl HPLC Grade H₂O to a final volume of 10 µl (Table 23). The PCR was run using the following program: initial denaturation of 4 min at 98°C was followed by 40 cycles of 30 s at 95°C, 30 s at 65°C, 30 s at 72°C and a final elongation step for 5 min at 72°C (Table 24). Positive and negative control was included in every PCR run. Primers sequences and amplicon length provided in Table 22.

Methods

Table 22. Endpoint PCR primers used to assess the presence of tag oligo in the WGA products.

Primer	Sequence (5' → 3')	Length (bp)	Amplicon length (bp)
Index #1	CAAGCAGAAGACGGCATAACGAGATCGCCTTAGTCTCGTGGGCTCGG	46	165
Index #2	AATGATACGGCGACCAACCGAGATCTACACTAGATCGCTCGTCGGCAGCGTC	51	

Table 23. Master mix composition for Endpoint PCR

Reagents	Volume per sample
10x FastStart PCR Buffer	1.0 µl
dNTPs (10 µM)	0.2 µl
Index #1 primer (10 µM)	0.5 µl
Index #2 primer (10 µM)	0.5 µl
FastStart Taq Pol (5 U/µl)	0.1 µl
PCR-Wasser	6.7 µl
Template	1.0 µl

Table 24. Endpoint PCR program

Step	Temperature	Duration	Cycles
Step 1	98.0 °C	4 min	GOTO 2, 40 times
Step 2	95.0 °C	10 s	
Step 3	65.0 °C	30 s	
Step 4	72.0 °C	30 s	
Step 5			
Step 6	72.0 °C	5 min	1
Step 7	4.0 °C	forever	-

3.9. Agarose gel electrophoresis

PCR products from WGA Multiplex QC PCR and Endpoint PCR were visualized with gel electrophoresis, on 1.5% agarose and 2% agarose respectively. The agarose gel was prepared in 1X TBE buffer with ethidium-bromide (0,5 µg/ml). PCR product (10 µl) was mixed with loading dye (3 µl), loaded into the wells of the gel and were allowed to run for 45 min at 160V. Finally, the gel was imaged on a UV transilluminator.

3.10. Purification of primary WGA product utilizing Agencourt AMPureXP beads

The purification of primary WGA products was done utilizing Agencourt AMPure XP beads, calculated as 1.8 times the reaction volume of the unpurified sample. The

Methods

beads were incubated at RT for at least 30 minutes and thoroughly resuspended by vortexing. Freshly prepared 80% ethanol was equilibrated to RT, as all steps needed to be performed at this temperature. For the purification protocol, 20 μ L of the unpurified sample was aliquoted into a 200 μ L PCR tube. Subsequently, 36 μ L of thoroughly resuspended AMPure XP beads was added, and the mixture was vortexed for at least 5 seconds. The samples were then incubated at RT for 5 minutes to allow the DNA to bind to the beads. After incubation, the samples were placed in a magnetic particle collector to capture the beads, with an incubation time of at least 2 minutes or until the liquid became clear. The supernatant was carefully removed and discarded. While keeping the samples on the magnetic particle collector, 200 μ L of freshly prepared 80% ethanol was added, followed by a room temperature incubation for at least 30 seconds. The ethanol was then removed, and this step was repeated with another 200 μ L of 80% ethanol, again incubating at room temperature for 30 seconds before discarding the ethanol, ensuring all residual ethanol was removed without disturbing the beads. The beads were allowed to dry at RT until all ethanol evaporated. Afterward, the samples were removed from the magnetic particle collector, and the beads were thoroughly resuspended in 22 μ L of elution buffer (10 mM Tris-HCl, pH 8.0, or Qiagen EB buffer, or 1x TE low EDTA buffer). The samples were incubated at RT for 2 minutes to allow the DNA to elute from the beads. Once again, the samples were placed on the magnetic particle collector for 2 minutes to capture the beads, and the 20 μ L supernatant was transferred to a fresh tube for downstream analyses or stored at -20°C.

3.11. Measurements of DNA concentration

Nucleic acid concentration of genomic DNA from cells and DNA tag was determined using Nanodrop 2000c spectrophotometer. For this, 1 μ L of the DNA sample was taken. Before measuring the samples, the device was calibrated by using 1 μ L of EB as blank. Pure DNA was determined by values of 1.8-2.0 for A260/280 and A260/230 larger than 2.0. Following the Nanodrop measurements, all samples were diluted to a concentration of 20 ng/ μ L for downstream qPCR quantification.

Methods

3.12. Normalizing the qPCR readout using exogenous Tag DNA spike-ins

3.12.1. Spike-in oligo preparation

The spike-in oligo used as a reference allowing us to normalize qPCR readout and compensate for bias that we might have introduced normalizing the qPCR with uniform quantities of WGA product (amplified single-cell DNA mixed with amplified tag DNA). To prepare a 100 μM stock solution, oligo resuspend by adding 63 μl of H_2O according to manufacture protocol. The solution further diluted by mixing 2 μl of the oligo stock with 48 μl of H_2O , resulting in a working solution. Next, the concentration of the oligo solution measured twice with nanodrop and the average concentration calculated which was determined to be 159.8 $\mu\text{g}/\mu\text{l}$. Given the concentration of 159.8 $\mu\text{g}/\mu\text{l}$ and the length of the oligo (138 bp of single-stranded DNA), DNA copy number per μl calculated using online copy number calculator. The resulting copy number was 2.113×10^{12} copies/ μl . To prepare the stock solution targeting a final concentration of 10^{12} copies/ μl , 1 μl of the concentrated DNA solution mixed with 1.113 μl of H_2O . Alternatively, a larger volume can be prepared by mixing 20 μl of DNA with 22.26 μl of H_2O . A serial dilution ranges from 10^{12} to 10^3 copies/ μl was prepared for further qPCR evaluation (Figure 10).

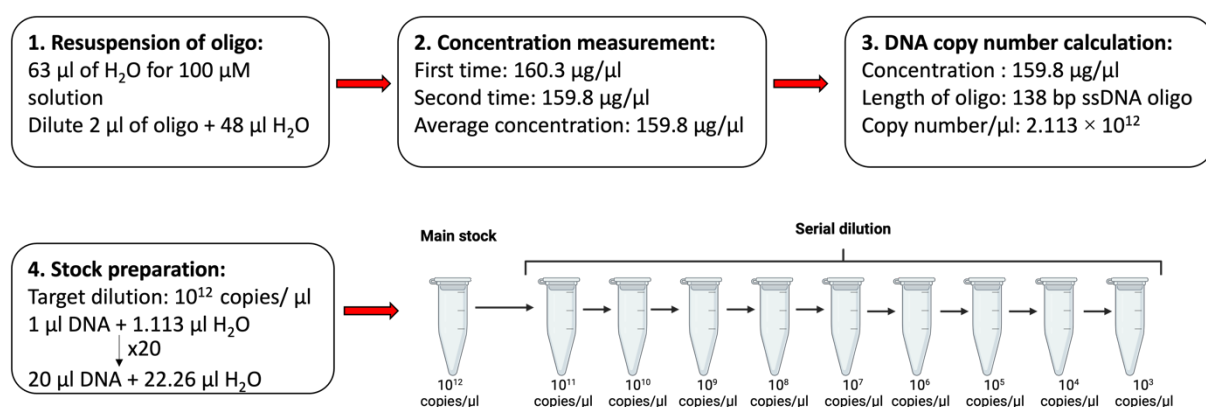


Figure 10. Spike-in oligo preparation. Resuspension and dilution of a spike-in oligo were performed by preparing a 100 μM stock solution and diluting it to a 2 μM working solution. The concentration was measured using a Nanodrop, and the DNA copy number was calculated based on the determined concentration. A main stock solution with 10^{12} copies/ μl was prepared and further serial dilutions were made for experimental use.

Methods

3.12.2. Optimization of DNA tag spike-in for accurate qPCR analysis

The primary goal of this experiment was to determine the optimal concentration of spike-in oligo that results in consistent and reliable Cp values during qPCR. To achieve this, quantification of spike-in oligo was done alongside single cell genome providing a measure of the efficiency and consistency of qPCR reactions across different samples. Spike-in oligo prepared in 10 different concentrations, ranging from 10^{12} to 10^3 copies/ μ l added to the Proteinase K (PK) solution of WGA (Table 16). For each concentration group of the spike-in oligos, three single PBMCs were isolated by micromanipulation and subjected to WGA. Subsequently, a dedicated endpoint PCR was done to confirm the presence of spike-in oligo in WGA sample and qPCR was conducted to quantify the amount of amplified spike-in oligos.

3.13. Quantitative Real-Time PCR

3.13.1. Standard qPCR

Quantitative PCR (qPCR) was performed using a LightCycler 480 instrument (Roche). Each qPCR comprised 1 μ l of the template DNA, 5 μ l of 2x probe mastermix (Roche), 1 μ l of Nextera i7 and 1 μ l of Nextera i5 oligo, 0.2 μ l TaqMan probe and 0.8 μ l PCR HPLC Gradient Grade H₂O (Table 25). The quantitative PCR was run using the following program: 1 cycle for 4 min at 98°C (temp. ramp of 4.4°C/s), 40 cycles for 10 s at 95°C (ramp 4.4°C/s), 30 s at 65°C (ramp 2.2°C/s) and 30 s at 72°C (ramp 4.4°C/s) and a final elongation step for 5 min at 72°C (Table 24). The crossing point (Cp) values were determined with the LightCycler 480 software using the second derivative maximum method applying the high sensitivity algorithm. All single-cell WGA products were analyzed in technical triplicates. Cp-values were averaged across the technical replicates before further data processing.

Table 25. Master mix composition for qPCR quantification of DNA tag (Standard protocol)

Reagents	Volume per sample
2x Probe MasterMix	10.0 μ l
Nextera i7 Oligo (10 μ M)	1.0 μ l
Nextera i5 Oligo (10 μ M)	1.0 μ l
TaqMan spike-in oligo (10 μ M)	0,2 μ l
PCR-Wasser	6,8 μ l
Template (20 ng/rnx)	1 μ l

Methods

3.13.2. qPCR reagent master mix optimization

The qPCR readout optimization initially was performed by testing 2x concentration of primers, 2x concentration of TaqMan, and addition of BSA individually (Optimization #1, table 26) as well as combination of 2x TaqMan probes with increasing primer concentrations (2x, 3x, and 4x) in both single and double readout (inclusion of both TaqMan probes, optimization #2, table 27 and 28). Mastermix reagents were adjusted according to the specific protocol outlined in the accompanying tables.

Table 26. qPCR optimization #1

Reagents	2x TaqMan probe 1x [µl]	2x Primer 1x [µl]	addition of BSA 1x [µl]
2x Probe MasterMix	10.0 µl	10.0 µl	10.0 µl
Nextera i7 Oligo (10 µM)	1.0 µl	2.0 µl	1.0 µl
Nextera i5 Oligo (10 µM)	1.0 µl	2.0 µl	1.0 µl
*TaqMan probe (10 µM)	0,4 µl	0,2 µl	0,2 µl
BSA	-	-	0,2 µl
PCR-Wasser	6,6 µl	4,8 µl	6,8 µl
Template (20 ng/rnx)	1 µl	1 µl	1 µl

*The qPCR optimization was done for 2 TaqMan probes including spike-in oligo and HER2 DNA oligo

Table 27. qPCR optimization #2 (Single readout)

Reagents	2x primer/2x TaqMan 1x [µl]	3x primer/2x TaqMan 1x [µl]	4x primer/2x TaqMan 1x [µl]
2x Probe MasterMix	10.0 µl	10.0 µl	10.0 µl
Nextera i7 Oligo (10 µM)	2.0 µl	3.0 µl	4.0 µl
Nextera i5 Oligo (10 µM)	2.0 µl	3.0 µl	4.0 µl
*TaqMan probe (10 µM)	0,4 µl	0,4 µl	0,4 µl
PCR-Wasser	4,6 µl	2,6 µl	0,6 µl
Template (20 ng/rnx)	1 µl	1 µl	1 µl

*The qPCR optimization was done for 2 TaqMan probes including spike-in oligo and HER2 DNA oligo

Table 28. qPCR optimization #2 (Double readout)

Reagents	2x primer/2x TaqMan 1x [µl]	3x primer/2x TaqMan 1x [µl]	4x primer/2x TaqMan 1x [µl]
2x Probe MasterMix	10.0 µl	10.0 µl	10.0 µl
Nextera i7 Oligo (10 µM)	2.0 µl	3.0 µl	4.0 µl
Nextera i5 Oligo (10 µM)	2.0 µl	3.0 µl	4.0 µl
TaqMan probe, spike-in (10 µM)	0,4 µl	0,4 µl	0,4 µl
TaqMan probe, HER2 (10 µM)	0,4 µl	0,4 µl	0,4 µl

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PCR-Wasser	4.2 µl	2.2 µl	0.2 µl
Template (20 ng/rnx)	1 µl	1 µl	1 µl

3.13.3. Relative quantification

The relative qPCR quantification analysis was performed using the spike-in oligo as a reference to calculate the fold change in protein expression in cell lines or DCC, normalized to the spike-in oligo relative to the control (PBMCs or non-tumor cells). For this, the Livak method, also known as the $\Delta\Delta C_p$ method [95], was applied. The analysis involved three steps: first, the C_p of the target gene was normalized to the C_p of the reference gene; second, the ΔC_p of the test sample was normalized to the ΔC_p of the calibrator; and third, the expression ratio or fold difference was calculated.

- 1- Normalize C_p (target gene) to C_p (reference gene)
- 2- Normalize ΔC_p of test sample to ΔC_p of calibrator
- 3- Calculate expression ratio, or fold difference

3.14. Ampli1 LowPass CNV Analysis

Genome wide copy number variations (CNV) sequencing was performed using *Ampli1*TM LowPass Kit according to the manufacturer's instructions (Menarini Silicon Biosystems). Briefly, 5 µl of *Ampli1*TM WGA products was diluted in 5 µl NGS water and purified with 1.8X AMPure XP beads (Beckman Coulter). The *Ampli1*TM LowPass workflow started from 3µl of purified primary *Ampli1*TM WGA products and included three main steps: first extension (FE) reaction with *Ampli1*TM D7xx index adapter, second extension (SE) reaction with *Ampli1*TM D5xx index adapter and final library amplification (LA). Obtained libraries were quantified using the Bioanalyzer 2100 HS DNA kit (Agilent) and Qubit HS DNA kit (ThermoFisher Scientific) according to manufacturer's instructions followed by pooling the libraries in equimolar amounts (4nM). Sequencing was performed in single-end mode using Illumina Miseq or Novaseq 6000 according to the manufacturer's instructions. A maximum of 32 samples were pooled in one Miseq run and 96 samples were pooled in one Novaseq 6000 run. Equimolar pools were sequenced in 150 bp single end mode using a MiSeq device at Fraunhofer ITEM-R on HiSeq2500 or NovaSeq6000 platforms using a custom read primer provided by *Ampli1*TM LowPass kit. To obtain CNV profile HIENA pipeline has been used. Briefly, for CNV profile analysis the human genome was

Methods

divided into non-overlapping bins, each with a size of 500 kb. After removing adapter sequences and poor-quality bases (BBduk 38.84) as well as decontamination of non-human reads originating from microbial and/or fungal fresh water and/or reagents (BioBloom Tools 2.0.13), remaining reads aligned with bwa mem 0.7.17 to the reference genome hg19 were counted per bin, corrected, filtered, normalized and segmented with the bioconductor package QDNAseq 1.34.0. The same package was used to create the log₂ (ratio) CNV-profiles. The minimum amounts of reads mapped to the reference genome and the measured standard deviation (σ_{Δ}) are important thresholds applied in the analysis pipeline. Samples with <100.000 mapped reads were rejected from CNV-analysis. Also, profiles with a measured standard deviation $\sigma_{\Delta} > 0.5$ were always omitted.

3.15. Drug treatment experiment

3.15.1. Optimization of seeding density

BT474 and MDA-MB-468 cell lines were grown to a confluency of 80-90% in T-75 flasks. Once confluency was reached, the cells were trypsinized and counted (See 3.2.1). A serial dilution was performed to obtain final cell concentrations of 14,000, 16,000, 18,000, 20,000, and 22,000 cells per well. This dilution series was prepared in a sterile container and mixed well before loading the wells. Next, 150 μ L of culture medium was added to each well of a 96-well plate. Using a multichannel pipette, 50 μ L of the prepared cell suspensions from each dilution were added to the respective wells in triplicate. The plates were then carefully placed in the Incucyte Live-Cell Imaging System and incubated for 7 days. The Incucyte system continuously monitored and recorded cell proliferation and confluency in real-time throughout the incubation period.

3.15.2. Optimization of drug concentration

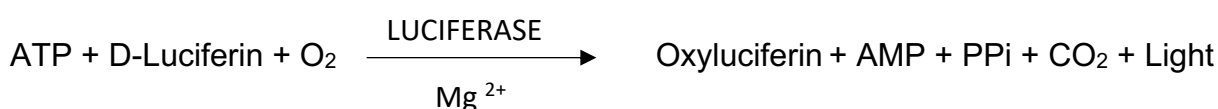
To optimize drug concentration, cells were grown to a confluency of 80-90% in T-75 flasks. Once confluency was reached, the cells were trypsinized and counted (See 3.2.1). A cell suspension was prepared with a seeding density of 25,000 cells per well for the BT474 cell line and 22,000 cells per well for the MDA-MB-468 cell line and seeded into a 96-well plate. After 24 hours of incubation, five different concentrations

Methods

of lapatinib (0.001, 0.01, 0.1, 1, and 10 μ M) were added to the wells using a digital dispenser. Each concentration was tested in five replicates and incubated for 48 hours for both cell lines to assess the drug's effect on cell proliferation and viability. In addition, control wells were included in the experiment using dimethyl sulfoxide (DMSO) as a vehicle control.

3.15.3. Viability measurements using ATPlite assay

Following 48 hours of drug treatment, cell viability was measured using the ATPlite assay. To begin, 100 μ L of the medium was carefully removed from each well. Next, 100 μ L of ATPlite 1step reagent was added to the wells and the plate was shaken for 2 minutes at 700 rpm using an IKA® MTS4 plate shaker. The resulting luminescence was monitored at 5 minutes intervals in a temperature controlled (22 °C) TopCount® NXT and expressed as counts per second (CPS). The ATPlite assay system is based on the production of light caused by the reaction of ATP with added luciferase and d-luciferin. This is illustrated in the following:



The emitted light is proportional to the ATP concentration within certain limits. To calculate normalized cell viability as a percentage, the following formula was used:

$$\text{Cell viability (\%)} = \left(\frac{\text{CPS measured in each well (treated with lapatinib)}}{\text{Median CPS measured (untreated, DMSO)}} \right) \times 100$$

3.16. Statistical analysis

Statistical analysis was performed with GraphPad Prism Software, version 10, using unpaired t-test, one-way ANOVA with multiple comparisons or Pearson correlation. Asterisks represent the significance as follows: ***P < .001, **P < .01, *P < .05 and ns: P $\geq$.05.

Results

4. Results

In this thesis, we established and optimized a method for isolating bone marrow-derived DCCs from archived and densely loaded cytopins. The optimized protocol incorporated IF staining for tumor cell identification, combined with AP-polymer staining to counteract laser-induced damage, followed by laser-assisted ablation of surrounding cells, manual micromanipulation of the target cells, whole genome amplification, quality control, and low-coverage sequencing to analyze CNVs (Figure 11A). Additionally, we developed the G&P workflow which utilizes antibodies conjugated with DNA oligonucleotides designed to enable their amplification alongside the single-cell genome facilitating parallel genome and proteome profiling. The amplified genome enables a comprehensive genomic analysis, allowing for applications such as genome-wide CNV profiling or lineage tree analysis. Concurrently, the amplified DNA tags attached to antibodies provide a quantitative readout of protein expression, which can be measured using qPCR or sequencing (Figure 11B). The optimization, validation, and application of these methods are detailed in section 4.1. and 4.2. respectively.

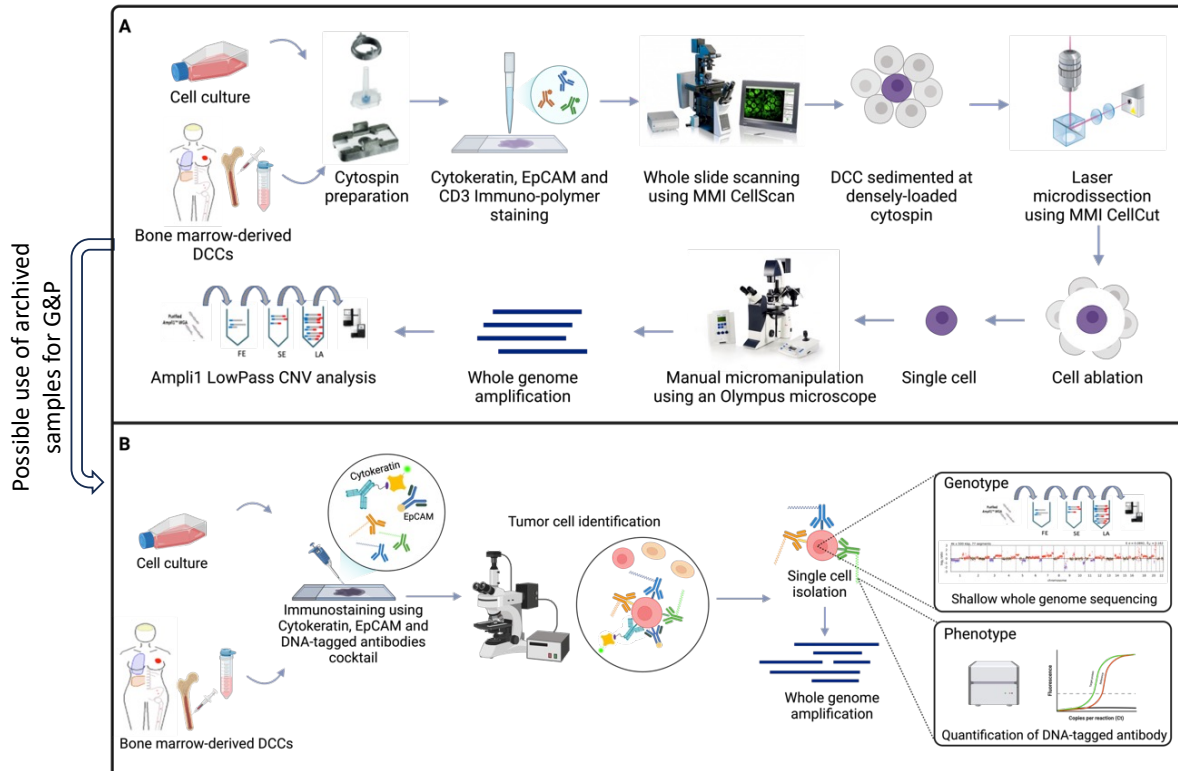


Figure 11. Workflows for DCCs Isolation and genomic-proteomic profiling. The graph generated using BioRender.

Results

4.1. Establishment of DCC isolation from archived cells using MMI CellCut Plus system

So far, studies involving single-cell DNA analysis of DCCs in our laboratory used standard micromanipulation to isolate single cells from adhesion slides. These slides contained cells sedimented at low cell densities, providing enough space for contamination-free isolation. However, this method was impractical for isolating single cells from cytopins due to the higher cell density and strong adherence to the slide. To address this, Dr. Manjusha Ghosh explored seven different protocols in her PhD thesis [96]. Ultimately, she developed and selected Laser Cutting Assisted Standard Micromanipulation (LCAM) as the most effective method. LCAM combines laser cutting with micromanipulation, where the laser ablates surrounding cells, creating enough space to manually isolate the target cell. In her project, LMD was performed using the Zeiss PALM Microbeam system, a well-established platform for single-cell isolation through laser-assisted ablation. While effective, the Zeiss PALM system has certain limitations in terms of precision and the fine-tuning of dissection parameters. However, this project aimed to enhance the LMD process by employing the MMI CellCut Plus system (Molecular Machines & Industries), which represents a significant advancement in LMD technology.

4.1.1. Laser microdissection (LMD) with MMI CellCut Plus system

To set up laser microdissection for isolating DCCs from cytopins using the MMI instrument, adjusting the laser parameters was essential for precise cutting, minimizing heat damage, and ensuring good DNA quality for downstream applications. To optimize the laser parameters for isolating single cell from cytopins, breast cancer cell line MCF7 and PBMCs combined onto cytopins. Pan cytokeratin A45-B/B45 staining was performed for the detection of MCF7 cell line (Figure 12B). The slides were screened using the MMI CellScan (See 3.4.2.2), where detected cells were marked and microdissected using the MMI cellCut. A range of laser power, speed, and frequency settings was tested to determine the ideal parameters for cell isolation (Figure 12A). The goal was to use the minimum laser energy necessary to achieve precise cuts while minimizing potential damage to the DNA. It was found that a laser power of 3.5%, combined with a speed of 30 $\mu\text{m}/\text{sec}$ and a frequency of 100 Hz, was sufficient for accurate cutting around the target cells. While no significant

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differences were observed between the various frequencies tested, a notable difference was found between laser power of 3.5% and 5.5% (Figure 12B). The higher laser intensity of 5.5% produced a wider and sharper cut compared to 3.5%. Due to this observed variation, MCF7 cells were subsequently isolated using both 3.5% and 5.5% laser intensities, keeping the laser speed at 30 $\mu\text{m}/\text{sec}$ and frequency at 100 Hz in both cases (Figure 12C). For the ablation step, which was performed at a distance from the target cell to reduce the risk of damage, a laser intensity of 15%, a speed of 60 $\mu\text{m}/\text{sec}$, and a frequency of 200 Hz were applied. The isolated cell was subjected to WGA to assess genome integrity. The Genome Integrity Index (GII), evaluated via WGA-QC multiplex PCR (see 3.7.) was used to predict DNA quality. A GII of 0, indicating no visible bands in the QC-PCR, suggests that the cell was not successfully collected or the DNA could not be amplified. In contrast, a GII greater than 2 defines a sample with sufficient DNA quality for further analysis [79]. The GII for all isolated cells was 4, confirming high and consistent DNA quality across both the 3.5% and 5.5% laser intensities (Figure 12D).

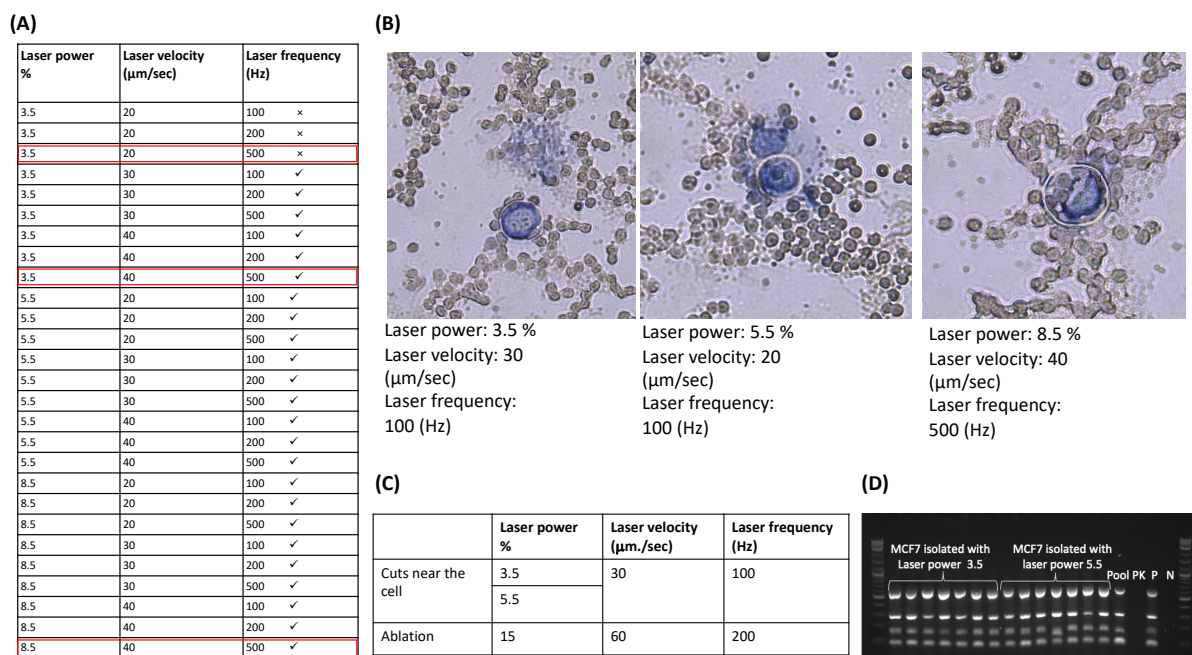


Figure 12. Laser parameters adjustment. (A) The table summarizes the laser parameters and various combinations utilized in the experimental setup, highlighting three conditions classified as minimum, medium, and high laser parameters. (B) Representative images corresponding to the selected laser conditions. (C) Two specific laser power settings (3.5% and 5.5%) were chosen for isolating cells, and their effects on genome quality were evaluated. (D) Results of WGA-QC PCR, demonstrate the quality of MCF7 single cell DNA subjected to laser treatments at 3.5% and 5.5% power. The "Pool" indicates a pooled sample of cells, "PK" refers to a control containing only the PK solution without cells, while "P"

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and “N” denote positive and negative controls for quality control, respectively. All other samples are single cells.

4.1.2. CNV analysis of cells subjected to LMD

To further evaluate the CNV analysis of the cells subjected to LMD, MCF7 spike in PBMCs adhesion slide was used as a control sample. This slide was stained with pan cytokeratin A45-B/B3 and CD3, developed using BCIP/NBT for detection of MCF7 and T cell, respectively. A total of 32 samples were isolated, consisting of 24 T cells and 8 MCF7 cells, using laser powers of 3.5% and 5.5% (Figure 13A). The isolated cells were assessed for quality using WGA-QC before being subjected to Ampli1™ LowPass sequencing. All 32 isolated cells, including both MCF7 and T cells, displayed GII of 3-4, indicating good DNA quality (Figure 13B).

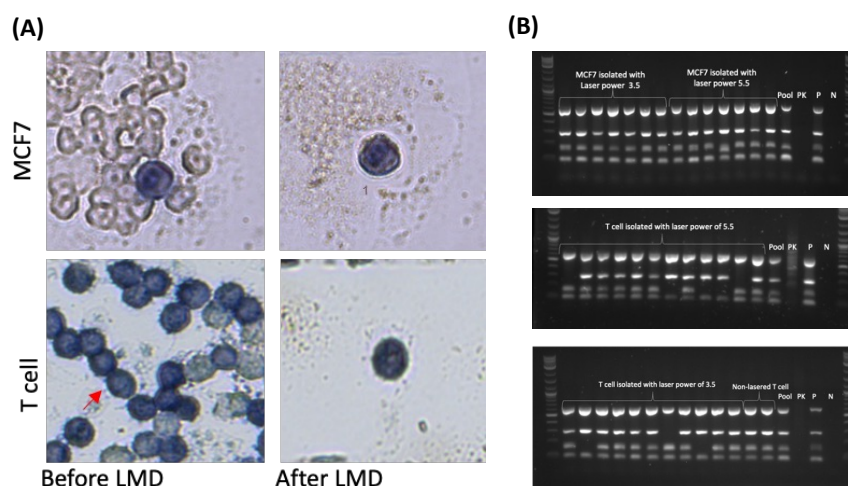


Figure 13. WGA-QC of MCF7 and T cell isolated using different laser setup. (A): Test cytopspins prepared of MCF7 cell line spiked in blood PBMCs, stained against CK and CD3 for MCF7 and T cell detection, screened and lasered using MMI. (B): WGA-QC results of MCF7 and T cells isolated with different laser setup.

Next, all 32 samples were subjected to *Ampli1* lowpass CNV analysis which revealed an aberrant profile for the MCF7 cells, as expected and balanced profile for T cells (Figure 14A). This suggests that if normal cells isolated using this protocol, display normal profile, the aberrations detected in tumor are likely true and accurately reflect the tumor's genetic alterations. Additionally, the derivative log ratio spread (DLRS), which measures hybridization noise, was compared between the two laser setups (3.5% vs. 5.5% laser power). The differences observed between the individual LMD protocol variants were marginal and not statistically significant (Figure 14B). To minimize the risk of laser-induced damage to the cells, a laser power of 3.5% was selected for further experiments, as this setting has been shown to reliably preserve

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DNA integrity while allowing precise cell isolation. In conclusion, the LMD protocol using MMI microscopy was effective for isolating single cells with sufficient quality for genome-wide CNV profiling.

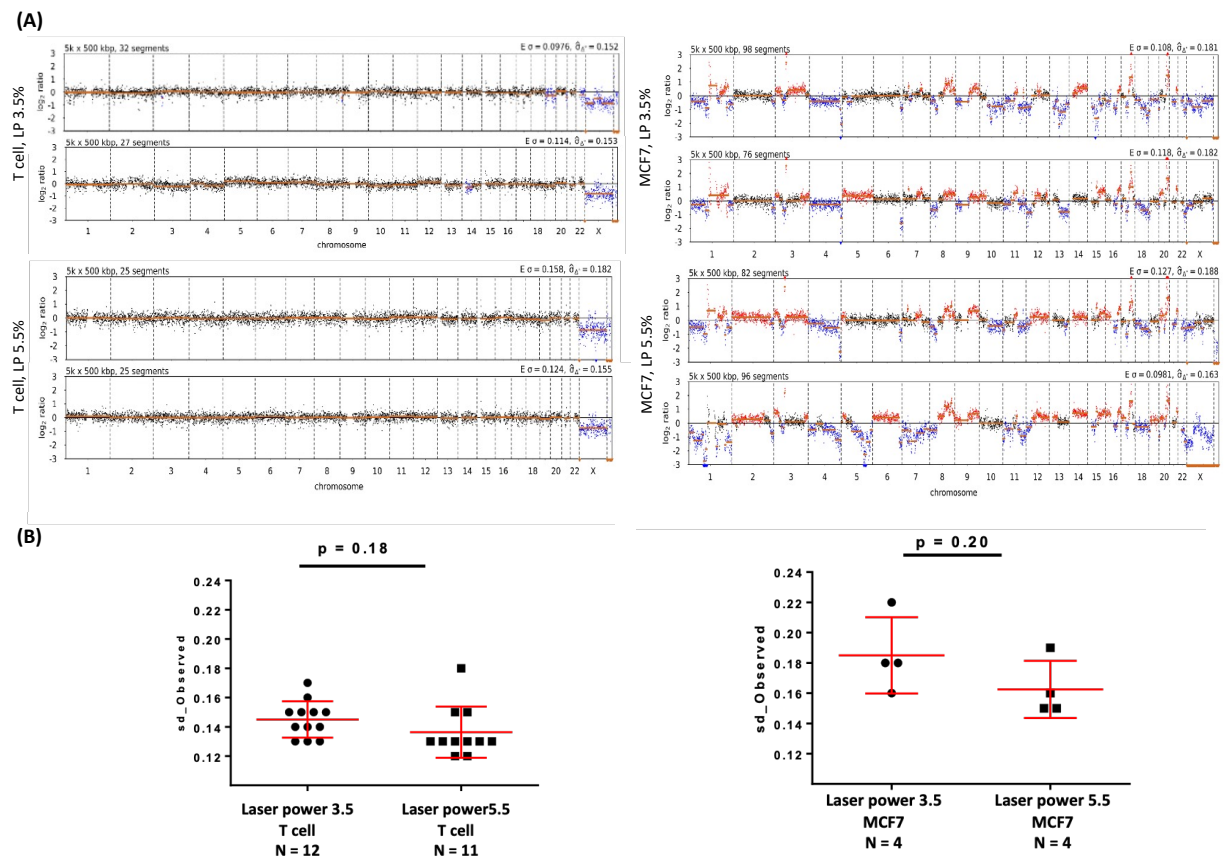


Figure 14. CNV analysis of cells subjected to LMD. (A): *Ampli1* Lowpass CNV analysis of T cells (left) and MCF 7 (right) isolated by different laser set up (2 CNV profiles of each are represented). (B): DLRS values compared between different laser setup, which lower values allow higher resolution analysis of CNV.

4.1.3. Establishment of IF staining in LMD protocol

In previous studies involving LMD in our lab, AP-Polymer staining was used to protect DNA from laser-induced damage during cell isolation, as the dark precipitate absorbs UV laser energy and shields the DNA from degradation. In the next phase, we aimed to improve tumor cell identification specificity by incorporating IF staining, ultimately developing a combined IF and AP-Polymer staining protocol that enabled high-quality DNA extraction from archived cytopins. This optimization was performed on MCF7 spike into PBMCs cytopins. The optimized protocol involved an initial IF staining step against CK and EpCAM for MCF7, and CD3 for T cells detection. Following staining, the slide was scanned and analyzed using the MMI microscope, during which the geometry and precise coordinates of the identified cells were recorded and exported.

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Then, the slide incubated overnight with primary antibodies and day after subjected to AP-Polymer staining. Following the staining procedures, the geometry and coordinates of the target cells were re-imported into the MMI system, enabling precise navigation for LMD. This relocating advantage of the MMI allows for combination of IF and AP-polymer staining. The target cells were then carefully isolated using LMD and subsequently isolated through manual micromanipulation under an Olympus microscope. Isolated cells subjected to WGA and the quality assessed. WGA-QC revealed good DNA quality in all tested WGA products (GII of 3-4), (Figure 15). Therefore, we concluded that good quality DNA can be obtained from laser-microdissected cells by combining 2 staining protocols (IF and AP-polymer staining). The finalized Immuno-polymer staining not only enhanced the identification of tumor cells through immunostaining but also maintained the structural integrity of the genomic DNA.

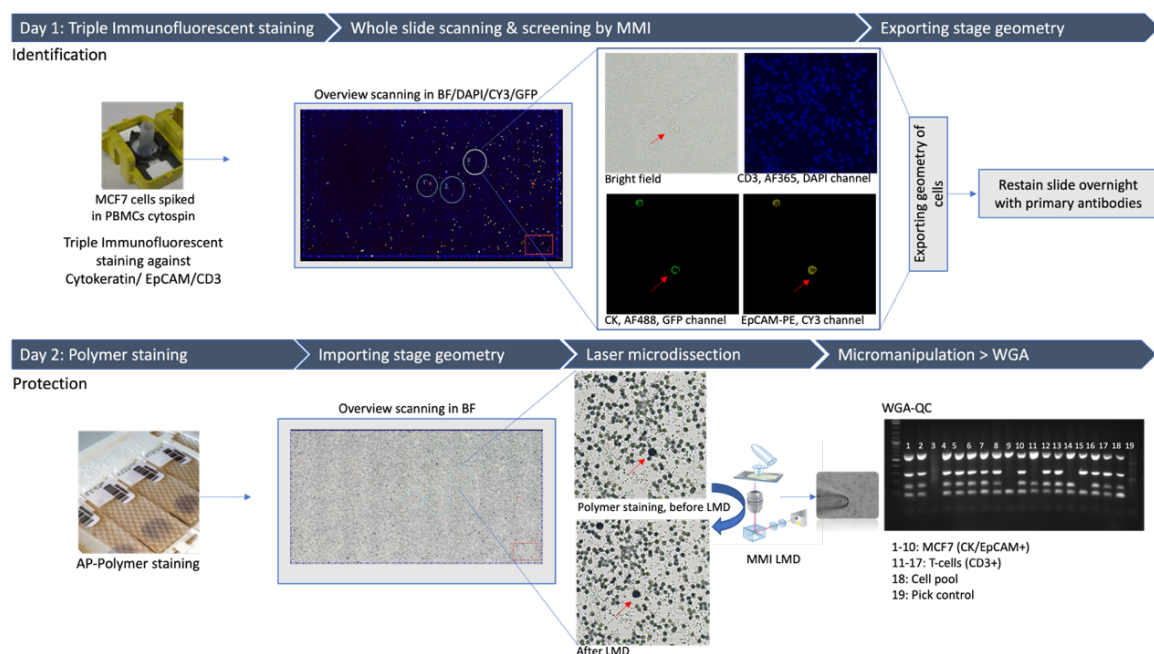
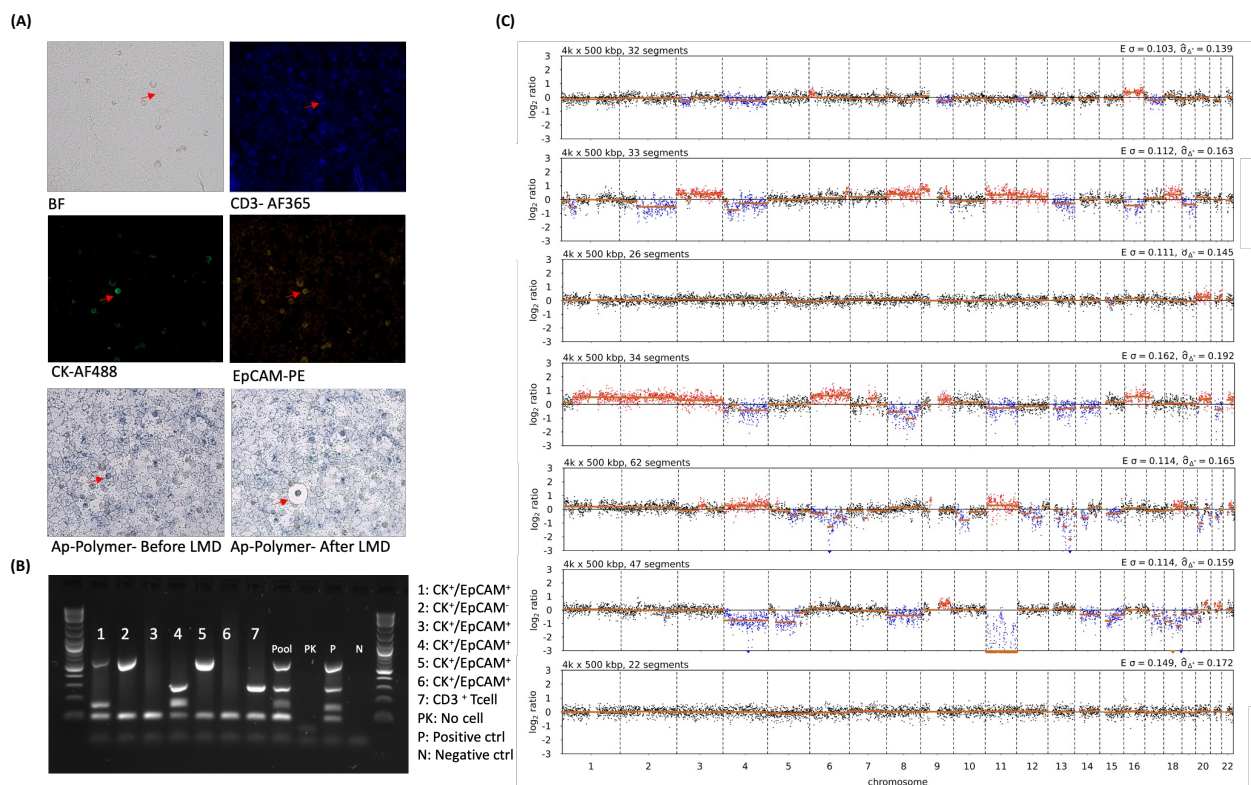


Figure 15. Schematic representation of the comprehensive workflow for immune-polymer staining. Day 1, involved triple IF staining, including CK, EpCAM, and CD3. The entire slide was then scanned and screened using MMI microscopy. The geometry of the scanned areas containing target cells was exported for later reference, and the slide was retained overnight with primary antibodies for optimal staining. Day 2, consisted of polymer staining, followed by the importation of the previously exported stage geometry. LMD was performed to isolate the target cells, which were then subjected to micromanipulation and WGA for further analysis. The final panel displayed a WGA-QC gel image, demonstrating good genome quality with the optimized staining protocol.

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4.1.4. Application of optimized LMD protocol in DCIS patients

To acquire bone-marrow (BM) DCCs we collaborated with the Department of Gynecology, University of Tübingen. Using the established protocol (See 4.1.3), we screened bone marrow derived archived cytopspins from four patients with DCIS. Cytopspins stained against CK and EpCAM for DCC detection and CD3 for T cells detection (Figure 16A). Among the patients analyzed, one was negative for DCCs, while two exhibited low genomic quality. However, from one patient, we successfully isolated a subset of cells, including single CK⁺, single EpCAM⁺, and double CK⁺/EpCAM⁺ cells. These isolated cells were subjected to WGA and CNV analysis. WGA-QC indicated a GII of 2-4 for most cells, except for two, which exhibited lower genomic quality (Figure 16B). Despite this, all isolated cells were further analyzed through CNV profiling. The CNV analysis revealed a balanced profile for the T cell, while the DCCs demonstrated aberrant CNV profiles, indicative of various level of genomic alterations. The patterns suggest the presence of genomic instability, particularly in certain regions, such as chromosomes 4, 8, 11, and 13, where alterations (both gains and losses) are more pronounced in multiple samples (Figure 16C).



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Figure 16. Application of optimized LMD protocol in DCIS patient. (A): Bone marrow of DCIS patient stained against CK, EpCAM and CD3 alongside AP-polymer staining, both before and after the application of LMD. (B) The resulting WGA-QC of the isolated cells. (C): *Ampli1* Lowpass CNV analysis of cells showing genomic profile. The order of the CNV profiles corresponds to the number of cells depicted in the WGA-QC gel image.

4.2. A method for the combined genomic and proteomic (G&P) analysis of DCCs

So far, molecular analysis of the DCCs involved profiling of CNVs using the previously established protocol [97]. In addition, we aimed to further characterize DCCs by profiling selected proteins of interest. For this purpose, we developed a workflow enabling the parallel analysis of the genome and proteome of single cells.

4.2.1. Principle of G&P workflow

In this workflow the basis for measuring protein expression in single cells relies on Tag DNA oligonucleotides conjugated to monoclonal antibodies, which are custom-designed to be compatible with the *Ampli1* whole-genome amplification (WGA) approach. The workflow initiates with IF staining to label target proteins in individual cells with DNA-tagged antibodies, followed by single cell isolation by manual micromanipulation. Isolated single cells are then subjected to WGA. During *Ampli1* WGA both the single-cell genome and the DNA tags attached to antibodies will be amplified, enabling simultaneous genotypic and phenotypic profiling of individual cells. The amplified genome enables a comprehensive genomic analysis, allowing for applications such as genome-wide CNV profiling or lineage tree analysis. Concurrently, the amplified DNA tags attached to antibodies provide a quantitative readout of protein expression, which can be measured using quantitative PCR (qPCR) or sequencing (Figure 17). To integrate DNA-conjugated antibodies into G&P assay, a systematic approach applied for individual markers as described below.

I: Cell Line collection: Cell lines exhibiting known differential expression levels of each target protein were collected.

II: Establishment of staining protocols with unconjugated antibodies: Optimized staining protocols were developed for each protein, employing either IF staining or flow cytometry, depending on the protein's specific requirements.

III: Antibody-DNA conjugation: Monoclonal antibodies (mAbs) were conjugated to DNA oligonucleotides, followed by validation to confirm conjugation efficiency.

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IV: G&P staining and protein quantification: Finally, we conducted G&P staining with DNA-tagged mAb and quantified the DNA tags (reflecting protein expression) to confirm the assay's ability to accurately measure distinct protein expression levels at the single-cell level.

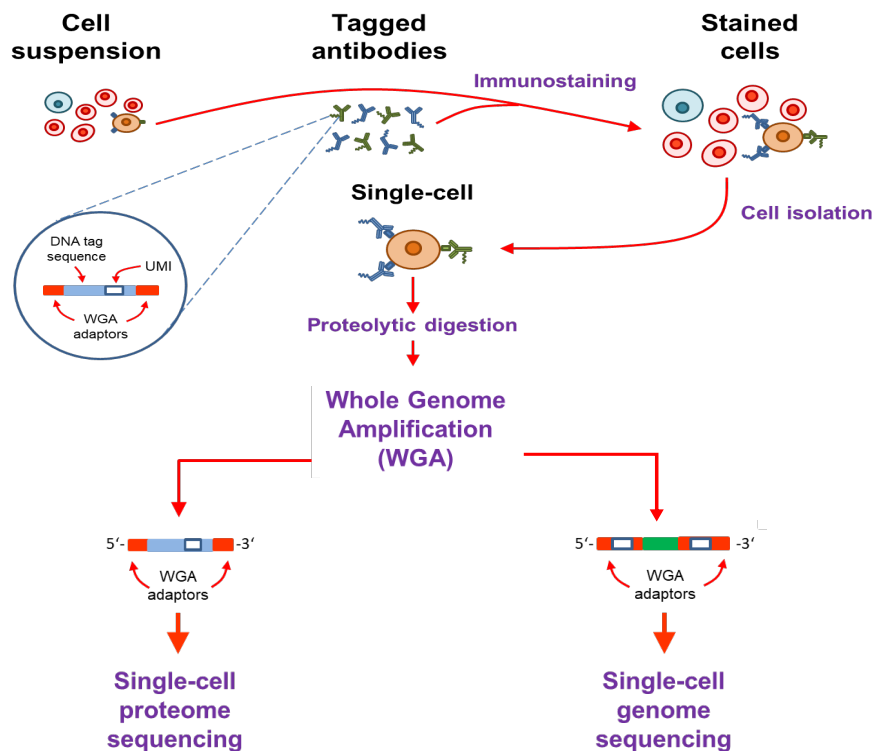


Figure 17. Overview of the concept of combined genome and proteome analysis of single cell.

4.2.2. Design of the tag DNA oligonucleotides

The design of the DNA tags comprised the following elements.

- (i) Chemical moieties allowing covalent binding of DNA tags to antibodies (e.g. 5'-thiol, biotin, amino (or similar) moieties allowing conjugation to antibodies)
- (ii) Chemical moieties preventing enzymatic degradation (e.g. phosphorothioate bonds preventing digestion of the Tag DNA oligos by exonucleases)
- (iii) Distinct DNA motifs (barcodes) tagging specifically different antibody species allowing discrimination and parallel quantification of multiple proteins in a single cell.
- (iv) Unique molecular identifies (UMIs), consisting of randomized sequence motifs, allowing discrimination and quantification of individual protein molecules.

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- (v) WGA-compatible adaptor sequences allowing co-amplification of DNA tags alongside with single-cell DNA (e.g. LIB1 sequence motif is implemented in the tag DNA oligos, when using the Ampli1 WGA approach)
- (vi) Sequencing adaptor sequences, e.g. Illumina TruSeq or Illumina Nextera adaptors, (or parts thereof) allowing selective amplification of tag DNA following the WGA and construction of libraries enabling quantification of tag DNA oligos by means of high-throughput sequencing.
- (vii) Optional offset sequence consisting of sequence blocks varying in length introduced in the sequence of tag DNA oligonucleotides to increase the sequence complexity of tag DNA sequences in experiments involving low levels of multiplexing, i.e. utilizing low number of DNA-tagged antibodies. Tag DNA oligos designated for various antibodies will be equipped with different offset sequence motifs. When pooling various antibodies, and thus also different tag DNA oligos, in a single experiment presence of offset sequences will increase the sequence complexity of the tag DNA oligo pool, thereby preventing failures of sequencing runs due to limited sequence complexity of the amplicons being sequenced (Figure 18).

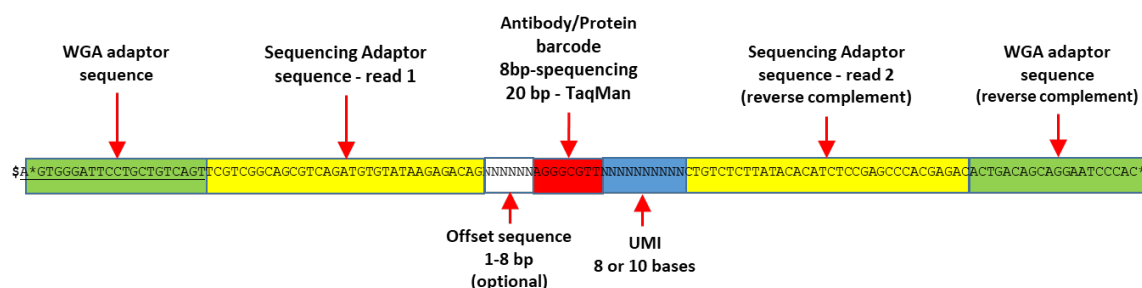


Figure 18. Design of the tag DNA oligonucleotides. The scheme comprises the functional components of the design. The functional group necessary for conjugation of DNA to antibodies (here placed on the 5'-end of the oligo sequence) is marked with a dollar sign. Chemical moieties preventing enzymatic degradation of DNA oligos (e.g. phosphorothioate bonds) were marked with asterisks.

4.2.3. First proof of principle of the G&P assay

Our primary goal was to demonstrate that the G&P assay enables the quantification of distinct levels of HER2, an oncogene over-expressed in 15-20% of human breast cancer carcinomas [98, 99], as well as its phosphorylated form as it can provide a

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more accurate representation of the signaling pathways and functional activity of the HER2 protein [100, 101].

4.2.3.1. Establishment of HER2 and phospho-HER2 staining

To establish HER2 and phospho-HER2 (pHER2) staining on adhesion slides, we used the BT474 cell line, which is known for its overexpression of HER2 protein along with PBMCs as a negative control. We focused on two key phosphorylation sites of HER2, Tyr1248 and Tyr1221/1222, as these are among the most commonly phosphorylated sites on the HER2 protein [100, 102, 103]. Frozen adhesion slides of BT474 cells spiked into PBMCs were stained individually with mouse anti-human CD340 (erbB2/HER2), mouse anti-pHER2 (Tyr1248), and Rabbit anti-pHER2 (Tyr1221/1222) mAb. We compared a standard staining protocol with a modified protocol, which included doubling the concentration of the primary antibody (pAb) (4 µg/ml), adding an initial fixation step using 1.36% paraformaldehyde (PFA), and a combination of both approaches. The comparative analysis revealed that fixation with PFA provided the best results, particularly for pHER2 staining. While increasing the pAb concentration was also beneficial, it was less effective than fixation, particularly for pHER2 staining. Furthermore, combining both increased antibody concentration and fixation did not yield better results than fixation alone. Consequently, we decided to proceed with the fixation protocol without increasing the antibody concentration for future experiments (Figure 19).

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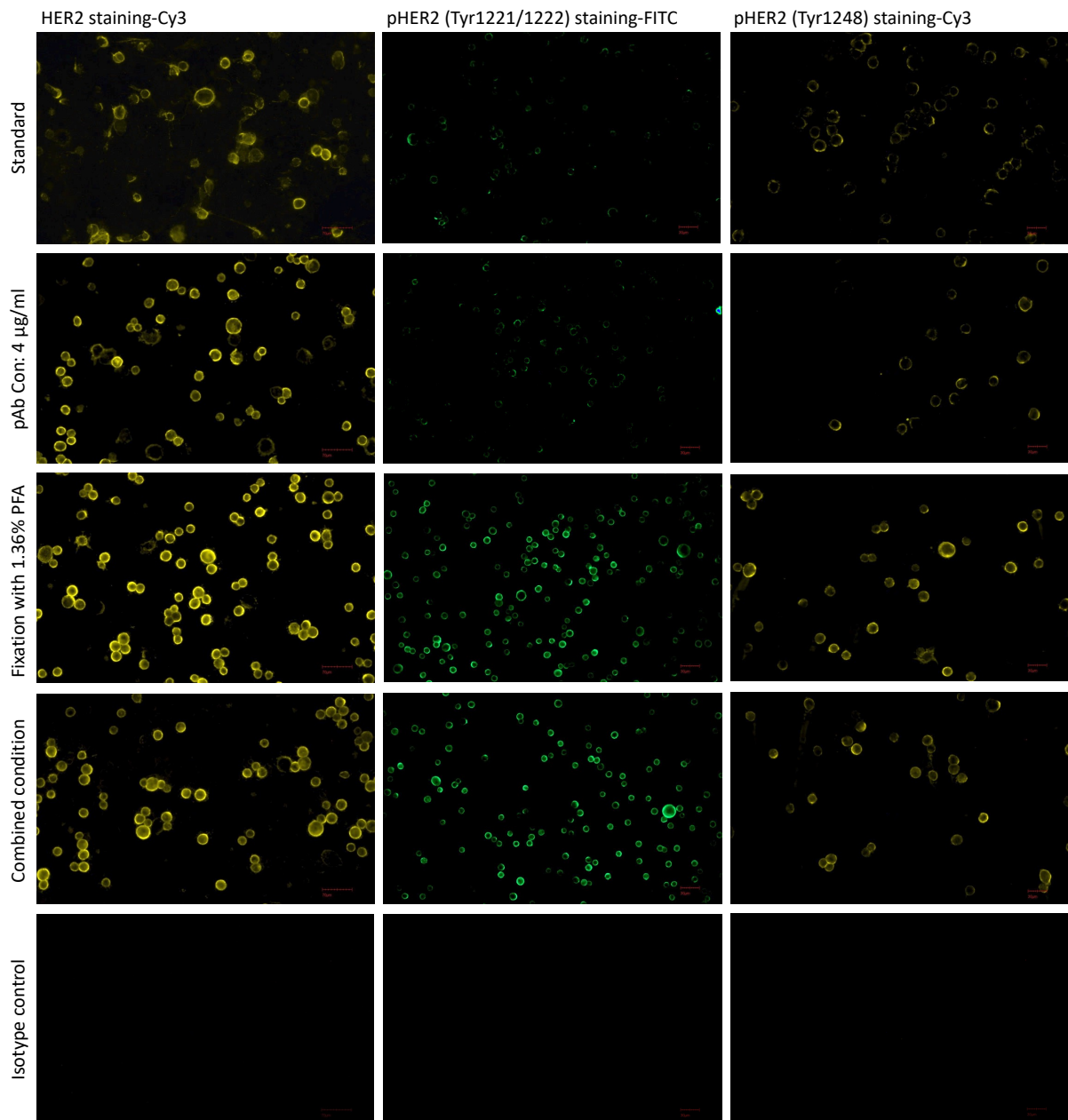


Figure 19. Optimization of HER2/pHER2 staining. Each row represents different staining conditions, with the left column displaying HER2 staining, the middle column showing pHER2 (Tyr1221/1222), and the right column showing pHER2 (Tyr1248). Individual conditions were tested, followed by a combined condition, which utilized a 2x concentration of pAb (4 µg/ml) along with fixation using 1.36% PFA. Isotype controls were performed separately for each marker, with only the control for the combined condition shown here. Mouse IgG isotype was used as the control for HER2 and pHER2 (Tyr1248), while Rabbit IgG isotype was used as the control for pHER2 (Tyr1221/1222). Images were captured using the MMI microscope (micrographs 20x magnification).

4.2.3.2. Quantifying HER2 expression levels using FACS and G&P assay

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Following successful establishment of staining, the next step was to integrate the HER2 DNA-tagged mAb into the G&P assay and further quantify protein level. The HER2 DNA-tagged mAb used in this experiment was conjugated through a commercial service provided by BioLegend. Three cell lines including MDA-MB-231, MDA-MB-453 and, BT474, alongside PBMCs, serving as a negative control were selected for this experiment. We selected these cell lines because (i) they were expected to exhibit different levels of HER2 expression, (ii) cell lines harbor distinct patterns of genetic alteration and, (iii) these cell lines represent different subtypes of breast cancer. Cells were stained with mouse anti-human HER2 mAb conjugated to DNA oligonucleotides, followed by incubation with secondary antibody (Alexa flour 555) for visualization. Fluorescence-activated cell sorting (FACS) was performed to isolate three distinct cell populations exhibiting non-overlapping levels of HER2 expression (Figure 20A). Following sorting, 12 single cells and 3 cell pools were isolated by manual micromanipulation to conduct WGA. All WGA products were assessed for genome quality using the WGA-QC workflow, and the presence of amplified tag DNA was confirmed with a dedicated endpoint PCR assay (See 3.8). To test the ability of the workflow to quantify the expression levels of HER2, the quantity of tag DNA oligonucleotides present in the WGA products was measured using qPCR. The WGA-QC analysis showed GII scores of 3-4 for nearly all cells and endpoint PCR confirmed the presence of tag DNA in all samples (Figure 20B). Notably, the quantities of the anti-HER2 mAb measured by qPCR in the single-cell WGA products were significantly different among the sorted cell populations, confirming that the workflow accurately quantified protein expression at the single-cell level and detected distinct HER2 expression levels (Figure 20C). Additionally, a linear correlation was observed between the quantities of HER2 DNA-tagged mAb and the mean fluorescence intensity measured by FACS (Figure 20D).

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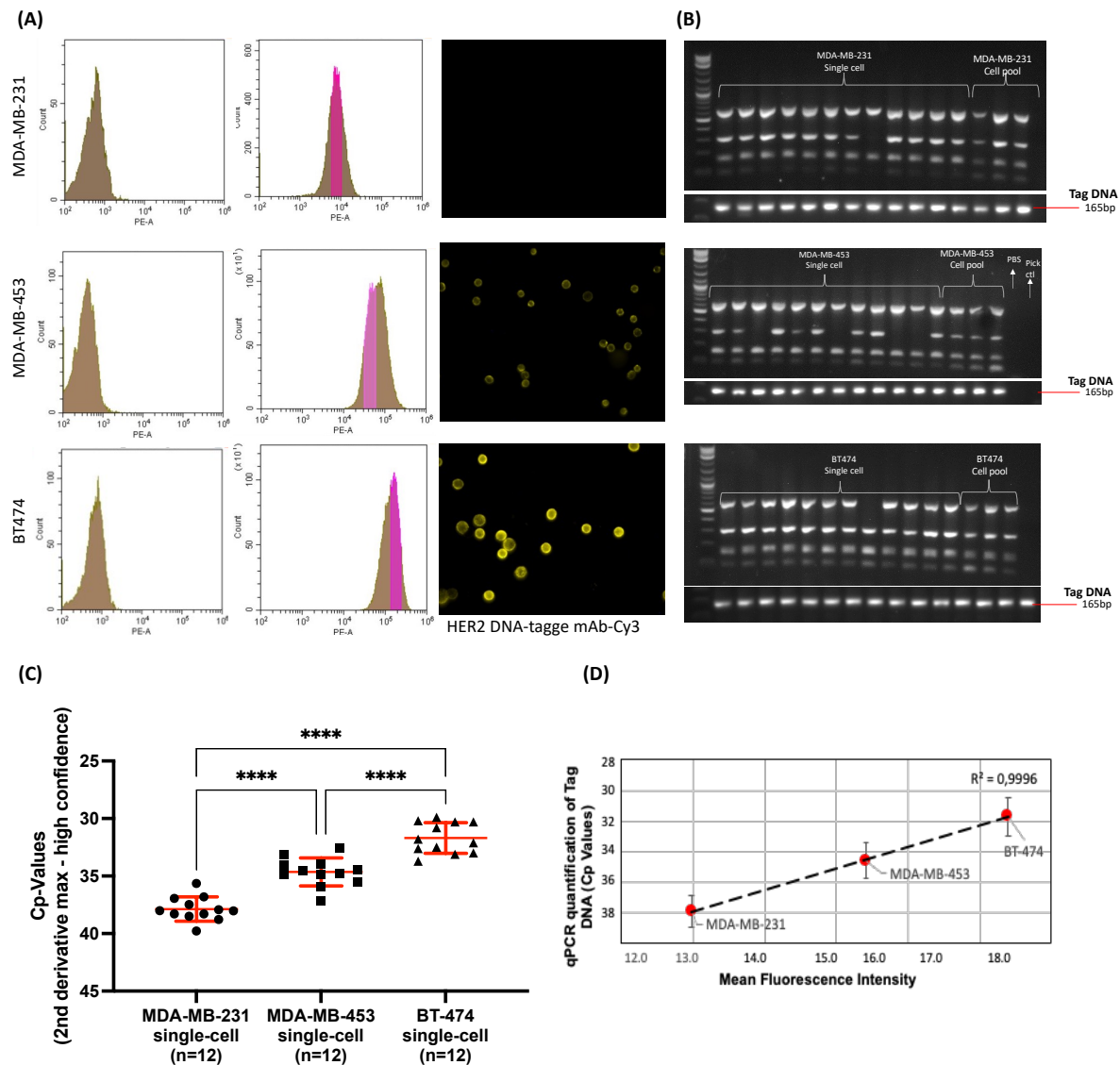


Figure 20. Quantifying different HER2 expression level at the single cell resolution using FACS and G&P assay. (A) HER2 expression analysis in three breast cancer cell lines (MDA-MB-231, MDA-MB-453 and BT474) assessed by flow cytometry and microscopically (micrographs 20x magnification). Three populations exhibiting different expression levels of HER2 were sorted for further analysis indicated with pink. (B) WGA-QC results of isolated single cells and cell pools. (C) qPCR-based quantification of HER2 DNA-tagged in sorted populations. (D) Correlation between DNA tag oligos quantities quantified by qPCR and average fluorescence intensity quantified by FACS. Asterisks denote statistical significance, with **** $P < 0.0001$. Mean $\pm$ SD, one-way ANOVA with Tukey's multiple comparisons test.

4.2.3.3. Challenges in G&P staining and qPCR readout

The G&P quantification for the cell lines was largely successful. However, unexpectedly, PBMCs used as negative controls for HER2 expression also showed the presence of HER2 tag DNA (PBMC quantification was added later as the experiment initially lacked a negative control) (Figure 21A). We reasoned that this anomaly could be attributed to either (i) passive carryover of DNA-tagged antibodies

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or unconjugated DNA tag oligo onto HER2-negative PBMCs or (ii) differences in cellular ploidy between the tested cell types (with PBMCs being diploid and cancer cells exhibiting higher level of ploidy). Given that polyploid cells contain more DNA and produce higher quantities of proteins than diploid counterparts [104, 105], we hypothesized that disparities in ploidy could influence the relative abundance of genomic and tag DNA, thereby skewing the qPCR readout (Figure 21B). Both these hypotheses were addressed experimentally.

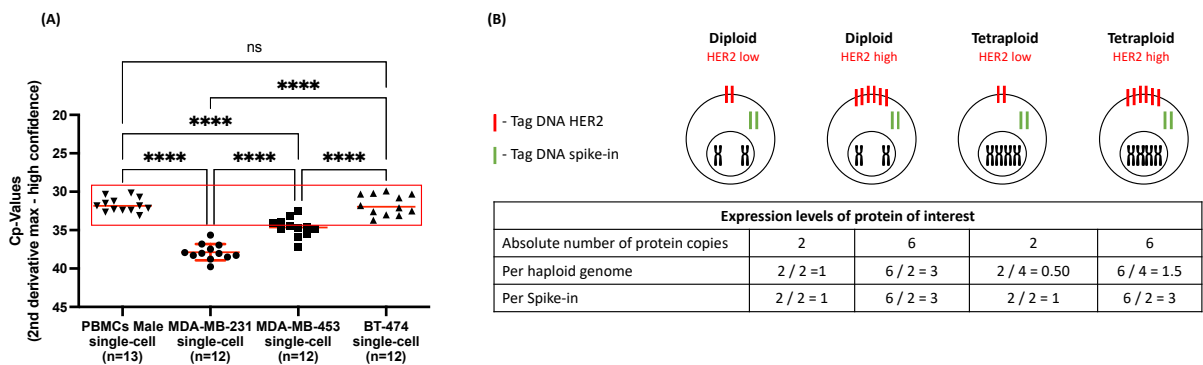


Figure 21. Difficulties in G&P quantification. (A) qPCR-based quantification of HER2 DNA-tagged in sorted populations including polyploid cancer cells and diploid PBMCs. Note that the measurement showed comparable levels of tag quantities in PBMCs compared to BT474. (B) A schematic of how quantification of DNA tags may be biased, when measurements are normalized based on the total DNA input, along with a potential solution by introducing spike-in oligonucleotides to normalize qPCR readouts.

4.2.3.4. Addressing unspecific binding of DNA-tagged antibody and ploidy variability in qPCR

To decrease non-specific background signals, blocking conditions were optimized by adding dextran sulfate (DS), which is a commonly used additive in microarray and in situ hybridization protocols. It blocks non-specific background binding and accelerates kinetics of the hybridization process [106, 107].

To address the issue of different ploidy, instead of adjusting the total quantity of DNA input (20 ng per reaction), normalization of the qPCR readout was achieved using an exogenous spike-in oligonucleotide as an internal reference and loading control. The spike-in consisting of DNA tag, designed similarly to oligonucleotide conjugated to anti-HER2 mAb but equipped with a distinct sequence, was added in defined quantity to each isolated cell and used as an internal control allowing for normalization of qPCR measurement independently of cellular DNA abundance.

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A: Optimization of DNA tag spike-in for accurate qPCR analysis

The primary goal of this experiment was to determine the optimal concentration of the spike-in oligo that yields consistent and reliable Cp values during qPCR. To achieve this, quantification of the spike-in oligo was done alongside single cell genome amplification providing a measure of the efficiency and consistency of qPCR reactions across different samples. Spike-in oligos from 10^{12} to 10^3 copies/ μ l were added to the Proteinase K (PK) solution of WGA. For each concentration of the spike-in oligo, three single PBMCs were isolated by micromanipulation and subjected to WGA. Subsequently, an endpoint PCR was performed to confirm the presence of spike-in oligo in WGA sample, followed by qPCR to quantify the amount of amplified spike-in oligos. The results of the endpoint PCR confirmed the presence of DNA tag (amplicon of 165 bp) in the WGA product (Figure 22A). The qPCR of the DNA tag showed that spike-ins with absolute copy numbers ranging from 10^3 and 10^5 could not be reliably measured as the obtained Cp values were outside of the linear area of the standard curve. The obtained Cp values for these samples were equal to approx. 36 cycles (Figure 22B). However, the qPCR measurements obtained for WGA samples with spike-in copy numbers ranging from 10^6 to 10^{12} exhibited a linear relationship between the spike-in concentration and Cp values and could be reliably measured by qPCR (Figure 22C). Finally, a spike-in oligo number of 10^8 per WGA was selected for the future experiments. This number provided Cp values of ~27, which was representing intermediate Cp values for the Tag DNA measurements expected.

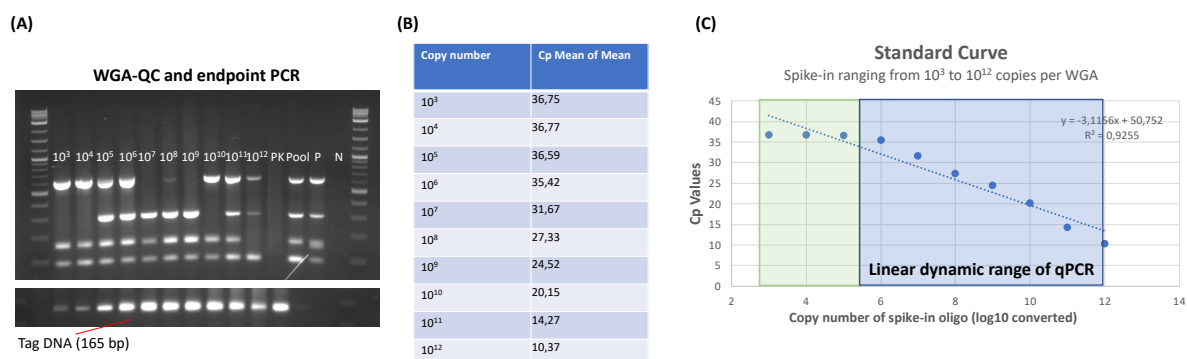


Figure 22. Optimization of spike-in oligo using qPCR. (A) The gel picture indicates WGA-QC and endpoint PCR for one isolated cell from each serial dilution. “PK” (Pick-Kontrolle) contains no single cell genome but all other reagents, “pool” contains DNA from picking 1 μ l of scratched cells, “P” shows positive control for WGA-QC which previously assessed good quality sample, but contains no spike-in oligo and “N” represents negative control for the WGA-QC which contains neither cell nor spike-in oligo. (B) The table provides the DNA copy number per μ l alongside the corresponding Cp Mean of Mean

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(three samples per copy number with three replicate). (C) The representative standard curve on the right correlates these Mean Cp values with log₁₀ converted of the DNA copy number.

B: Application of the optimization measures

To test the optimized staining condition, we selected MDA-MB-231 (HER2^{L_o}) and BT-474 (HER2^{H_i}) cell lines along with PBMCs as negative control. Frozen adhesion slides of these cell lines stained with HER2 DNA-tagged mAb under optimized condition (0.1% DS+2%BSA as blocking agent), (See 3.3.4). The optimized spike-in oligo concentration (10⁸ DNA copies/μl) introduced to PK solution of WGA (See Table 17). Five single cells were isolated from cell lines and PBMCs adhesion slides and subjected to WGA. qPCR-based quantification was done to assess the effect of the protein detection optimization and normalization using spike-in oligo.

The optimized protein detection condition (Protocol 2) was compared with our standard detection protocol (Protocol 1, using only 2%BSA as blocking). Incorporating DS as a blocking agent significantly improved the IF staining results by reducing non-specific binding in MDA-MB-231 and PBMCs compared to benchmark staining where only BSA 2% was used as blocking (Figure 23A). We then compared the absolute and relative quantification methods.

qPCR-based quantification of HER2 tag DNA showed a significant difference between quantities of HER2 tag DNA between MDA-MB-231 (HER2^{L_o}) and BT-474 (HER2^{H_i}) (Figure 23B). Therefore, the absolute quantification allowed us to correctly uncover the expression levels in HER2^{H_i} and HER2^{L_o} cell lines. Interestingly, in PBMCs treated with DS the Cp value ranges (value obtained in the qPCR inversely correlated with the abundance of tag DNA) changed by 6-7 cycles compared to PBMCs treated with blocking solution comprising only 2% BSA. The increased Cp values in PBMCs treated with DS indicates a 64-126-fold decreased background staining reflected by lower abundance of HER2-associated tag. However, the absolute quantification did not reveal the difference in HER2 expression level between PBMCs and BT474. We reasoned that this was caused by the different ploidy of the cell lines and PBMCs. To make the G&P measurement independent of the ploidy of tested cells, we normalized the readout using spike-in oligo.

Using the spike in oligo as a reference, allowed us to calculate fold change in HER2 expression in cell lines normalized to the spike in oligo relative to PBMCs (used here as a control sample group). For relative quantification, we used Livak method (also

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called $\Delta\Delta C_p$ method), [108]. Relative quantification showed around 16-fold higher abundance of HER2 tag DNA in BT474 compared to MDA-MB231 and PBMCs (Figure 23C). In addition, we calculated a 0.5-fold increase in HER2 expression in MDA-MB231 relative to PBMCs but this was not statistically significant. In conclusion, the incorporation of DS into the staining protocol and the introduction of spike-in oligo as a reference enabled a more accurate quantification of different HER2 expression levels in the G&P assay.

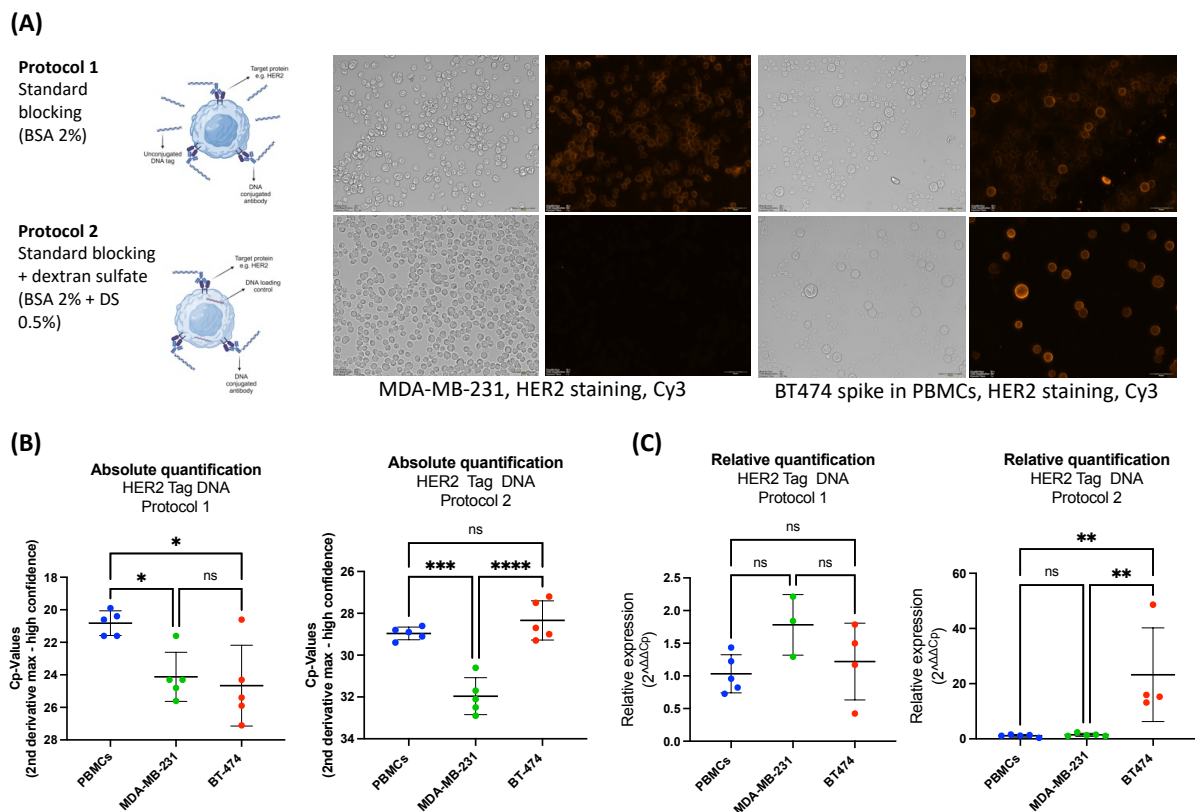


Figure 23. Blocking of unspecific antibody binding and ploidy-correction enables protein quantification. (A) Introducing dextran-sulfate as blocking agent and exogenous DNA spike-ins (protocol 2) compared with using only BSA 2% as blocking (protocol 1). The figure presents representative images for two staining protocols performed on MDA-MB-231 and BT474 cells spiked into PBMCs (micrographs 20x magnification). (B) Absolute and (C) relative quantification of HER2 DNA-tagged mAb comparing two staining protocols. Asterisks represent the significance as follows: *** $P < .001$, ** $P < .01$, * $P < .05$ and ns: $P \geq .05$. Mean $\pm$ SD, one-way ANOVA with Tukey's multiple comparisons test.

C: Optimization of qPCR reagent master mix for improved amplification efficiency

Since we began incorporating the spike-in oligo alongside the tag DNA oligo bound to the anti-HER2 ab, we observed significant differences in the amplification curves for certain samples. For instance, PBMCs isolated using protocol 2 (stained with BSA 5%)

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+ DS 0.5%) showed distinct amplification patterns compared to those isolated with protocol 1 (stained with control conditions, blocking with BSA 5%). In particular, the curves reach their plateau phase at a much lower fluorescence intensity than before, suggesting that the overall efficiency of the amplification may be compromised. In the case of PBMCs isolated using protocol 2, the amplification of the HER2 tag exhibited a noticeable delay of approximately 10 cycles, along with reduced fluorescence intensity. In contrast, C_p values of the spike-in oligo remained unchanged across samples, as equal amounts were introduced into all reactions. However, inefficiencies in qPCR still affected the spike-in oligo (Figure 24).

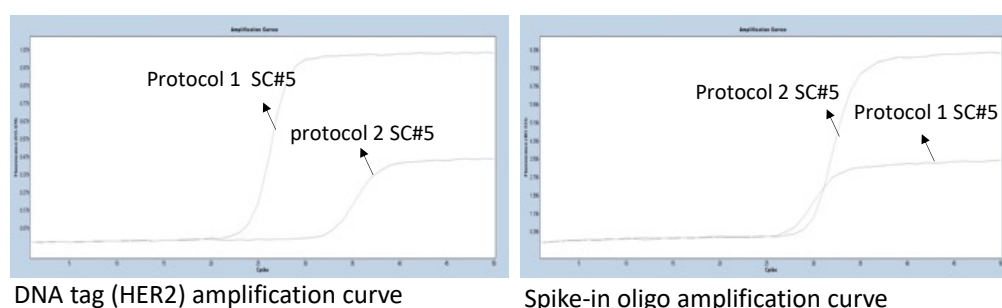


Figure 24. Amplification inefficiency observed in qPCR curves. Amplification curves comparing PBMCs isolated using protocol 1 (BSA 5%) and protocol 2 (BSA 5% + DS 0.5%). Protocol 2 showed a 10-cycle delay and lower fluorescence intensity, indicating reduced amplification efficiency, while spike-in oligo C_p values remained almost consistent across both protocols. However, qPCR inefficiencies were also observed in the spike-in oligo amplification.

To address the observed issues and optimize the PCR conditions, several strategies were employed. First, the concentration of TaqMan probes was adjusted to improve the detection of the target, as low probe concentrations may contribute to weak signal intensities. Additionally, primer concentrations were optimized, since inappropriate levels can lead to inefficient amplification. Furthermore, bovine serum albumin (BSA) was added to the PCR reaction to help alleviate the impact of potential inhibitors by stabilizing the reaction and enhancing enzyme activity. Together, these strategies provided a comprehensive approach for improving PCR performance and addressing the previous amplification inconsistencies. Initially, qPCR readout was optimized under multiple conditions individually, including the use of 2x concentration of TaqMan probes, 2x concentration of primer, and the addition of BSA to the qPCR reagent (Figure 24, optimization #1), (Table 24). For this experiment one sample of PBMCs isolated using protocol 2 was used as the template (qPCR inefficiencies were observed in the samples isolated with this protocol). The results of this experiment showed that

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2x TaqMan and 2x primer improved the signal intensity, while BSA also contributed to signal improvement, though not as effectively as increasing TaqMan and primer concentrations (Figure 25).

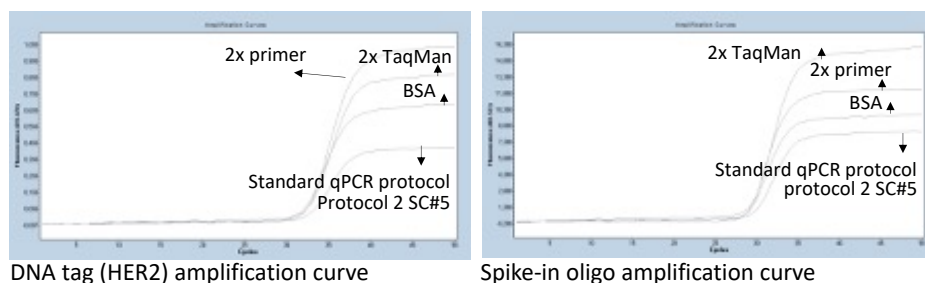


Figure 25. Optimization of qPCR reagent master mix #1. The amplification curve of the HER2 DNA tag and spike-in oligonucleotide illustrates the initial improvement of qPCR readouts using 2x primer concentration, 2x TaqMan concentration, and the addition of BSA. This optimization was performed using one PBMC isolated through protocol 2.

To further refine the qPCR readout and strengthen this effect, experiments were conducted using combinations of 2x TaqMan with increasing primer concentrations, including 2x, 3x and 4x, under both single-channel and double-channel read configurations (Table 25 and 26). The combinations of 2x TaqMan with increasing primer concentrations (2x, 3x and 4x) did not significantly alter C_p values (changes ranging from 0.5 to 1 cycle), while signal intensity improved in proportion to the increase in primer concentration (for both HER2 and spike-in oligo) (Figure 26A). To prevent the amplification of a secondary band, we selected the combination of 2x TaqMan and 3x primer for future qPCR experiments. The enhanced signal suggests that the optimized conditions, including adjustments to primer and probe concentrations, increased the overall assay sensitivity and target detection without affecting the C_p values. Furthermore, we also tested multiplex qPCR readouts; however, these did not demonstrate the same level of efficiency as single qPCR readouts (Figure 26B). Consequently, we prioritized single qPCR readouts in our experiments to maximize efficiency and improve assay performance.

Results

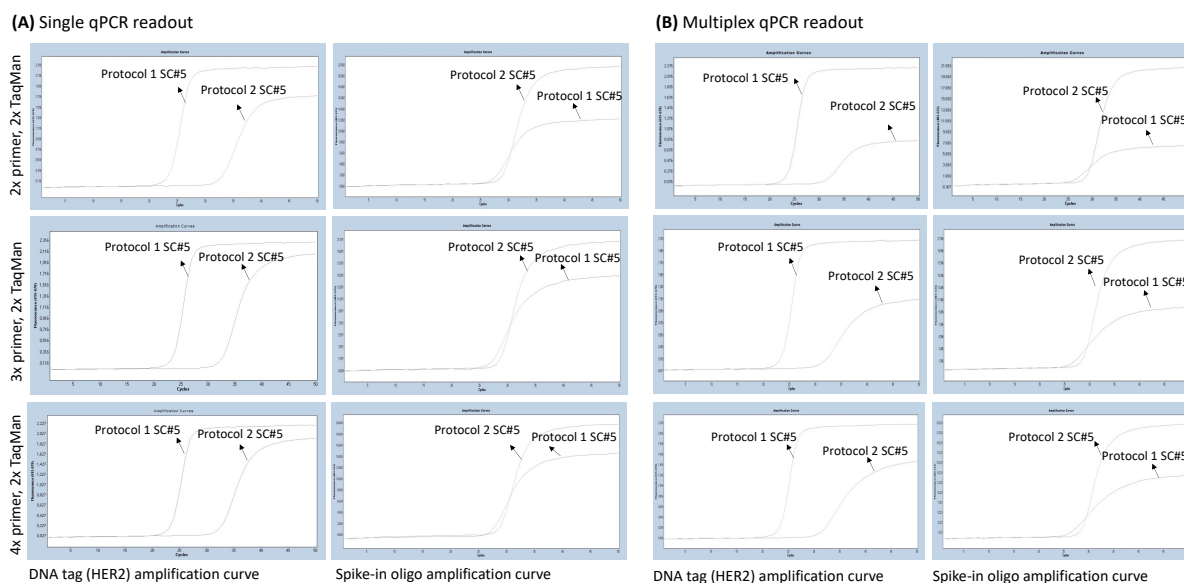


Figure 26. Optimization of qPCR reagent master mix #2. (A) Amplification curves generated using a combination of 2x TaqMan probes with increasing primer concentrations (2x, 3x and 4x). (B) The same optimized protocols applied in a multiplex qPCR readout.

4.2.4. Antibody-DNA conjugation for the G&P assay

Following the successful establishment and optimization of the G&P assay for measuring HER2 expression in single cells, we aimed to test additional target proteins, including pHER2, which was established in parallel with HER2, (see 4.2.3.1) as well as CK, known for its reliable detection of DCCs [48] and CD45, which serves as a marker for non-tumor cell detection. For the conjugation of the HER2 DNA-tagged mAb, we utilized a commercial service provided by Biolegend. However, widespread application and use of the G&P approach called for more efficient and cost-effective methods to conjugate tag DNA oligonucleotides with antibodies. To this end, we explored two conjugation techniques: Light-Activated Site-Specific Conjugation (LASIC) and the maleimide method. The results of both approaches are detailed below.

4.2.4.1. LASIC method

This approach utilizes chemistry-modified oligos which upon illumination with non-damaging Black-light (365 nm), forms a covalent bond with the antibody (see 3.5.1). We used this method to conjugate oligonucleotides to two antibodies: CK and pHER2.

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For CK Ab-DNA conjugation, we provided the selected sequence tag for the CK antibodies to AlphaThera, and they supplied us their oYo-Link oligo reagent (a chemically modified oligo). Upon receiving the reagents, CK antibody were mixed with the oYo-Link oligo and photo-crosslinking was performed using a handheld LED device. Following the conjugation, the samples were reduced with 2% β -mercaptoethanol and run on 10% Bis-Tris denaturing gel and stained with coomassie blue and ethidium bromide for protein and DNA visualization respectively. An upward shift of the heavy chains was observed in the gel indicating successful conjugation of the oYo-Link oligo to the heavy chain (HC) of the CK mAb (Figure 27A). However, the conjugation efficiency was low, with a significant portion of non-labeled antibodies present in the product (Figure 27A). To improve the conjugation efficiency, we sent CK antibody to AlphaThera and requested their conjugation service. Consequently, all subsequent experiments with CK antibody were conducted by the conjugated product generated by AlphaThera. The results of their quality control (QC) indicated successful conjugation of the oYo-Link oligo to the HC of the CK mAb (Figure 27B). Notably, the conjugation efficiency was significantly higher when using the AlphaThera LEDPX2 device compared to our handheld LED device. To enhance our independence in conjugating oligonucleotides to future antibodies and to eliminate the need for shipping antibodies, we opted to purchase AlphaThera's LEDPX2 device for future conjugation experiments.

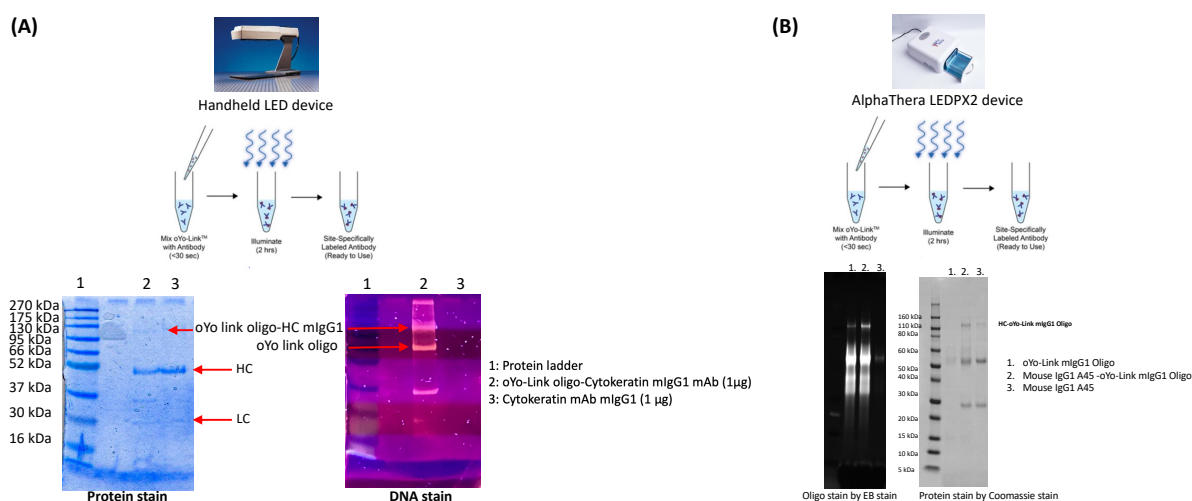


Figure 27. CK mAb-DNA conjugation using the LASIC method. (A) The top panel illustrated the procedure for conjugating CK mAb with DNA using a handheld LED device (the figure designed by AlphaThera). Below, a representative SDS-PAGE gel image compared the conjugated antibody (second lane) with the unconjugated antibody (third lane). (B) The conjugation process was performed

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by AlphaThera using a LEDPX2 device, and the provided quality control gel image confirmed the efficiency of the conjugation.

For pHER2 mAb DNA conjugation, all procedures were conducted as outlined in 4.2.2.1.1, with the exception that the conjugation was performed using AlphaThera's LEDPX2 device in our laboratory. The SDS-PAGE gel staining results demonstrated an upward shift of the heavy chain, confirming the successful conjugation of the oYo-Link oligo to the pHER2 mAb, as visualized by both protein and DNA staining. This antibody also contained 0.2% BSA, which was visualized through protein staining (Figure 28).

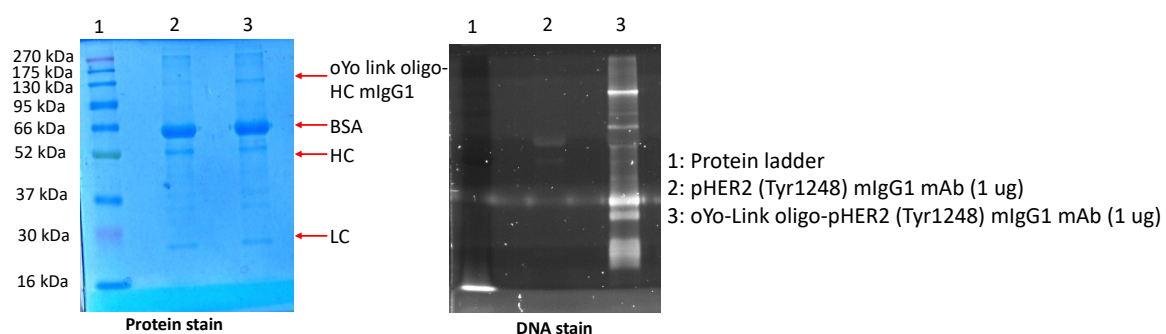


Figure 28. pHER2 mAb-DNA conjugation. The results of an SDS-PAGE gel showing the conjugation efficiency of the pHER2 mAb-DNA conjugate (third lane) compared to the unconjugated antibody (second lane). HC: heavy chain, LC: light chain, BSA: bovine serum albumin.

4.2.4.2. Maleimide conjugation method

Antibody conjugation in this method relies on the partial reduction of disulfide bonds in the IgG antibody, followed by incubation with a unique maleimide-modified DNA oligonucleotide (see section 3.5.2). To test this conjugation method, we selected CD45 mAb, which serves as a negative selection marker for tumor cell detection. The conjugation initially was assessed by using flow cytometry. This only demonstrated whether the conjugated antibody has oligonucleotide conjugated, not the number or efficiency of oligonucleotide conjugation. To evaluate the efficiency of conjugation, the CD45 mAb DNA-tagged ran on SDS-PAGE gel. Finally in situ staining was done to validate the staining efficiency.

To perform the antibody-oligonucleotide conjugation, we designed a sequence specific to CD45 and chemically modified it with a maleimide group, synthesized by Metabion. The entire conjugation procedure was conducted in collaboration with Yannick Palmowski, a PhD student in Prof. Dr. med. Christian Schürch's lab in

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Tübingen (see 3.5.2). To evaluate the success of the conjugation, we utilized flow cytometry with complementary fluorescently labeled DNA probes (also called bottom oligo) to determine whether the antibody was effectively conjugated to the oligonucleotide. Flow cytometry analysis of CD45 DNA-tagged mAb was done using a negative control (compensation beads + bottom oligo), tested antibody (compensation beads + CD45 DNA-tagged mAb + bottom oligo) and a positive control (compensation beads + CD45 antibody conjugated to APC). The negative control showed 22.08% positivity, indicating background signal due to non-specific binding from reagents. This population was used to adjust for fluorescence spillover and correct for any background signal. The CD45 DNA-tagged antibody read at 35.95%, while the positive control with CD45-APC showed 49.68% (Figure 29A). Accounting for the background signal from the negative control, the corrected signal for the CD45 DNA-tagged antibody was 13.87%, indicating a low conjugation efficiency. To further evaluate the efficiency of the antibody-DNA conjugation, we ran the samples on an SDS-PAGE gel and compared the CD45 DNA-tagged antibody with the identical unconjugated antibody. The protein stain revealed several bands above the expected size of the antibody HC, which were not visible with the DNA stain (appeared as a smear). This suggests that the conjugation was not entirely successful (Figure 29B). Additionally, in situ staining was carried out on tonsil tissue to validate the functionality of the conjugated antibody (this staining was performed by Yannick Palmowski). This staining was compared to a previously established CODEX CD45 DNA-tagged mAb, which showed a clear membranous staining pattern. However, G&P CD45 DNA-tagged mAb staining was unsuccessful (Figure 29C). Given that the G&P oligo used for conjugation was significantly longer (142 bp) compared to the oligos typically used in the CODEX method (23 bp), we suspected that this conjugation method may be less effective when long oligonucleotides are used.

Results

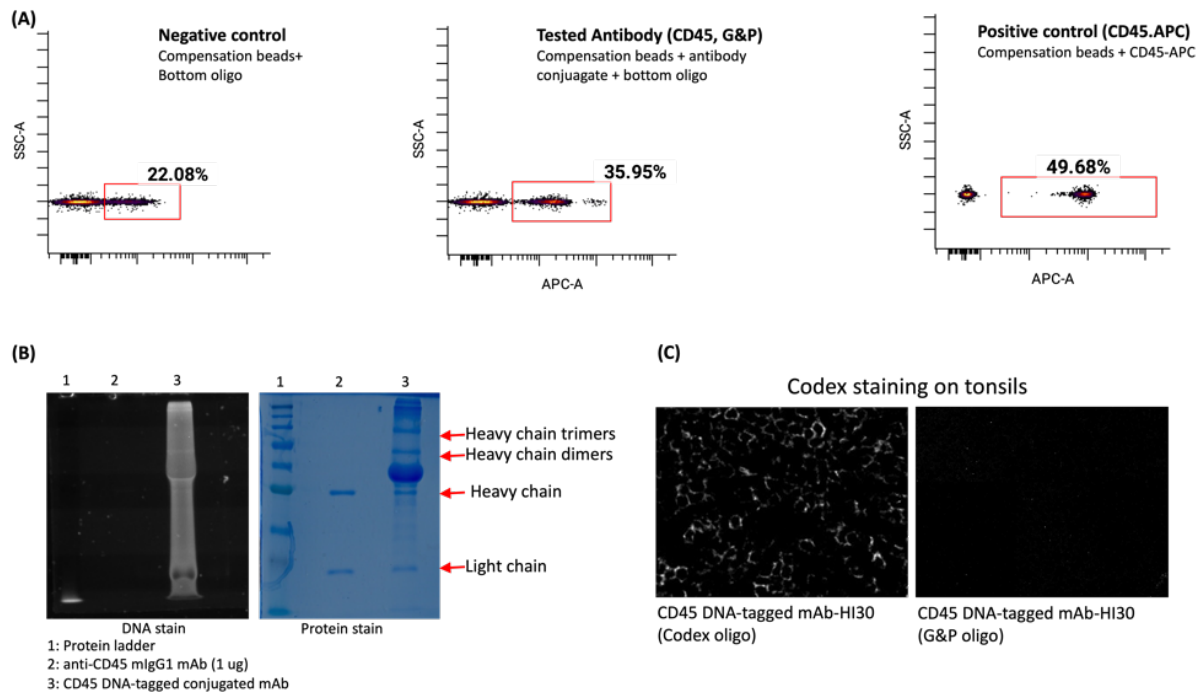


Figure 29. CD45 mAb-DNA conjugation using maleimide conjugation method. (A) Validation of antibody-DNA conjugation by flow cytometry. Compensation beads have a negative population that enables the detection of background binding from reagents. (B) SDS-PAGE gel results compared unconjugated CD45 with conjugated antibody. (C) Validation of conjugation's functionality using CODEX staining on tonsil tissue using CD45 DNA-tagged mAb, codex oligo (left panel) with CD45 DNA-tagged, G&P oligo (right panel).

4.2.5. Staining performance of CK DNA-tagged mAb

To validate the staining performance of the CK DNA-conjugated antibody, we initially conducted flow cytometry measurements and IF staining to evaluate CK expression levels in breast cancer (BC) cell lines, enabling us to select those with varying levels of CK expression (using unconjugated antibody). Subsequently, we performed G&P staining using CK DNA-tagged mAb, followed by qPCR-based quantification of CK DNA-tagged mAb.

Flow cytometry analysis was performed to identify cell lines with low, medium, and high CK expression. Based on this, we selected the MDA-MB-231, MDA-MB-453, SKBR3, and MCF7 cell lines. The cells were cultured to 80-90% confluency, harvested, and stained with mouse anti-human CK and Alexa fluor 555 for visualization. Flow cytometry analysis was done using a Gallios device (Beckman). The cell lines displayed different expression levels, with MDA-MB-231 showing the lowest levels and MCF7 the highest (Figure 30A). An overlay comparison was

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employed to further distinguish the cell lines based on their CK expression differences (Figure 30B). To ensure consistency between the methods, we also performed the same staining on frozen adhesion slides of all four cell lines, in order to test the comparability of the two approaches.

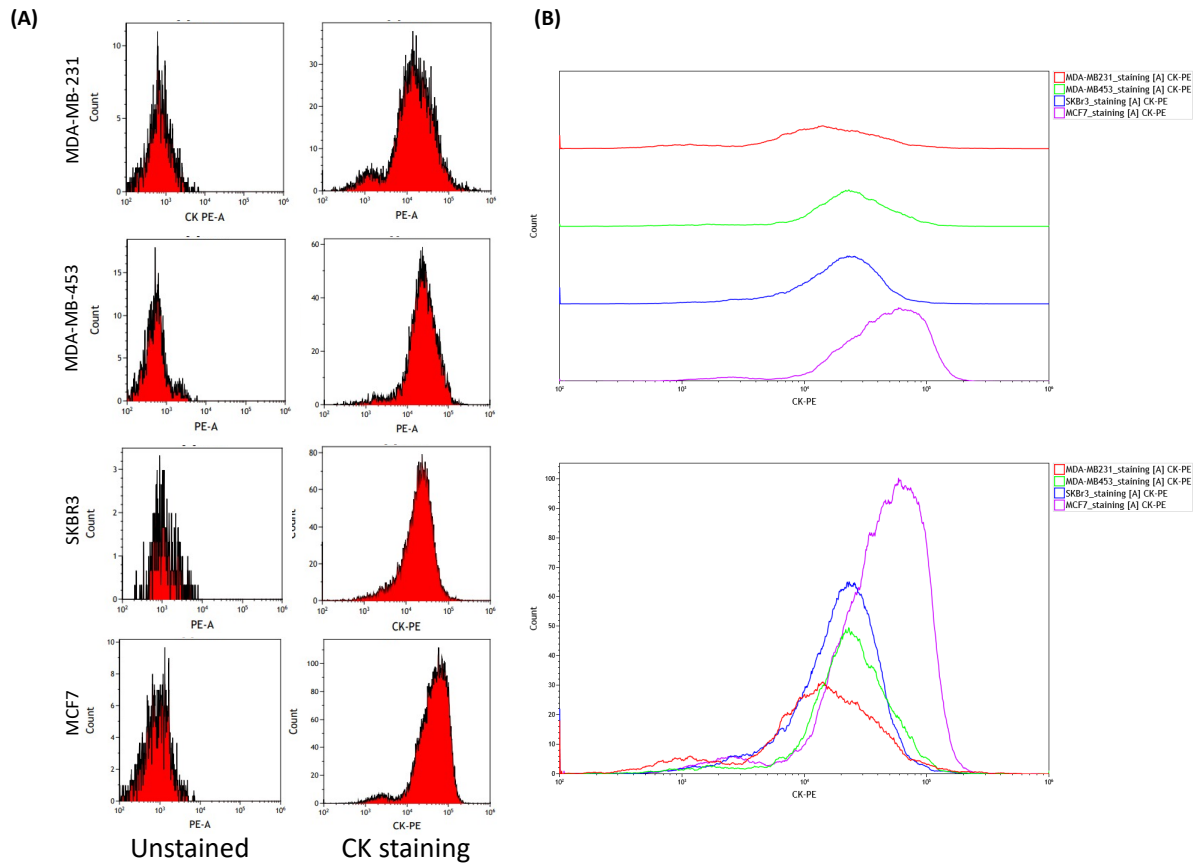


Figure 30. Flow cytometry measurements of CK expression. (A) Histograms of CK expression in four cell lines including MDA-MB-231, MDA-MB-453, SKBR3, and MCF7 compared in unstained and stained samples. (B) Overlay expression showing a gradient expression of CK.

For microscopic analysis of CK expression, adhesion slides of MDA-MB-231, MDA-MB-453, SKBR3, and MCF7 stained with mouse anti-human CK Ab and Alexa fluor 555 for visualization. The results from this staining revealed comparable CK protein expression levels as the flow cytometry analysis. Specifically, MDA-MB-231 exhibited the lowest CK levels, while MCF7 had the highest expression, with SKBR3 and MDA-MB-453 displaying intermediate and comparable levels of CK expression (Figure 31). Since MDA-MB-453 was used for HER2 protein quantification, we opted to continue with this cell line for subsequent experiments.

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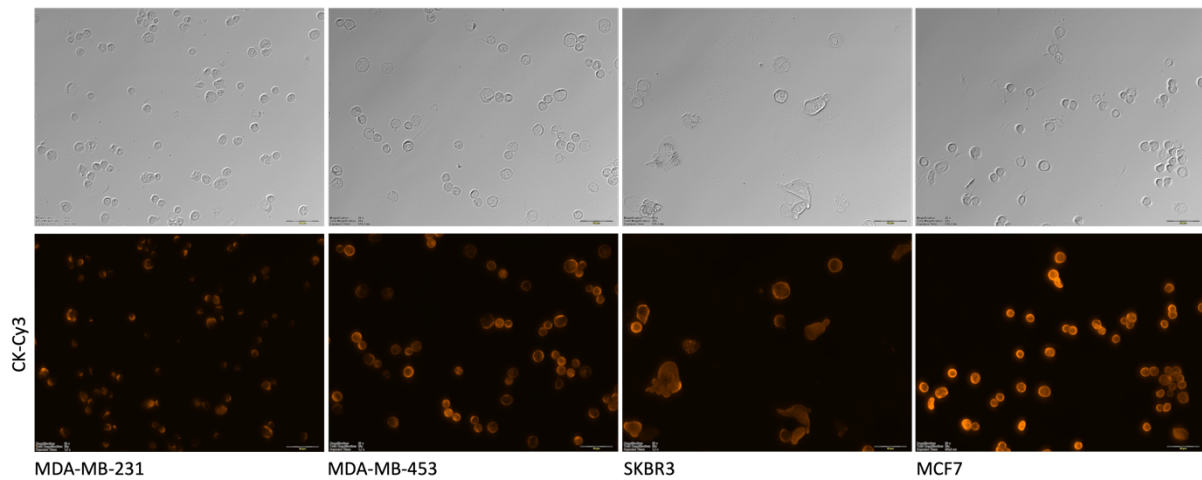
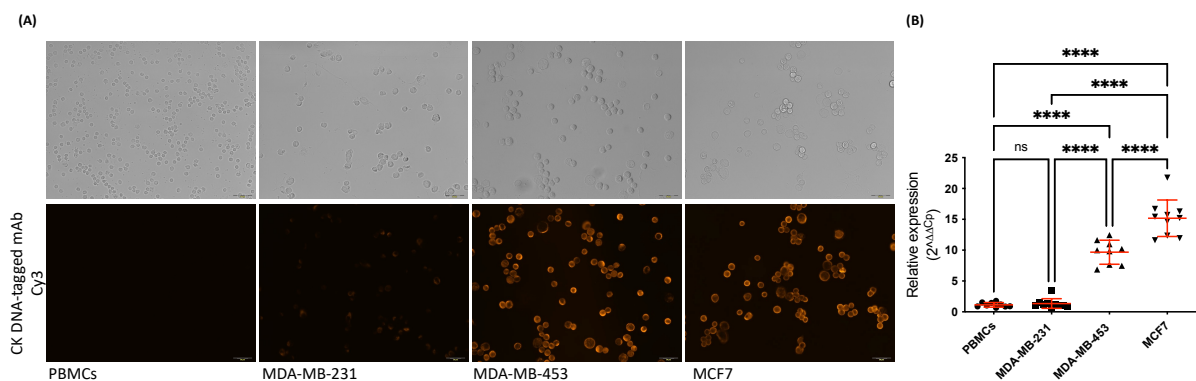


Figure 31. Microscopic analysis of CK expression on BC cell lines. Staining of BC cell lines against CK was performed on adhesion slides indicating different CK expression levels. Images were captured using an Olympus microscope (micrographs 20x magnification).

To quantify CK protein expression using G&P assay, adhesion slides of MDA-MB-231, MDA-MB-453 and MCF7 were stained with CK DNA-tagged mAb and Alexa fluor 555 for visualization, alongside PBMCs as a negative control (Figure 32A). Following screening, single cells were isolated by manual micromanipulation to conduct WGA. All samples were assessed for WGA quality using the WGA-QC workflow, and the presence of tag DNA was verified with a dedicated endpoint PCR assay. Finally, the samples were quantified for quantities of CK DNA-tagged in WGA products. Data analysis using the relative quantification approach revealed significant differences in DNA tag quantities between CK^{Hi} and CK^{Medium} expressing cell lines compared to CK^{Lo} and cytokeratin-negative populations (Figure 32B). While CK^{Lo} cells exhibited a slight increase in CK expression relative to the CK-negative cells, this difference was not statistically significant. Overall, these results demonstrated feasibility of the quantification of CK expression using the G&P assay, which effectively differentiates between three distinct levels of CK protein expression.



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Figure 32. Quantification of CK protein expression using the G&P assay. IF staining was done using CK DNA-tagged mAb with mlgG1 Alexa fluor 555 detected in the Cy3 channel (micrographs 20x magnification). (B) qPCR quantification of CK DNA-tagged mAb demonstrated distinct expression of CK among cell lines. Asterisks represent the significance as follows: **** $P < .0001$, ns: $P \geq .05$. Mean $\pm$ SD, one-way ANOVA with Tukey's multiple comparisons test.

4.2.6. Staining performance of pHER2 DNA-tagged antibody

The same approach was applied to a pHER2 (Tyr1248) DNA-tagged mAb. pHER2 staining was established alongside HER2 mAb (see 4.2.3.1.) for two HER2 phosphorylation sites Tyr1248 and tyr1221/1222, ensuring the robustness and consistency of the staining. However, only pHER2 (Tyr1248) was conjugated to DNA and integrated into the G&P assay. We selected this phosphorylation site as it known to become constitutively activated as a result of HER2 homodimerization [109].

To establish pHER2 quantification using an anti-pHER2 (Tyr1248)-DNA tagged mAb we utilized the same cell lines as for HER2 protein quantification, including MDA-MB-231, MDA-MB-453, and BT474, along with PBMCs as negative controls. Adhesion slides of these cell lines were incubated with the pHER2 (Tyr1248)-DNA tagged mAb and pHER2 binding was visualized via an anti-mouse Ig antibody conjugated with Alexa fluor 555. Screening was performed using an Olympus microscope, and several single cells were isolated from each cell line for further analysis. After WGA, high quality samples were quantified for pHER2 DNA tag presence, reflecting protein expression levels. IF staining results showed high expression of pHER2 in BT474 cell line, while MDA-MB-453, MDA-MB-231, and PBMCs exhibited low or no expression (Figure 33A). Relative quantification results indicated significant differences in pHER2 (Tyr1248) expression between BT474, and both the pHER2^{Lo} expressing cells and the negative cell population. Notably, MDA-MB-453, which demonstrated medium-high HER2 expression, exhibited very low pHER2 levels, consistent with IF staining results. Although a trend suggested that MDA-MB-453 and MDA-MB-231 exhibited low and no pHER2 expression respectively, the differences were not statistically significant (Figure 33B). Overall, we concluded that pHER2 protein expression can be accurately measured in single cells using DNA-tagged antibodies.

Results

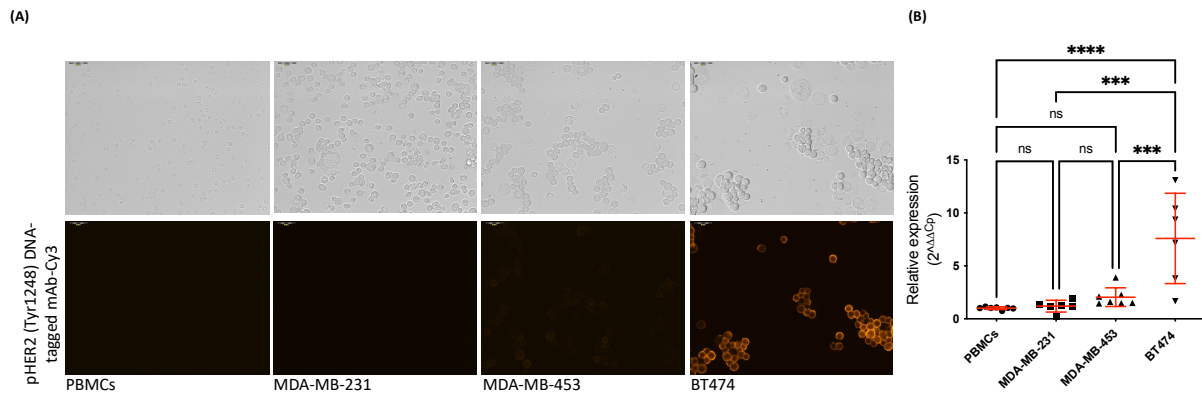


Figure 33. Quantification of pHER2 protein expression using G&P assay. (A) IF staining was done using pHER2 DNA-tagged mAb with mIgG1 Alexa fluor 555 detected in the Cy3 channel (micrographs 20x magnification). (B) qPCR-based quantification of pHER2 DNA-tagged mAb demonstrated distinct expression of pHER2 among cell lines. Asterisks represent the significance as follows: **** $P < .0001$, *** $P < .001$ ns: $P \geq .05$. Mean $\pm$ SD, one-way ANOVA with Tukey's multiple comparisons test.

4.2.7. Staining performance of CD45 DNA-conjugated antibody

The CD45 DNA-tagged mAb was generated using the maleimide conjugation method (see above). To establish the efficacy of CD45 detection with the G&P assay and to compare the performance of the distinct conjugation methods, we implemented the same experimental approach as used for the other markers. This involved establishment of CD45 mAb IF staining on adhesion slide and further G&P staining using CD45 DNA-tagged mAb, followed by CD45 protein quantification.

CD45 is a pan-leukocyte marker commonly expressed on all hematopoietic cells, including lymphocytes, monocytes, and granulocytes [110]. Therefore, we selected PBMCs as a positive control for the establishment of the CD45 marker and the BT474 cell line as a negative control. Adhesion slides of PBMCs were stained using mouse anti-CD45 mAb and visualized via an anti-mouse Ig antibody conjugated with Alexa fluor 555. To optimize antibody concentration, a titration was performed using concentrations of 0.1, 0.5, 1, and 2 $\mu\text{g/ml}$. At 0.1 $\mu\text{g/ml}$, the signal was insufficient for CD45 detection. However, clear CD45 expression was observed starting from 0.5 $\mu\text{g/ml}$, with a significant enhancement in signal intensity at 1 $\mu\text{g/ml}$. No marked improvement was seen when the concentration increased from 1 $\mu\text{g/ml}$ to 2 $\mu\text{g/ml}$ (Figure 34). These findings identified 1 $\mu\text{g/ml}$ as the optimal concentration for subsequent experiments. To confirm specificity, an isotype control was conducted, which showed no detectable signal. Additionally, BT474 cells were stained with the

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highest antibody concentration (2 $\mu\text{g/ml}$), and no signal was observed, further affirming the specificity of the CD45 antibody for hematopoietic cells.

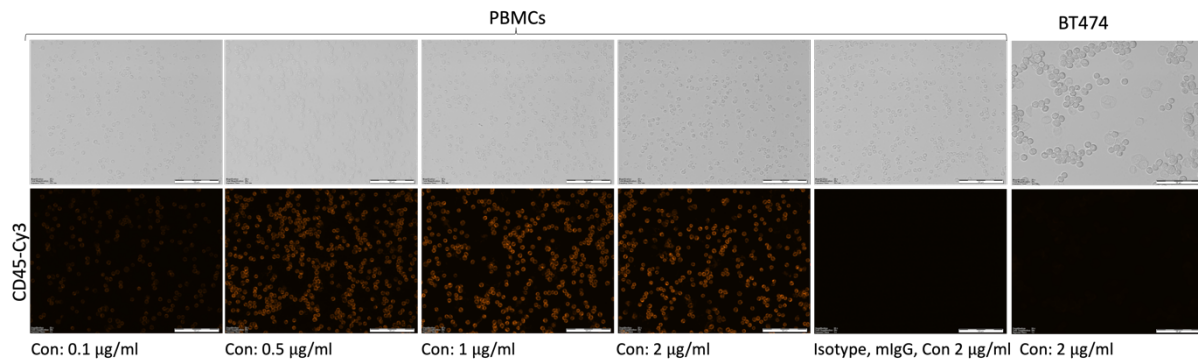


Figure 34. Anti-CD45 staining was established on PBMCs and BT474. Adhesion slides of PBMCs were stained with four concentrations of CD45 (0, 0.1, 0.5, and 1 $\mu\text{g/ml}$), with mIgG1 Alexa Fluor 555 detected in the Cy3 channel. A mouse IgG isotype control was included at the highest concentration, and the BT474 cell line was used as a negative control. Images were captured using an Olympus microscope (micrographs 20x magnification).

Following the successful establishment of CD45 staining, we aimed to test whether CD45 protein expression could be quantified using the G&P assay. To achieve this, adhesion slides of BT474 and PBMCs stained with CD45 DNA-tagged mAb and anti-mouse Ig antibody conjugated with Alexa fluor 555 for visualization and screened using Olympus (Figure 35A). Eight single cells from BT474 and PBMCs were then isolated, subjected to WGA, and high-quality samples were selected for CD45 protein quantification. qPCR analysis of the CD45 DNA tag revealed unexpected results: the average relative quantification value for BT474 was around 1, indicating no expression, while the quantification for PBMCs (expected to show high CD45 expression) was also below 1, suggesting a failure in accurately quantifying the CD45 DNA tag in this instance (Figure 35B). Given the unsuccessful in situ staining for CD45 and the inability to confirm the DNA conjugation of this marker through SDS-PAGE analysis, we concluded that the protein quantification was not achievable in this case, most likely due to insufficient amount of DNA-tagged antibody.

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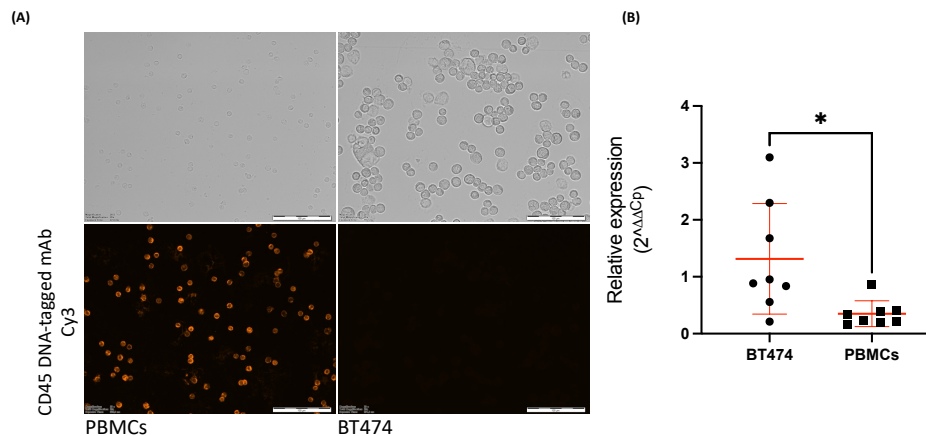


Figure 35. Quantification of CD45 protein expression. (A) IF staining was done using CD45 DNA-tagged mAb with mlgG1 Alexa Fluor 555 detected in Cy3 channel (micrographs 20x magnification). (B) qPCR-based quantification of CD45 DNA-tagged mAb. * $P < .05$. Mean $\pm$ SD, one-way ANOVA with Tukey's T test.

4.2.8. Multiplex G&P staining and qPCR quantification of HER2, pHER2 and CK DNA-tagged mAbs in BC cell lines

Following the establishment of each DNA-tagged in the G&P assay, the next objective was to determine if we could simultaneously quantify multiple protein expression levels. To achieve this, we selected cell lines previously used for the establishment of each marker. Specifically, MDA-MB-231, MDA-MB-453, and PBMCs were chosen for quantification of all three markers, as they exhibited low to medium expression levels for CK, HER2, and pHER2. The BT474 cell line was selected for its high expression of both HER2 and pHER2, while the MCF7 cell line displayed high CK expression. Adhesion slides of the cell lines were stained with CK, HER2 and pHER2 DNA-tagged mAbs. Since all three antibodies were from the same species (mlgG1), we used one secondary Ab for detection, resulting in a compound signal in IF staining (Figure 36A). Additionally, because multiple DNA-conjugated antibodies were used, we doubled the concentration of the blocking reagent to minimize background signal (5%BSA+ %DS1). Eight single cells from each cell line were isolated and subjected to WGA. Good quality samples were quantified for expression of proteins. To prevent overlapping signals of the TaqMan fluorophores, the qPCR for each marker was performed separately using specific TaqMan probes. The qPCR-based quantification of CK, HER2 and pHER2 revealed three distinct expression level for each protein and the same trend in protein expression, which were consistent with previous results when assessed individually (Figure 36B). This experiment demonstrated that multiplex

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G&P staining performed as effectively as the single staining workflow and proved several target proteins can be quantified in a multiplex G&P analysis.

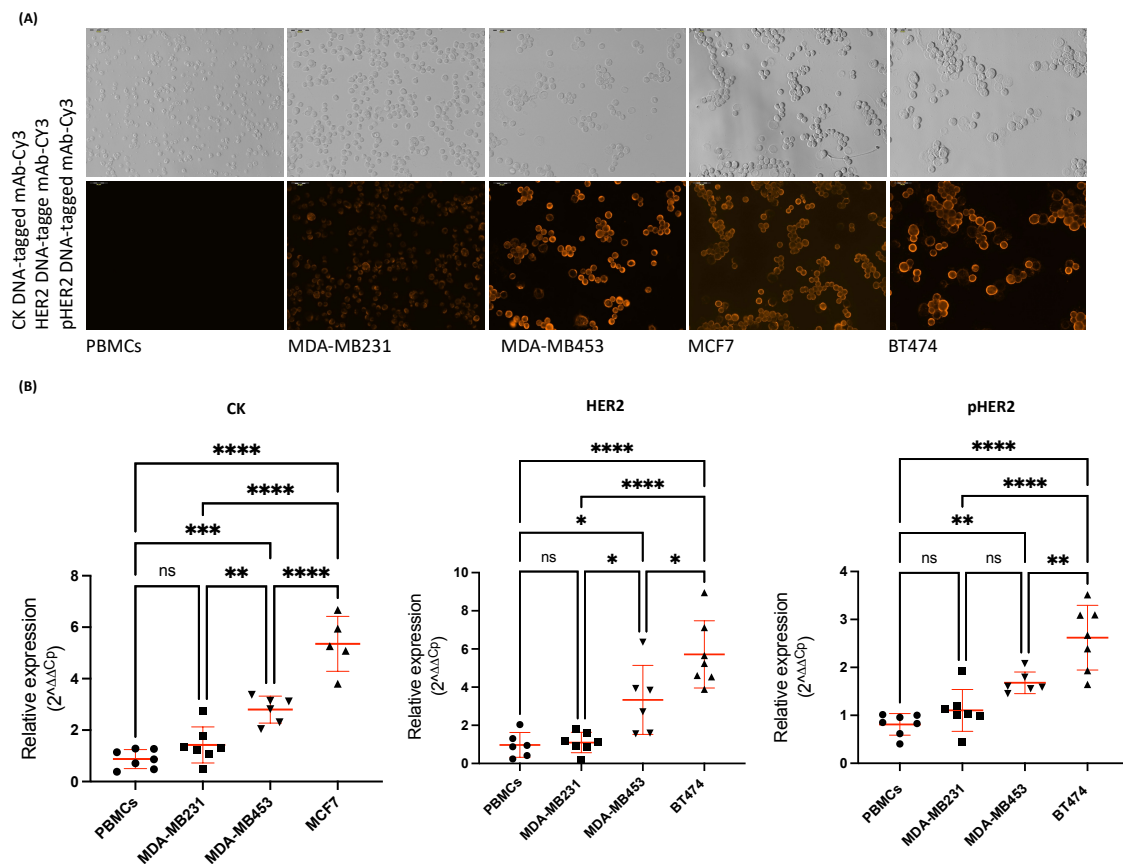


Figure 36. Multiplex G&P staining using CK, HER2 and pHER2 DNA-tagged mAb. (A) IF staining results for multiplex staining utilized CK, HER2, pHER2 DNA-tagged mAb with mlgG1 AF555 producing a compound signal detected in Cy3 channel (micrographs 20x magnification). (B) qPCR quantification of CK, HER2 and pHER2 showing different level of protein expression in BC cell lines. Asterisks represent the significance as follows: *** $P < .001$, ** $P < .01$, * $P < .05$ and ns: $P \geq .05$. Mean $\pm$ SD, one-way ANOVA with Tukey's multiple comparisons test.

4.2.9. Combined analysis of copy number changes and protein expression of the HER2 gene in single cells

Following the successful integration of DNA-tagged antibodies into the G&P assay and the subsequent verification of its robustness for precise protein quantification, the next aim was to assess the CNV profiles of WGA samples generated through the G&P assay. This analysis aimed to (i) assess the performance of the WGA and integrity of the single-cell genomes as well as (ii) correlate the CNV-derived copy numbers of the *ERBB2* gene with HER2 protein expression levels obtained via qPCR. To evaluate the accuracy of the measurements, cancer cell lines were selected that exhibited

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distinct levels of the copy number of the *ERBB2* gene and expression levels of HER2. For this purpose, we used MDA-MB-231 (HER2^{Lo}), MDA-MB-453 (HER2^{Medium}) and BT474 (HER2^{Hi}), consistent with those used in the FACS analysis. Adhesion slides of these cell lines were stained with HER2 DNA-tagged mAb (Figure 37A). Five single cells were isolated from each from each cell line and subsequently subjected to WGA. Samples with good genome quality were also quantified for the DNA-tag bound to the HER2 mAb in the WGA products. In parallel, isolated cells (two single cells per cell line) were subjected to CNV analysis using Ampli1 lowpass kit (Menarini Silicon Biosystems). Result of qPCR-based protein quantification showed that the quantities of anti-HER2 mAb measured in single-cell WGA product were significantly different between high, medium and low/negative HER2 expressing cell populations (Figure 37B). The CNV analysis conducted using Ampli1 low-pass sequencing further validated the feasibility of performing CNV profiling on cells subjected to G&P staining. In this context, the CNV profiles revealed a balanced profile for PBMCs and aberrant profile for cell lines consistent with their oncogenic alterations (Figure 37C). Additionally, we correlated CNV data with HER2 protein expression levels, as measured by qPCR. To achieve this, we extracted log2 values representing the *ERBB2* locus from the output of the CNV and performed three types of correlations. We correlated qPCR results with i: the average Log2 values calculated by the CNV pipeline, ii: raw Log2 values computed specifically for the *ERBB2* locus, along with genomic segments located ± 1 megabase (Mb) from it and iii: raw Log2 values calculated exclusively for the *ERBB2* locus. This comparative analysis showed that log2 lowpass values, computed specifically for *ERBB2* locus (iii), exhibited strongest correlation with qPCR measurements compared to log2 values computed across the region adjacent to *ERBB2* locus (i and ii). The first 2 correlations proved problematic primarily due to the MDA-MB-453 cell line. Although MDA-MB453 cells harbor a copy number of the *ERBB2* gene, due to its focal character (small size), presence of copy number losses in the vicinity and overall low levels of amplification, this event cannot be detected consistently by all type of assays (Figure 37D). Therefore, we proceeded with the last approach, focusing specifically on the *ERBB2* locus for more accurate correlation with protein expression levels. These findings highlighted the capacity of the G&P-assay to generate comprehensive, multiparametric measurements that integrate DNA copy number variations with protein expression levels, providing a

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robust method for linking genomic alterations with functional proteomic outcomes at the single-cell resolution.

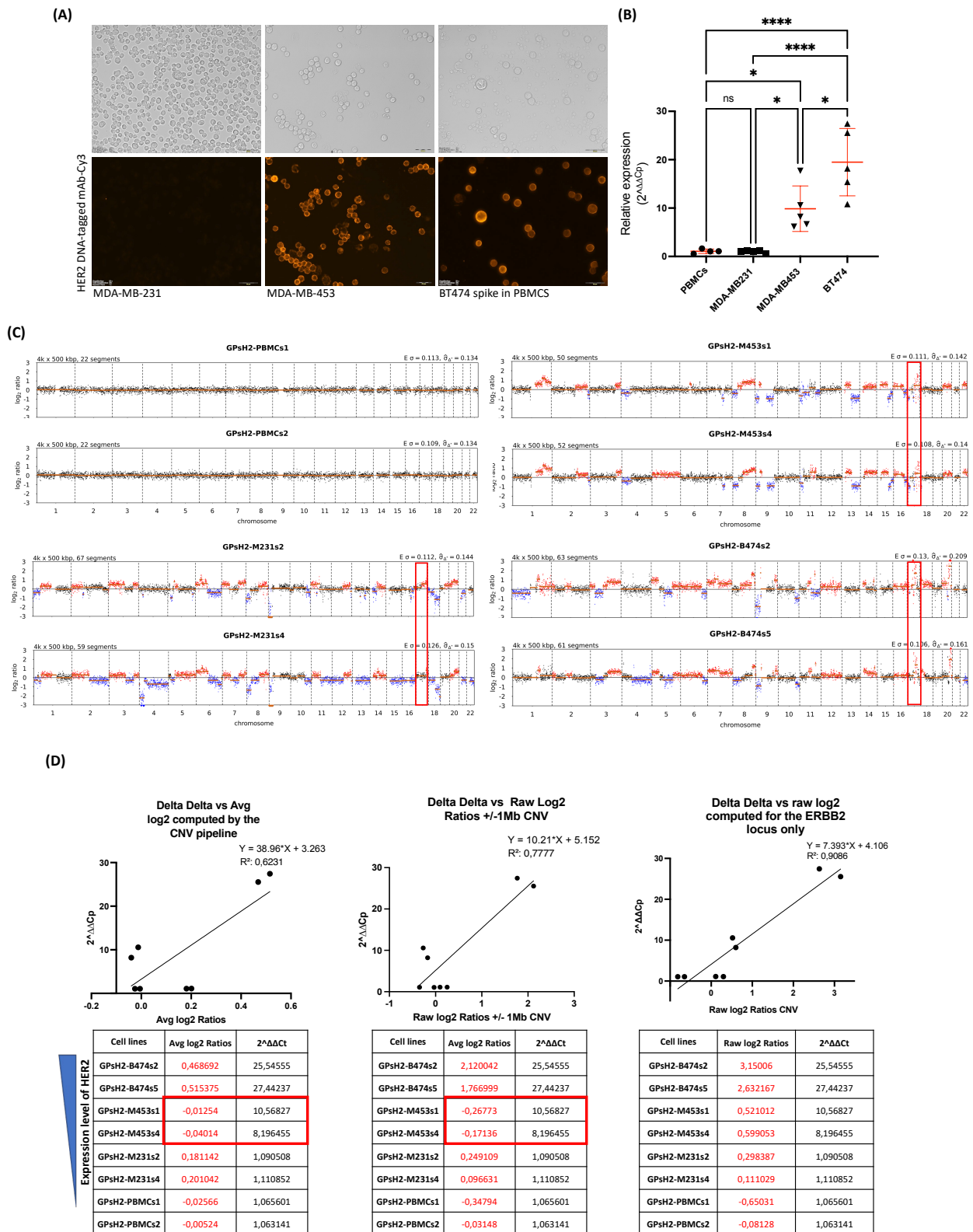


Figure 37. Comparative analysis of HER2 gene amplification and quantitative HER2 protein expression. (A) IF staining results of bound HER2 DNA-tagged mAb using anti-mouse Ig Alexa fluor 555-labelled antibodies (micrographs 20x magnification). (B) qPCR showing distinct level of HER2

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protein expression in BC cell lines. Asterisks represent the significance as follows: *** $P < .001$, ** $P < .01$, * $P < .05$ and ns: $P \geq .05$. Mean $\pm$ SD, one-way ANOVA with Tukey's multiple comparisons test. (C) 2 samples from each cell lines were used for CNV analysis. Histogram of genomic gains (red) and losses (blue) of PBMCs and cell lines with increasing copy number of *ERBB2* locus. Red brackets indicate the *ERBB2* locus on chromosome 17. (D) Three types of correlations between protein expression measured by qPCR and *ERBB2* gene amplification using values derived from the CNV analysis by low pass sequencing. Scatter plots indicate the linear correlation between protein expression levels of HER2 (Y-axis) vs. corresponding average/raw Log2 ratios extracted from CNV analysis (X-axis). The accompanying table provides the corresponding data values.

4.2.10. Validation of G&P assay using anti-HER2 drug

To further test whether dynamic changes in protein phosphorylation can be captured by the G&P assay, we used the tyrosin-kinase inhibitor lapatinib, which reversibly binds to the ATP-binding sites within the cytoplasmic domains of EGFR/HER1 and HER2 receptors, inhibiting tyrosine kinase phosphorylation. We hypothesized that the effects of the drug treatment on the expression of active/phosphorylated HER2 will be detectable at the single-cell level using the G&P assay.

For this, we selected two breast cancer cell lines: BT474 (HER2-amplified/overexpressing, lapatinib-sensitive) and MDA-MB-468 (HER2-negative, lapatinib insensitive). First, we determined the optimal cell density by culturing the cell lines in 96-well plates at different concentrations, from 14K to 22K cells per well, and allowing them to grow for 7 days. Cell proliferation and confluency were continuously monitored using the Incucyte system (BioScience), which provided real-time tracking of cell growth dynamics. The optimal cell density that resulted in roughly 30-40% confluency after 24 hours was chosen based on the growth curve. For the MDA-MB-468 cell line, the highest seeding concentration of 22K cells per well resulted in approximately 35% confluency after 24 hours, making this concentration suitable for further experiment (Figure 38A). In contrast, for the BT474 cell line, the same concentration (22K cells per well) yielded only 20% confluency at the 24-hour mark (Figure 38B). To achieve the desired 30-40% confluency, we increased the seeding density of BT474 to 25K cells per well for subsequent experiments. This adjustment ensured optimal cell growth conditions, enabling accurate assessment of lapatinib's effects in both cell lines.

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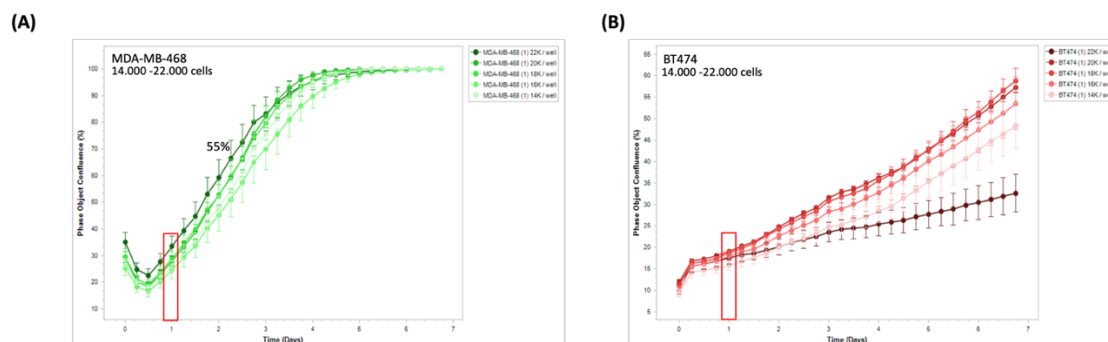


Figure 38. Optimization of seeding density. Cell confluency measurements for BT474 (A) and MDA-MB-468 (B) cell lines over 7 days. Cells seeded at five different densities into 2 plates and incubated for 7 days. Based on the results of this experiment, a seeding density of 22K cells for a 24-hour experiment was determined to be optimal for MDA-MB-468. However, an increased density of 25K cells was required to achieve optimal growth for BT474 after 24 hours.

Next, we aimed to identify the drug concentration, at which cells were not killed but HER2 phosphorylation likely to be inhibited. Cells at the selected density were cultured in two separate 96-well plates. After 24 hours of incubation, the cells were treated with lapatinib concentrations of 0.001, 0.01, 0.1, 1, and 10 μM , and cultured for an additional 48 hours. At the time of harvesting, one plate was used to assess confluency and apoptosis by measuring ATPase activity using the ATPlite assay, while the second plate was harvested to collect cells, which were then deposited on adhesion slides for subsequent IF staining.

Cell growth and apoptosis measurements of the BT474 cell line revealed that the lower concentrations of 0.001 and 0.01 μM had no significant effect on cell growth, as their growth patterns closely resembled those of the untreated control. In contrast, the highest concentration of 10 μM was found to be highly cytotoxic, resulting in approximately 80% cell death (Figure 39A). Given the lack of efficacy at the lowest concentrations and the excessive toxicity at 10 μM , these three concentrations were excluded from IF staining and further analysis. MDA-MB-468 cells used as control were insensitive to lapatinib at all concentrations, with the exception of 10 μM (Figure 39B). At this concentration, lapatinib reduced cell growth; however, it is likely that this concentration was cytotoxic, as approximately 60% of the cells were killed.

Results

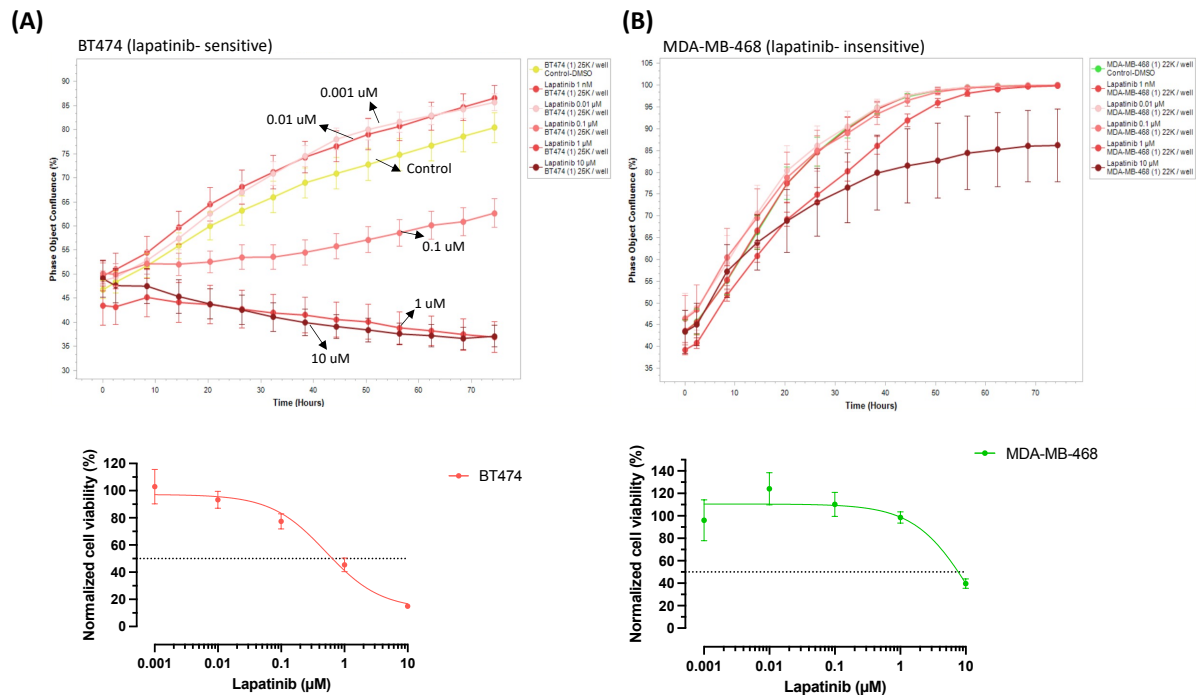


Figure 39. Identification of suitable drug concentrations to assess phosphorylation changes. Confluency measurements following drug treatment at five different concentrations over 72 hours in BT474 (A) and MDA-MB-468 (B) cell lines. ATPase activity was measured using the ATPlite assay, which assessed cell viability based on the light production resulting from the reaction of ATP with added luciferase and D-luciferin.

To assess HER2 phosphorylation sites, Tyr1248 and Tyr1221/1222, as well as total HER2, were evaluated. Double IF staining was conducted on BT474 cells untreated and treated with 0.1 μM and 1 μM lapatinib using mouse anti-HER2 and rabbit anti-pHER2 at Tyr1221/1222 monoclonal antibodies. Visualization was achieved with secondary antibodies, including anti-mouse Ig Alexa Fluor 555 and anti-rabbit Ig Alexa Fluor 488 respectively. Additionally, a separate staining procedure was performed using mouse anti-pHER2 (Tyr1248) and anti-mouse Ig Alexa Fluor 555 for visualization, as this antibody shared the same species as the HER2 antibody. IF staining showed that 0.1 μM lapatinib reduced phosphorylation at both HER2 sites without affecting total HER2 expression, while the reduction in phosphorylation became more pronounced with the 1 μM concentration. Notably, 1 μM lapatinib significantly diminished HER2 phosphorylation while maintaining 80% cell viability, making it the optimal concentration for further experiments. (Figure 40).

Results

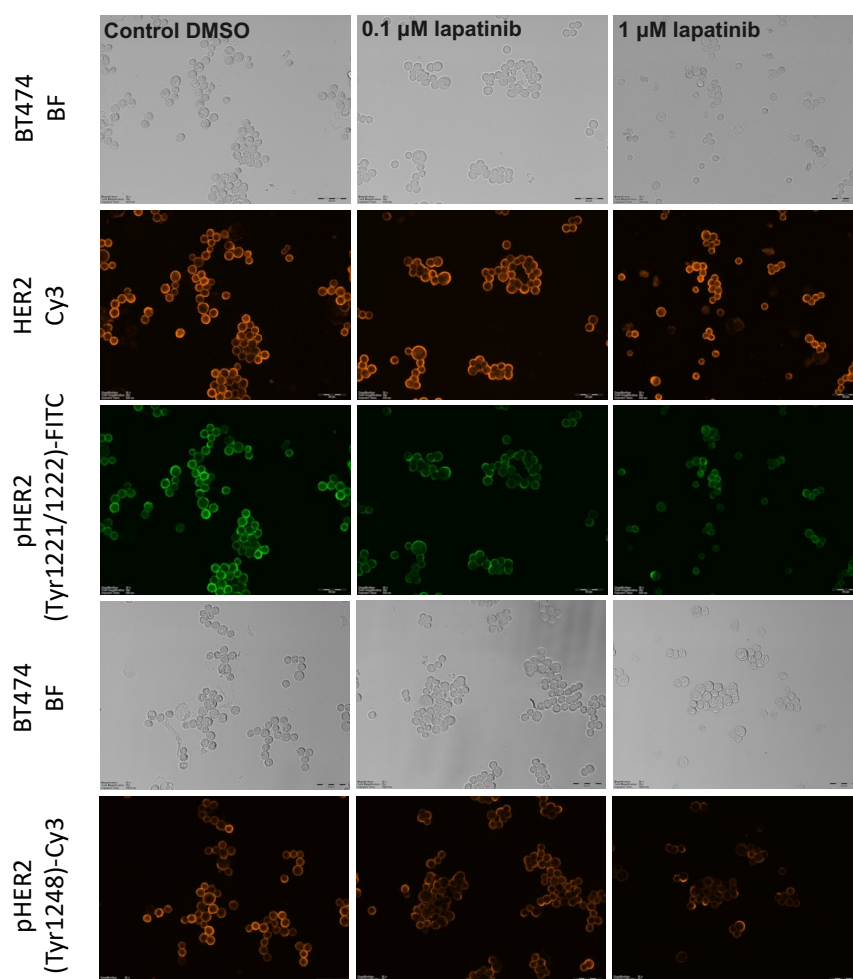


Figure 40. Effect of lapatinib on HER2/pHER2 protein expression. Double IF staining against HER2 and pHER2 (Tyr1221/1222) on untreated and treated BT474 cells (0.1 μ M and 1 μ M Lapatinib). A separate staining was also conducted against pHER2 (Tyr1248). While total HER2 expression remained unaffected by Lapatinib treatment, the impact on pHER2 expression at both phosphorylation sites became apparent at 0.1 μ M, with more pronounced effects observed at 1 μ M. Screening was conducted using an Olympus microscope (20x magnification).

Following the optimization of cell density for each cell line and drug concentration (1 μ M lapatinib), the experiment was repeated for the G&P assay. In contrast to the first experiment, only one phosphorylation site (Tyr1248) was evaluated in the G&P assay. It is important to note that since both markers share the same antibody species, IF detection was performed using anti-mouse Ig Alexa fluor 555. Consequently, the signal obtained was a compound signal, reflecting the combined expression of both HER2 and pHER2 (Tyr1248) (Figure 41A). Following staining, single cells were isolated and subjected to WGA. Subsequent qPCR analysis using HER2 and pHER2 DNA-tagged mAb revealed a significant reduction in pHER2 expression in BT474 cells treated with lapatinib, while total HER2 expression remained unchanged. In contrast,

Results

the lapatinib-insensitive MDA-MB-468 cell line, exhibited no alterations in either HER2 or pHER2 expression following treatment (Figure 41B). These findings indicated that the inhibitory effects of lapatinib can be effectively quantified by the G&P assay in sensitive cells.

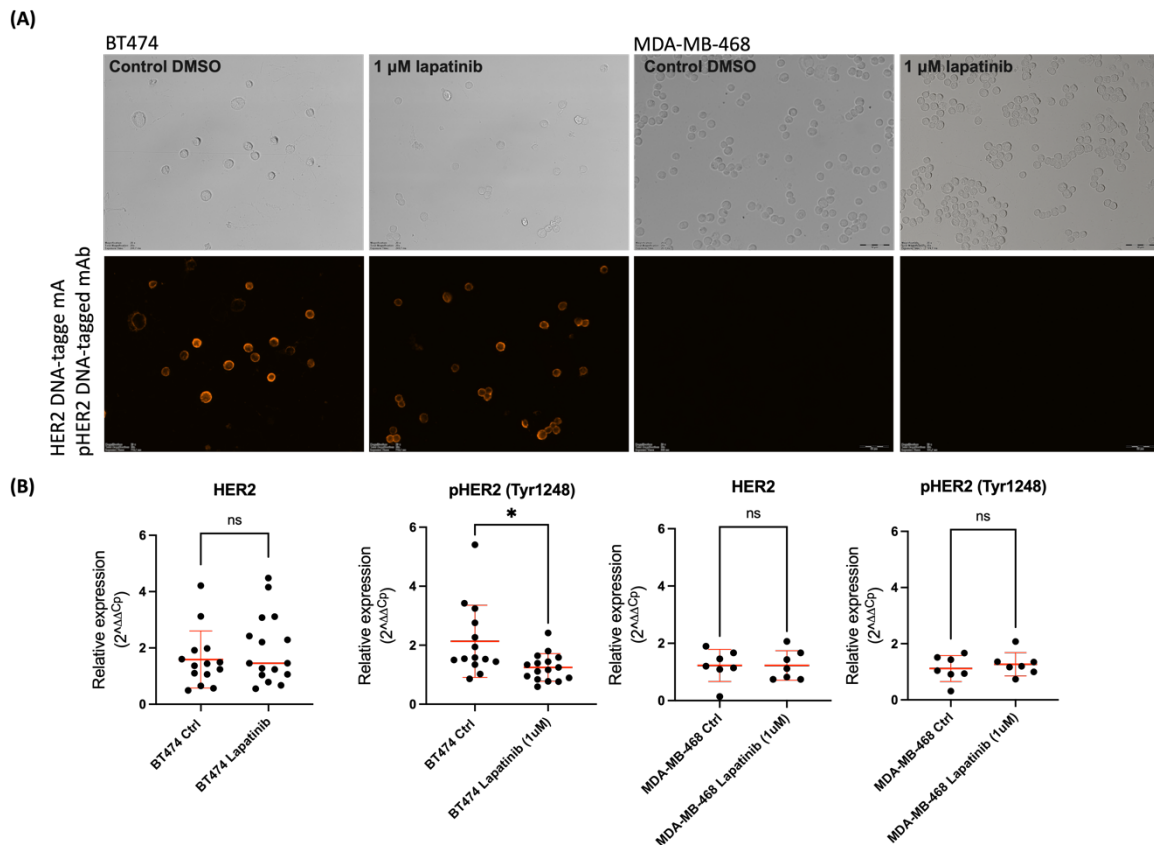


Figure 41. G&P staining and qPCR-based quantification of HER2 and pHER2 expression. (A) G&P staining using HER2 and pHER2 (Tyr1248) DNA-tagged mAb on BT474 and MDA-MB-468 cells, both untreated and treated with 1 μM lapatinib. Visualization was done using Alexa fluor 555. (B) Representative qPCR-based quantification of HER2 and pHER2 DNA-tagged mAb showing the consistent drug effect as observed with IF staining. Asterisk represent the significance as follows: * $P < .05$ and ns: $P \geq .05$. Mean $\pm$ SD, one-way ANOVA with Tukey's multiple comparisons test.

4.2.11. Expanding the G&P assay for pathway activation analysis

Following the successful establishment and optimization of a G&P assay to measure expression levels of HER2 and pHER2 in single cells, we aimed to expand the analysis to additional target proteins for a deeper understanding of cellular signaling. Since protein quantification alone may not accurately reflect the protein's active state, we focused on the phosphorylation sites that indicate proteins activation. Specifically, we selected phospho-ERK1/2 (Thr202/Tyr204; pERK1/2), phospho-AKT (Ser473; pAKT),

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and phospho-STAT3 (Tyr705; pSTAT3) as informative signaling molecules of the activated pathways.

4.2.11.1. Establishment of IF staining for pAKT, pERK and pSTAT3

To establish phospho-protein staining, we started with the anti-mouse pERK antibody. Since intracellular phospho-protein staining is known to be challenging, I performed a series of experiments, as outlined below:

To optimize pERK antibody concentration, a titration was performed using concentrations of 0.5, 1, and 2 µg/ml. For this, we used adhesion slides of the MDA-MB-231 cell line, which is reported in the literature to express pERK [111]. However, our initial attempts yielded no detectable signal. (Figure 42A).

To evaluate the expression of total ERK in MDA-MB-231, we selected an antibody targeting the endogenous levels of total p44/42 MAP kinase (ERK1/2) protein, along with pERK1/2 Ab. The results revealed robust expression of total ERK in this cell line, but no detectable pERK expression (Figure 42B).

To further assess the expression of pERK1/2 by other cell lines, we selected the MCF10A-HER2-tet-inducible model (generated previously in our lab). As a control, we included MCF10A cells without doxycycline treatment. Additionally, we utilized BT474 cell line, which is highly HER2-positive and known to express pERK1/2 [111]. However, we were unable to detect any signal in either of the cell lines. These results highlighted the need for further optimization of the staining protocol (Figure 42C).

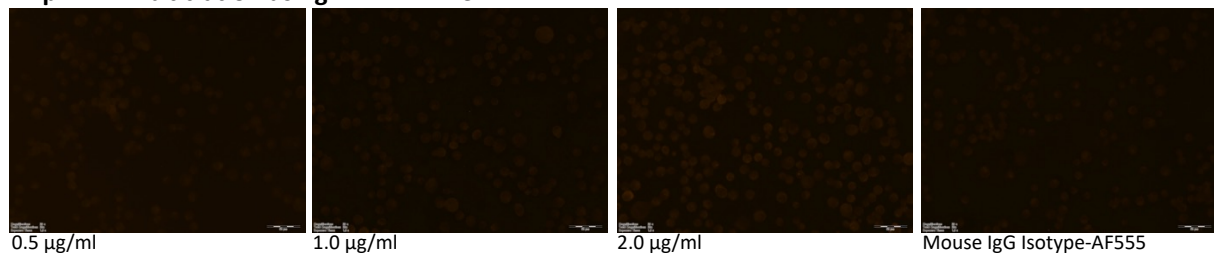
Consulting the literature on phospho-protein staining we found a study, which demonstrated that a combination of fixation with paraformaldehyde (PFA) and permeabilization with ice-cold methanol yielded the most effective phospho-protein staining [112]. We utilized the previously tested cell line, MDA-MB-231, as well as an additional TNBC cell line, MDA-MB-468. The results were significantly improved, showing clear intracellular pERK staining patterns in both cell lines and clean isotype controls (Figure 42D).

To establish pAKT staining, we selected the MCF7 and MDA-MB-468 cell lines, both of which known to express pAKT [113]. The cell lines were stained using Rabbit IgG anti-pAKT Ab and an anti-rabbit Ig Alexa fluor 488, which resulted in successful visualization of intracellular pAKT staining (Figure 42E).

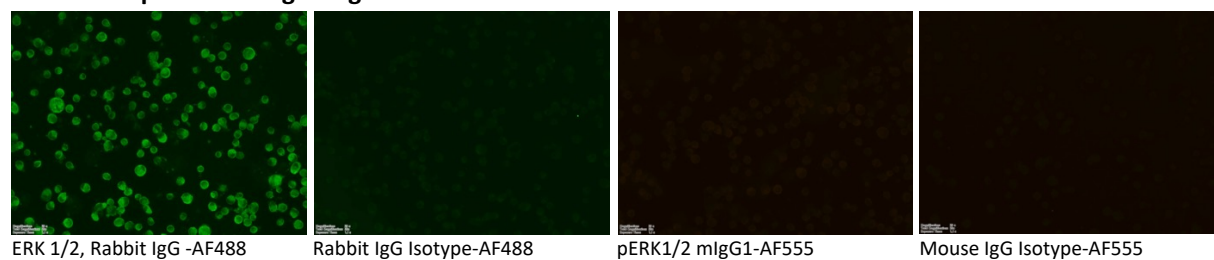
Results

To establish pSTAT3 staining, we selected two TNBCs including MDA-MB-231 and MDA-MB-468 known to express pSTAT3 [114]. The cell lines were stained with mouse IgG anti-pSTAT3 antibody and an anti-mouse Ig Alexa fluor 555 for visualization. The staining results demonstrated consistent intracellular pSTAT3 expression, revealing lower levels in MDA-MB-231 and significantly higher expression in MDA-MB-468 (Figure 42F). Overall, we successfully established phospho-protein staining protocols for pERK, pAKT, and pSTAT3, allowing their incorporation into the G&P assay.

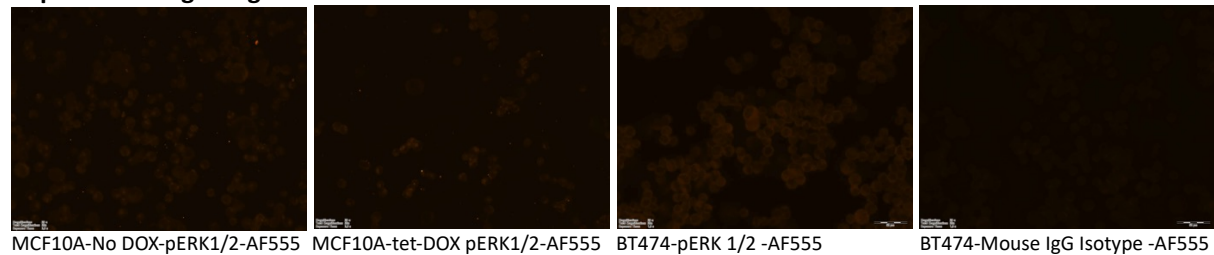
A: pERK mAb titration using MDA-MB-231



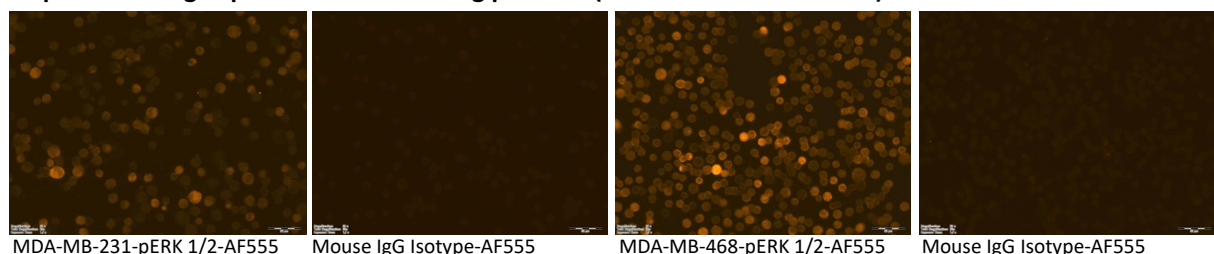
B: ERK and pERK staining using MDA-MB-231



C: pERK staining using MCF10A-tet-inducibel and BT474

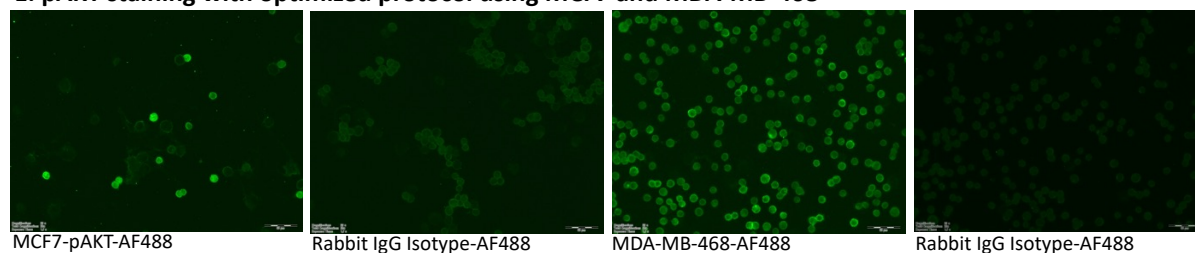


D: pERK staining- Optimization of staining protocol (PFA + Ice-cold methanol)



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E: pAKT staining with optimized protocol using MCF7 and MDA-MB-468



F: pSTAT3 staining with optimized protocol using MDA-MB-231 and MDA-MB-468

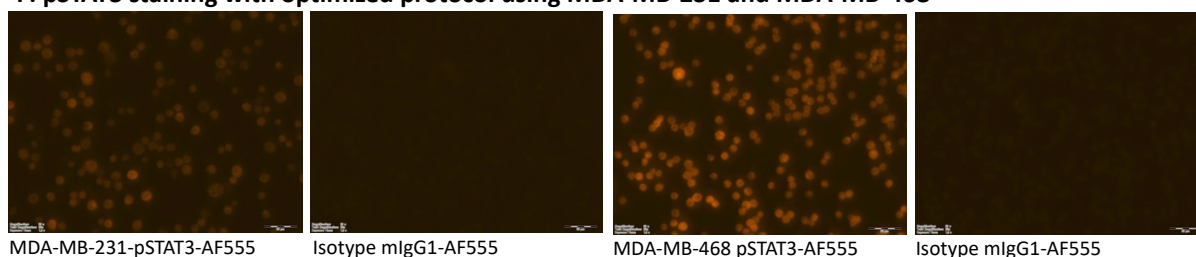


Figure 42. Phospho-protein detection of pERK1/2, pAKT and pSTAT3. (A-D) IF staining results using mouse anti-pERK1/2. Three concentrations of antibody including 0.5, 1, and 2 $\mu\text{g/ml}$ were tested. (E and F) IF staining results against anti-AKT and anti-STAT3 using the optimized protocol. Screening was performed with an Olympus microscope (micrographs 20x magnification).

4.2.11.2. Antibody-DNA conjugation of pAKT, pERK and pSTAT3

mAb

We next performed antibody-oligo conjugation using the LASIC method. For each antibody, we designed a specific oligonucleotide with a distinct tag sequence and ordered the chemistry kit from Alphathera. After receiving the oYo-link oligos, each antibody was conjugated to its corresponding oligo (see 3.5.1). Validation of each antibody-oligo was done using SDS-PAGE gel electrophoresis. In all three cases, we observed an upward shift of the heavy chain, confirming the successful conjugation of the heavy chain to the oYo-link oligo comparing to its unconjugated counterpart. However, the efficiency of conjugation varied based on the antibody species and isotype and the purity of the oYo-link oligo. The pERK1/2 DNA-tagged (mIgG2b) antibody showed a clear band above the heavy chains (HC) and a significant reduction in HC intensity in the conjugated antibody compared to the unconjugated antibody (Figure 43A). The pAKT DNA-conjugated antibody displayed two bands above the HC. According to the manufacturer, the chemically modified oligo was a mixture of oYo-link oligos and oYo-link alone, both of which could conjugate to the heavy chain, resulting in the appearance of two bands (Figure 43B). Lastly, for pSTAT3 DNA-tagged mAb, a band above the HC indicated less efficient conjugation compared to

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the other markers (Figure 43C). This reduced efficiency was attributed to the mouse IgG1 isotype, which according to the manufacturer has a low conjugation efficiency. However, all DNA-tagged mAb were then tested in the G&P assay.

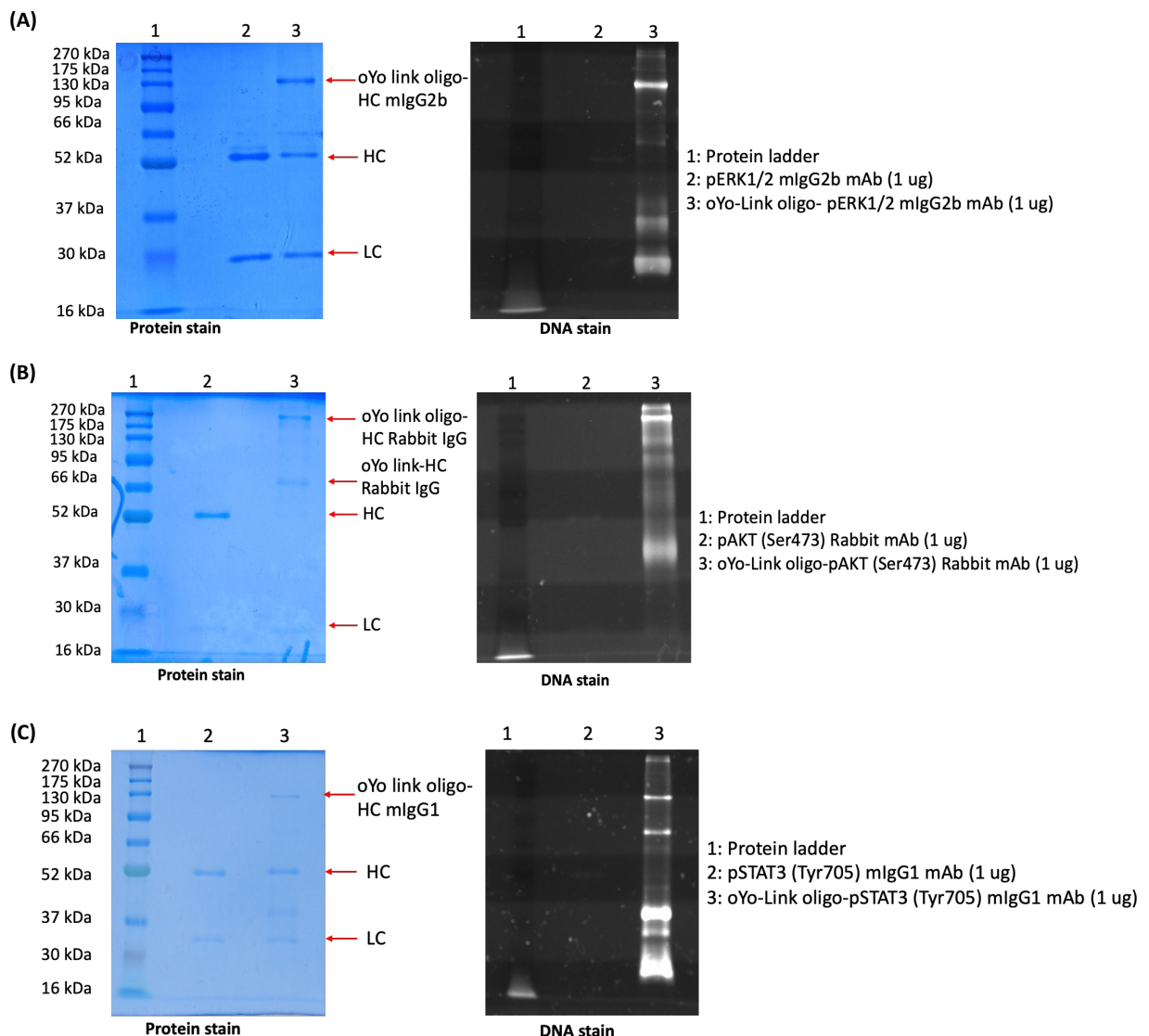


Figure 43. SDS-PAGE gel of pERK, pAKT and pSTAT3 Ab-DNA conjugation using LASIC method. (A-C): Gels were stained with coomassie blue and ethidium bromide for protein and DNA visualization comparing the conjugated antibody (third lane) to the unconjugated antibody (second lane). HC: heavy chain, LC: light chain.

4.2.11.3. Comparative pERK1/2, pAKT and pSTAT3 quantification in single cells

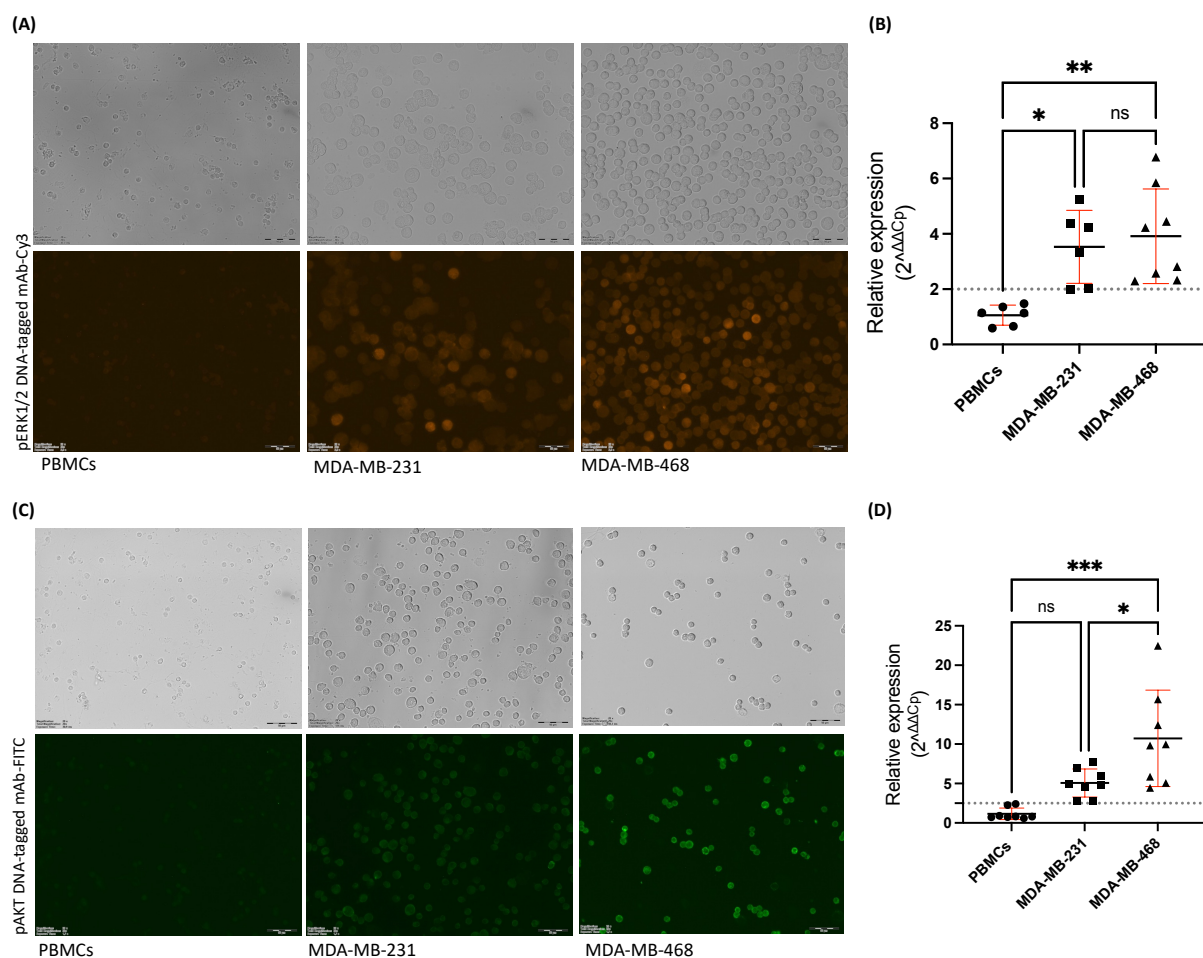
To establish phospho DNA-tagged antibodies in the G&P assay, we applied the same approach used for HER2 and pHER2 across MDA-MB-231, MDA-MB-468, and PBMCs as controls.

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For pERK1/2, IF staining showed intracellular staining in MDA-MB-231 and MDA-MB-468 compared to PBMCs (Figure 44A), with relative quantification revealing significant expression differences between the cell lines and PBMCs (Figure 44B).

For pAKT, IF staining revealed a low expression in MDA-MB-231 and higher expression in MDA-MB-468 (Figure 44C). Relative quantification confirmed significant differences in pAKT expression between MDA-MB-468 and MDA-MB-231, but no significant differences were observed between MDA-MB-231 and PBMCs (Figure 44D).

For pSTAT3, IF staining showed higher expression in MDA-MB-468 compared to MDA-MB-231 (Figure 44E). Relative quantification also demonstrated significant differences in pSTAT3 expression between MDA-MB-468 and MDA-MB-231 consistent with IF staining (Figure 44F).



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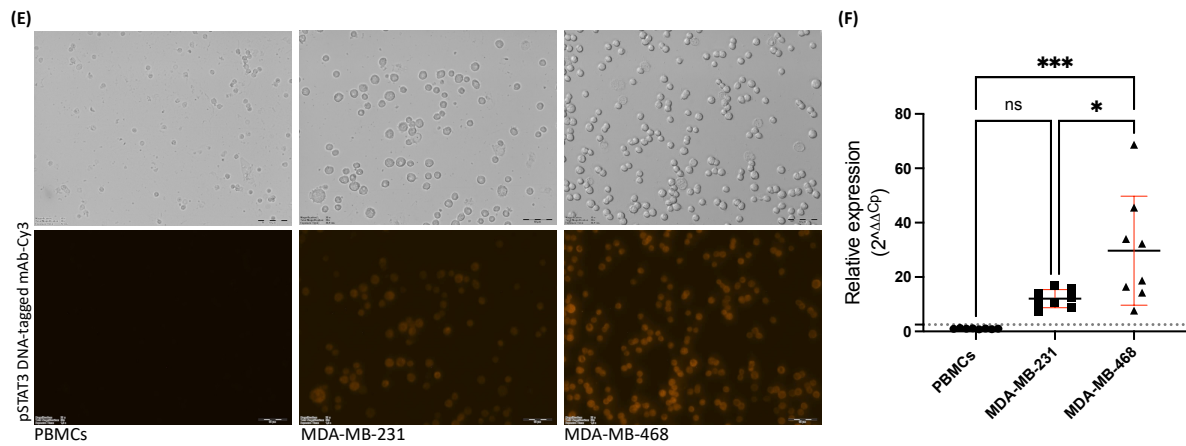


Figure 44. Quantification of pERK1/2, pAKT and pSTAT3 expression via DNA-conjugated antibodies. (A) IF staining of bound pERK1/2 DNA-tagged mAb with mouse IgG Alexa Fluor 555. (B) qPCR-based quantification of pERK1/2 indicating different levels of protein expression. (C) IF staining of bound pAKT DNA-tagged mAb with rabbit IgG Alexa Fluor 488. (D) qPCR-based quantification of pAKT. (E) IF staining of bound pSTAT3 DNA-tagged mAb with mouse IgG Alexa Fluor 555. (F) qPCR-based quantification of pSTAT3. Screening was performed with an Olympus microscope (micrographs 20x magnification). Asterisks represent the significance as follows: *** $P < .001$, ** $P < .01$, * $P < .05$ and ns: $P \geq .05$. Mean $\pm$ SD, one-way ANOVA with Tukey's multiple comparisons test.

4.2.12. Multiplex phenotyping in single cancer cells using G&P assay

We next aimed to test the method for the analysis of several signaling pathways critical for cancer cell proliferation and survival. Adhesion slides of cell lines were stained using biotinylated CK-streptavidin Alexa fluor 488 and EpCAM-PE and microscopically screened. A mix of DNA-tagged mAbs including HER2, pHER2 (Tyr1248), pERK1/2, pAKT, pSTAT3 DNA-tagged mAbs was used in the staining for subsequent quantification. Since most of the markers were derived from the same species (mouse IgG), their differentiation in IF staining was limited. IF staining revealed a high expression of both CK and EpCAM in BT474 cells, while MDA-MB-231 cells exhibited very low levels of CK and no positive signal for EpCAM. The MDA-MB-468 cells displayed low to medium expression of both CK and EpCAM. PBMCs, used as a negative control, further validated the specificity of these expressions (Figure 45).

Results

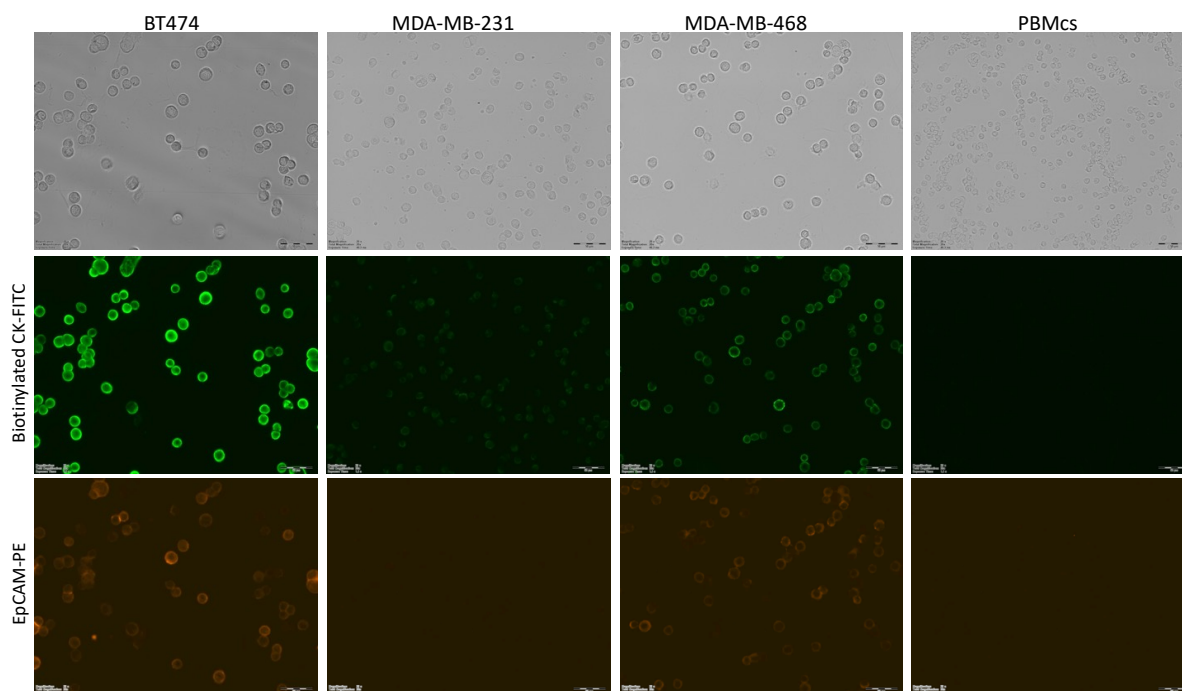


Figure 45. Multiplex G&P staining on BC cell lines. IF staining of BC cell lines and PBMCs using biotinylated CK-Streptavidin Alexa Fluor 488 and EpCAM-PE markers as detection marker. DNA-tagged mAbs used in the staining were quantified using qPCR.

Next ten to fifteen individual cells were isolated and subjected to WGA. Good quality samples were selected for protein quantification. To assess the protein expression level, specific TaqMan probes were designed for each DNA tag and qPCR was conducted separately. The results are summarized in figure 45. BT474 cell line showed high HER2 expression and phosphorylation (pHER2), while the TNBC cell lines showed minimal HER2 expression and lacked HER2-mediated signaling (Figure 46A, B). MDA-MB-468 cells exhibited moderate levels of pERK and pAKT, while MDA-MB-231 displayed moderate pERK but low pAKT expression (Figure 46C, D). BT474 showed significantly higher pERK, pAKT, and pSTAT3 levels compared to the TNBCs (Figure 46E). The table visually highlights these differential protein expression patterns (Figure 46F). Overall, the multiplex G&P staining allowed for the simultaneous identification and characterization of single cancer cells, providing a potential application of established assay for signaling pathway analysis.

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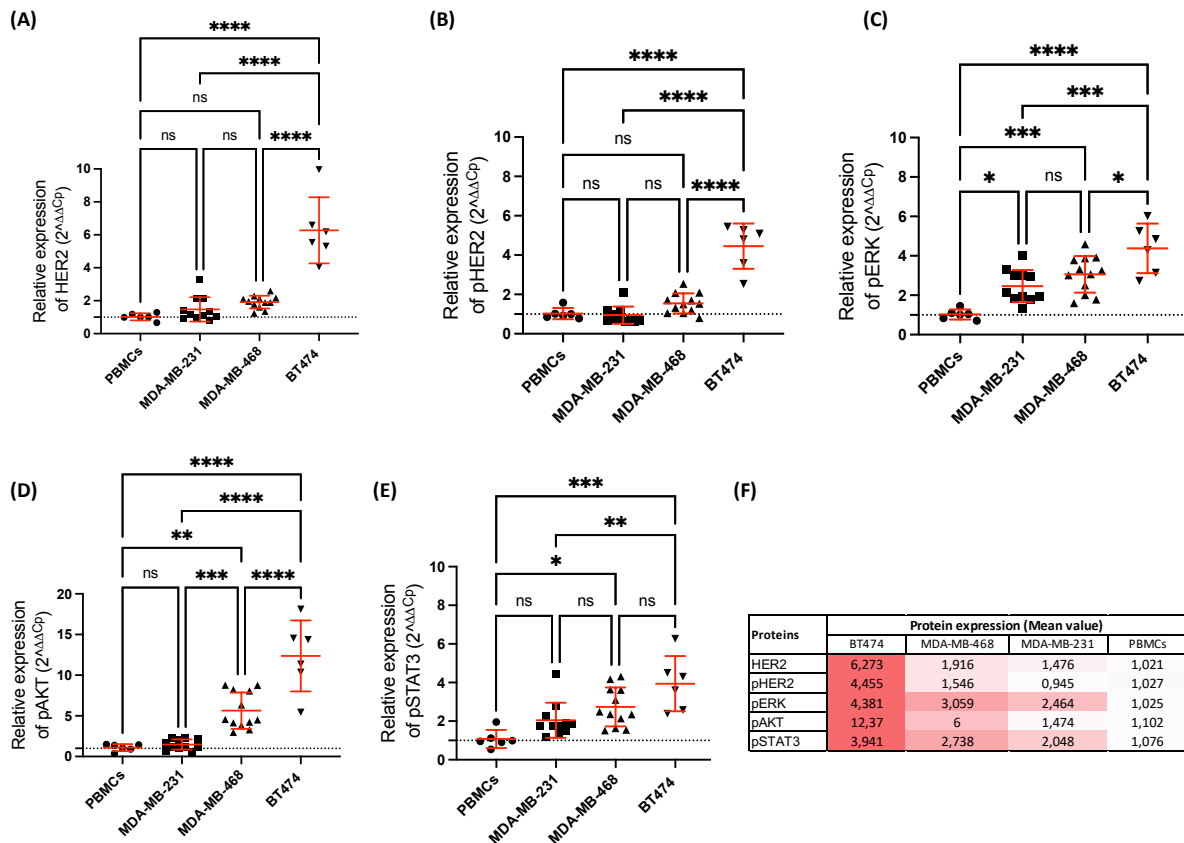


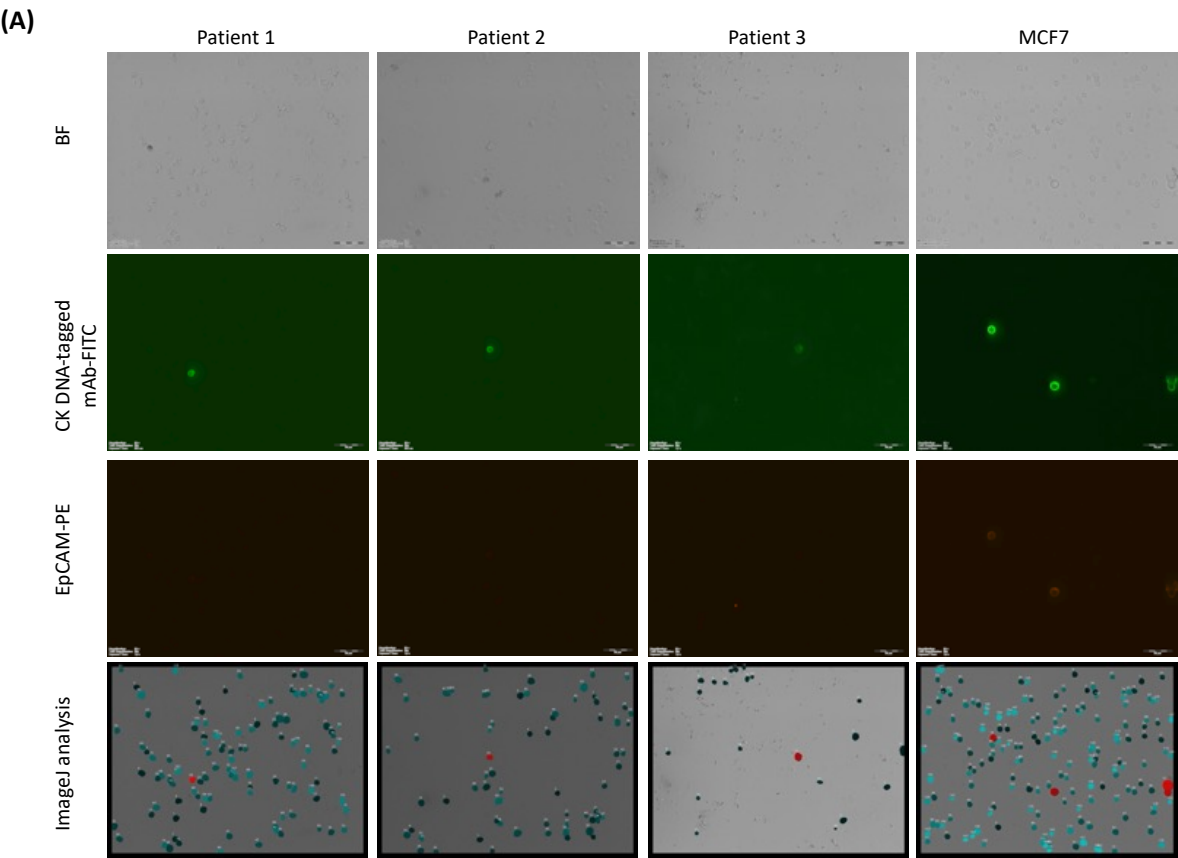
Figure 46. Multiplex phenotyping in single cancer cells using qPCR. (A-E): qPCR quantification of HER2, pHER2, pERK1/2, pAKT and pSTAT3 in BC cell lines and PBMCs. F: The heatmap illustrates the mean expression levels of all proteins obtained through qPCR across PBMCs and the cell lines. Asterisks represent the significance as follows: *** $P < .001$, ** $P < .01$, * $P < .05$ and ns: $P \geq .05$. Mean $\pm$ SD, one-way ANOVA with Tukey's multiple comparisons test.

4.2.13. Quantification of CK expression in single BM-derived DCC

To assess the feasibility and reliability of the G&P assay in patient samples, we aimed to determine its sensitivity, specificity, and robustness in detecting and quantifying CK expression in bone marrow-derived DCCs from breast cancer patients. To achieve this, BM-derived adhesion slides of three breast cancer patients were stained for CK DNA-tagged mAb and EpCAM-PE. Anti-CK antibody binding was detected using an anti-mouse Ig Alexa Fluor 488. Additionally, MCF7 cells were used as a positive control. The CK antibody was conjugated to a DNA-tagged mAb, allowing for subsequent quantification of the protein using qPCR. The stained samples were then screened with an Olympus microscope, and single cells positive for CK (FITC) and EpCAM (Cy3) were isolated using micromanipulation and subjected to WGA for further analysis.

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One adhesion slide per patient was screened; however, no EpCAM⁺ cells were detected (Figure 47A). Despite this, we successfully isolated CK⁺ DCCs and CK⁻/EpCAM⁻ normal cells from each patient for WGA analysis. The disseminated cancer cell density (DCCD, DCC number per million mononuclear cells) for each patient and number of isolated cells are summarized in the table (Figure 47B). qPCR analysis involved two separate comparisons. The first comparison showed a significant difference in CK expression between DCCs and normal cells comparing only these two groups (Figure 47C). In a multiple comparison, MCF7 cells demonstrated significantly higher CK expression compared to DCCs. While DCCs exhibited a 3.1-fold increase in CK expression relative to normal cells, this difference did not reach statistical significance (Figure 47D). ImageJ analysis was also performed, with a larger number of MCF7 (N=27) and normal cells (N=685) analyzed for CK expression. Image-based analysis showed similar trend in CK protein expression, revealing a significant difference in fluorescent intensity of CK expression between normal cells and both DCCs and MCF7 cells (Figure 47D).



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(B)

Sample	DCCD	Number of CK ⁺ cells isolated	Number of CK/EpCAM ⁺ cells isolated
Patient 1	4	6 cells isolated > 4 cells passed QC	10 cells isolated > 3 cells passed QC
Patient 2	3,5	6 cells isolated > 1 cells passed QC	5 cells isolated > 2 cells passed QC
Patient 3	3,5	6 cells isolated > 3 cells passed QC	8 cells isolated > 4 cells passed QC
MCF7 cell line	N/A	15 cells isolated > 10 cells analysed in qPCR	N/A

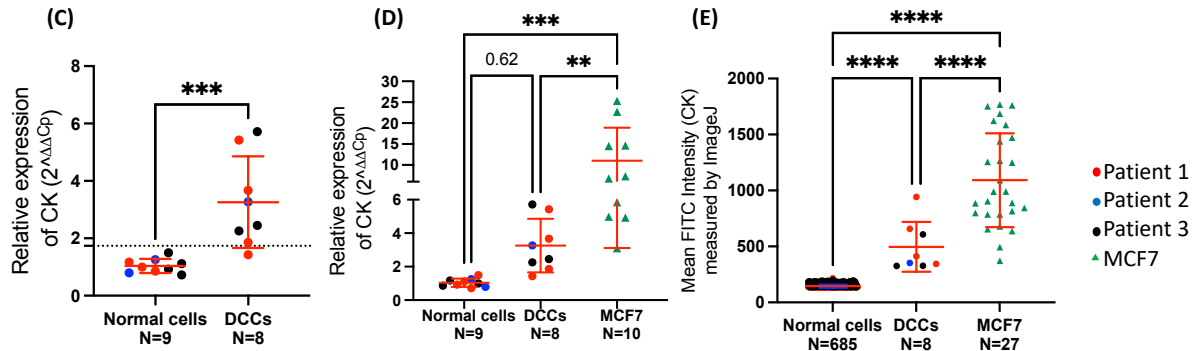


Figure 47. Quantification of CK in single DCCs using qPCR and ImageJ Analysis. (A): IF staining of bone marrow adhesion slides of three patients using CK DNA-tagged mAb-Alexa Fluor 488 and EpCAM-PE. One representative CK⁺ cell from each patient is shown (micrographs 20x magnification). MCF7 cells were also stained as a positive control against CK and EpCAM expression. (B) Table summarized DCCD, number of cells isolated and passed QC for each patient. (C) qPCR analysis showing relative CK expression in normal cells vs. DCCs. ****P* < .001, Mean ± SD, t-test (D); qPCR analysis comparing relative CK expression between normal cells, DCCs and MCF7 cell line. ****P* < .001, ***P* < .01, Mean ± SD, one-way ANOVA with Tukey's multiple comparisons test. (E) ImageJ analysis displays the FI of CK across the three groups.

To evaluate the accuracy of CK quantification in DCCs, we measured the sensitivity and specificity of the method. To determine these metrics, a threshold was defined based on the mean value of normal cells plus three times the standard deviation (3xSD). This threshold ensured the reliability of the method by minimizing the detection of false positives. Based on this threshold, we calculated the number of true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN). These values were then used to calculate the specificity, sensitivity, and positive predictive value (PPV) of the G&P assay. The results showed a specificity of 100%, a sensitivity of 94%, (with detection of one FN) and a PPV of 100%. The final qPCR graph mirrored the initial qPCR analysis, but with the newly defined threshold applied, clearly distinguishing DCCs from normal cells (Figure 48).

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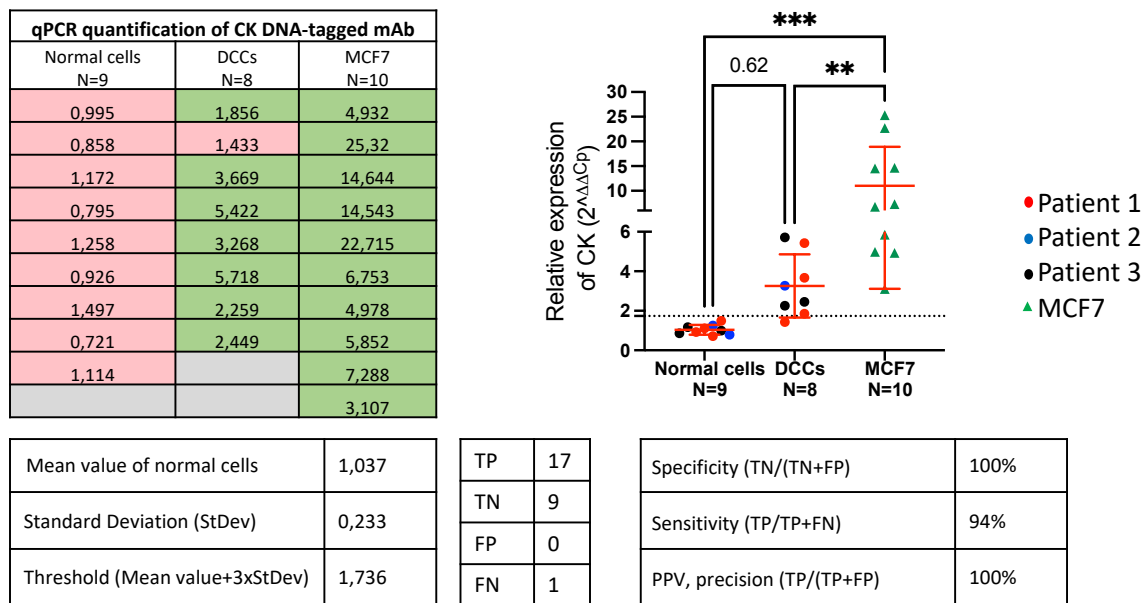


Figure 48. Sensitivity and specificity measurements of G&P assay. The threshold for distinguishing between normal cells and DCCs (or positive cells) was determined by adding three times the standard deviation (3xSD) to the mean CK expression of normal cells. Based on this threshold, true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN) were calculated, followed by the measurement of specificity, sensitivity, and positive predictive value (PPV). The qPCR quantification of CK in normal cells, CK+ DCCs, and MCF7 cells is shown. Statistical significance is indicated by asterisks as follows: ***P < 0.001, **P < 0.01, and ns: P ≥ 0.05. Data are presented as mean ± SD and were analyzed using one-way ANOVA with Tukey's multiple comparisons test.

4.2.14. Assessment of signaling pathway activity in single DCC

The next aim was to apply the G&P assay in a multiplex phenotyping approach using DCC of a HER2-positive breast cancer patient. This subsequent experiment aimed to showcase the signaling pathways in HER2-positive breast cancer, integrating phenotypic profiling with genomic data analysis.

A HER2-positive non-metastatic breast cancer patient was selected to apply the study method, with two timepoints examined, prior and after administration of a neoadjuvant therapy. The patient was diagnosed with right-sided breast cancer, classified as no special type (NST), Grade 2 (G2), with a clinical tumor size of cT2 and evidence of regional lymph node involvement (cN+). Hormone receptor testing revealed ER/PR-negative and HER2-positive status, with a Ki67 proliferation index of 23%. A bone marrow sample obtained at this timepoint was stained with CK antibody and visualized via AP-polymer antibody for detection and quantification of DCCs. The DCCD was determined to be 0.5 DCCs per million mononuclear cells, based on staining

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conducted and reported by technicians of the SCPL (Figure 49A). The patient received neoadjuvant chemotherapy, including carboplatin/taxol with Trastuzumab and Pertuzumab. After completing the final chemotherapy cycle at 3.5 months, followed by targeted therapy with Trastuzumab and Pertuzumab, surgery was performed 4 weeks later to remove the primary tumor. At the second timepoint, a postoperative bone marrow sample was collected and processed similarly to the first timepoint. The DCCD at this stage had increased to 6 DCCs per million mononuclear cells (Figure 49B).

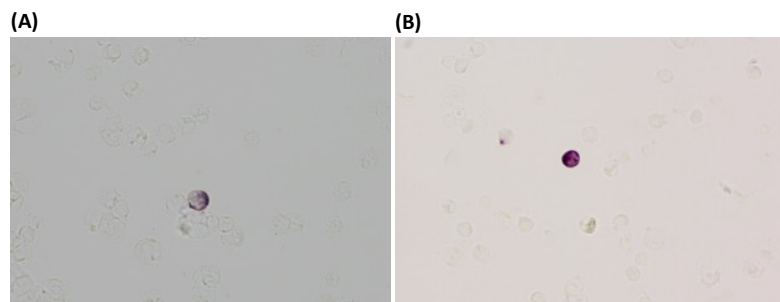
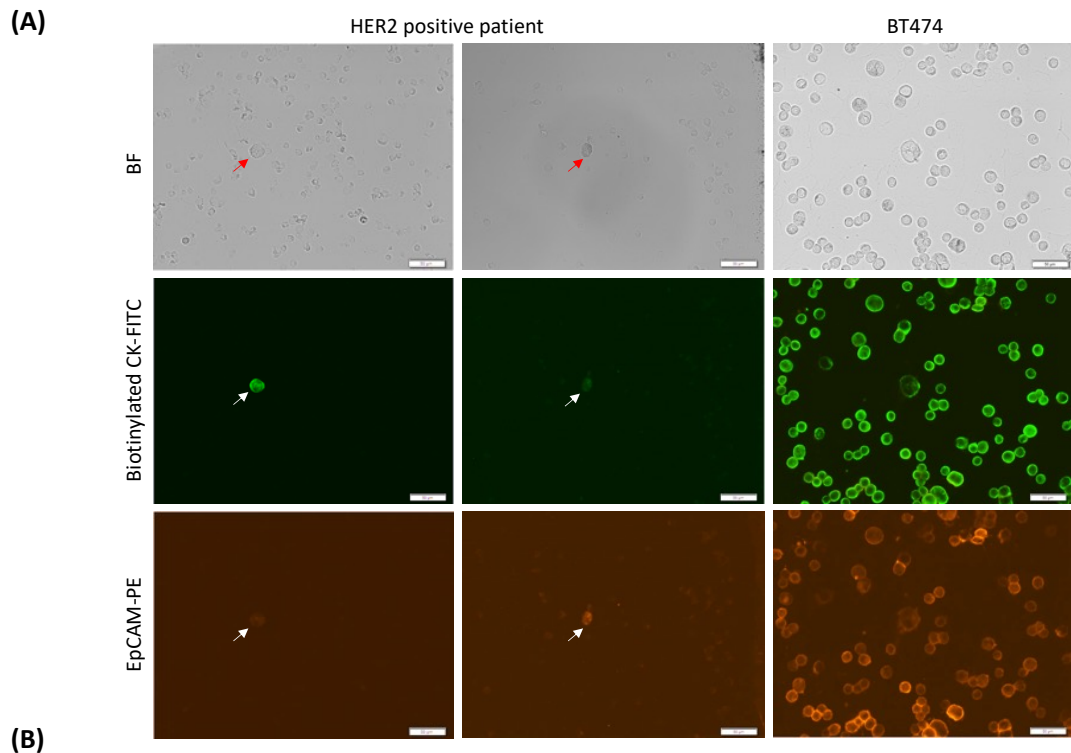


Figure 49. AP-polymer staining of bone marrow-derived adhesion slides of HER2 positive breast cancer patient. A: M0 Neoadjuvant1 (first timepoint). DCC detected by A45-B/B3 PAN-cytokeratin staining; B: M0 Neoadjuvant2 (second timepoint). The staining was done by technicians of the SCPL.

Bone marrow samples from both timepoints underwent G&P staining to identify and characterize tumor cells. CK and EpCAM antibodies were used for tumor cell identification, while HER2, pHER2, pERK1/2, pAKT, and pSTAT3 were targeted for subsequent molecular characterization. Bone marrow-derived adhesion slides were stained using biotinylated CK-streptavidin Alexa fluor 488 and EpCAM-PE and screened using the Olympus microscope (Figure 50A). BT474 cells served as a positive control. DNA-tagged mAbs included in the staining were quantified subsequently using qPCR. One adhesion slide from the first timepoint was stained, but no DCCs were found. Two adhesion slides from the second timepoint were stained and screened, leading to the isolation of 14 CK/EpCAM double positive cells, which were subsequently subjected to WGA. Of these, 4 cells were found to have a GII of 4 in WGA-QC and a DNA concentration exceeding 20 ng/ μ L following DNA purification. These 4 cells were subsequently analyzed using qPCR to quantify DNA-tagged mAbs (Figure 50B).

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Sample	DCCD	Number of CK ⁺ /EpCAM ⁺ cells isolated	Number of CK ⁻ /EpCAM ⁻ cells isolated
First timepoint	0.5	0	0
Second timepoint	6	14 cells isolated > 4 cells passed QC	15 cells isolated > 7 cells analysed in the qPCR
BT474 cell line	N/A	14 cells isolated > 7 cells analysed in the qPCR	N/A

Figure 50. Multiplex G&P staining of bone marrow-derived adhesion slides of HER2 positive breast cancer patient. (A) IF staining was performed using biotinylated CK-Streptavidin Alexa Fluor 488 and EpCAM-PE markers as detection marker. DNA-tagged mAbs used in the staining were quantified later using qPCR. Two examples of CK/EpCAM double-positive cells are shown. BT474 cells were used as a positive control for the staining procedure. (B) Table summarized DCCD, number of cells isolated and passed QC.

The 4 isolated cells were analyzed for the expression of HER2, pHER2, pERK1/2, pAKT, and pSTAT3. The first cell (T4) exhibited positive expression for HER2, pHER2, pERK1/2, and pAKT, indicating active signaling pathways associated with HER2-driven breast cancer progression. The second cell (T6) also expressed HER2, pHER2, pERK, and pAKT, though at lower levels compared to the first cell. The remaining two cells (T8 and T10) displayed low HER2/pHER2 expression but low to medium expression of pERK, pAKT, and pSTAT3 (Figure 51A-E). Normal cells indicated in the table (N1-N4) were assessed for CNV analysis. The table summarized relative expression of markers in the samples (Figure 51F). Overall, the analysis of the isolated putative DCCs revealed heterogeneity in signaling profiles, with some cells maintaining active HER2-driven pathways, while others showed activation of alternative survival pathways despite being HER2-negative.

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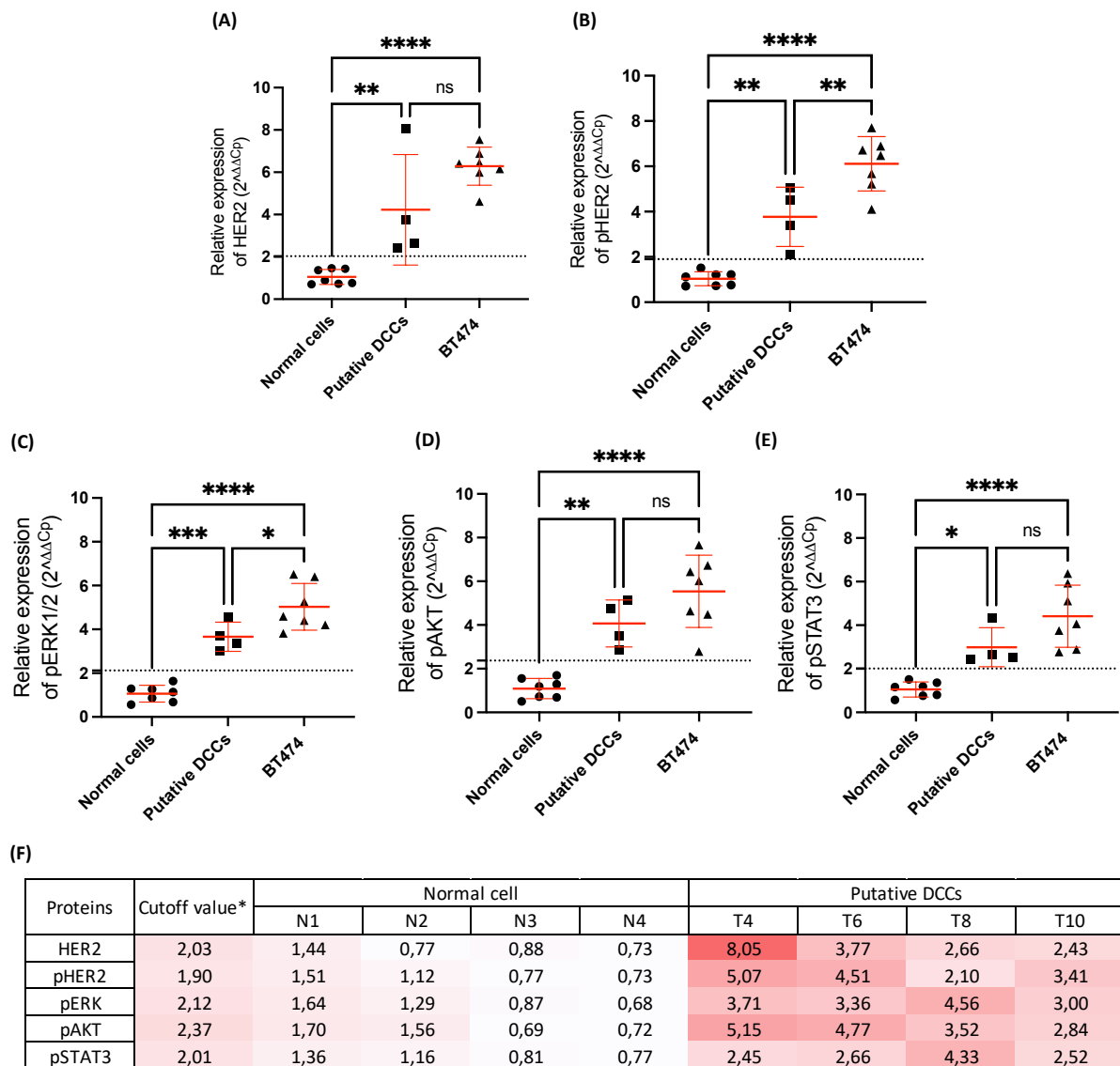


Figure 51. Multiplex phenotyping of candidate DCC. (A-E) qPCR quantification of HER2, pHER2, pERK1/2, pAKT and pSTAT3 in normal cells, putative DCCs and BT474 cell line. Asterisks represent the significance as follows: *** $P < .001$, ** $P < .01$, * $P < .05$ and ns: $P \geq .05$. Data are presented as mean $\pm$ SD, analyzed by one-way ANOVA with Tukey's multiple comparisons test. (F) Heat map showing relative expression of markers in 4 putative DCCs (T4, T6, T8, T10) and normal cell that assessed for CNV analysis (N1-N4) compared to cutoff which defined as mean value for normal cell (N=7) plus three times the standard deviation.

Four normal cells and the four candidate DCCs were subjected to CNV analysis. The normal cells exhibited a balanced CNV profile, while one of the DCCs displayed distinct genomic aberrations. The first putative DCC, characterized as most phenotypically altered profile, demonstrated an aberrant CNV pattern, indicative of genomic instability and malignancy. In contrast, the remaining three cells did not show the clear genomic abnormalities typically associated with fully transformed tumor cells (Figure 52A). Further examination of the aberrant CNV profile revealed amplification

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of key genes associated with signaling pathways, including *ERBB2*, *MYC*, *ZNF21* and *AURKA*. Conversely, other relevant signaling genes such as *PTEN*, *PIK3CA*, *TP53*, and *MAPK1/3* retained normal genomic profiles (Figure 52B). Moreover, the CNV-derived copy numbers of the *ERBB2* gene and HER2 protein expression levels obtained via qPCR showed a linear correlation (Figure 52C).

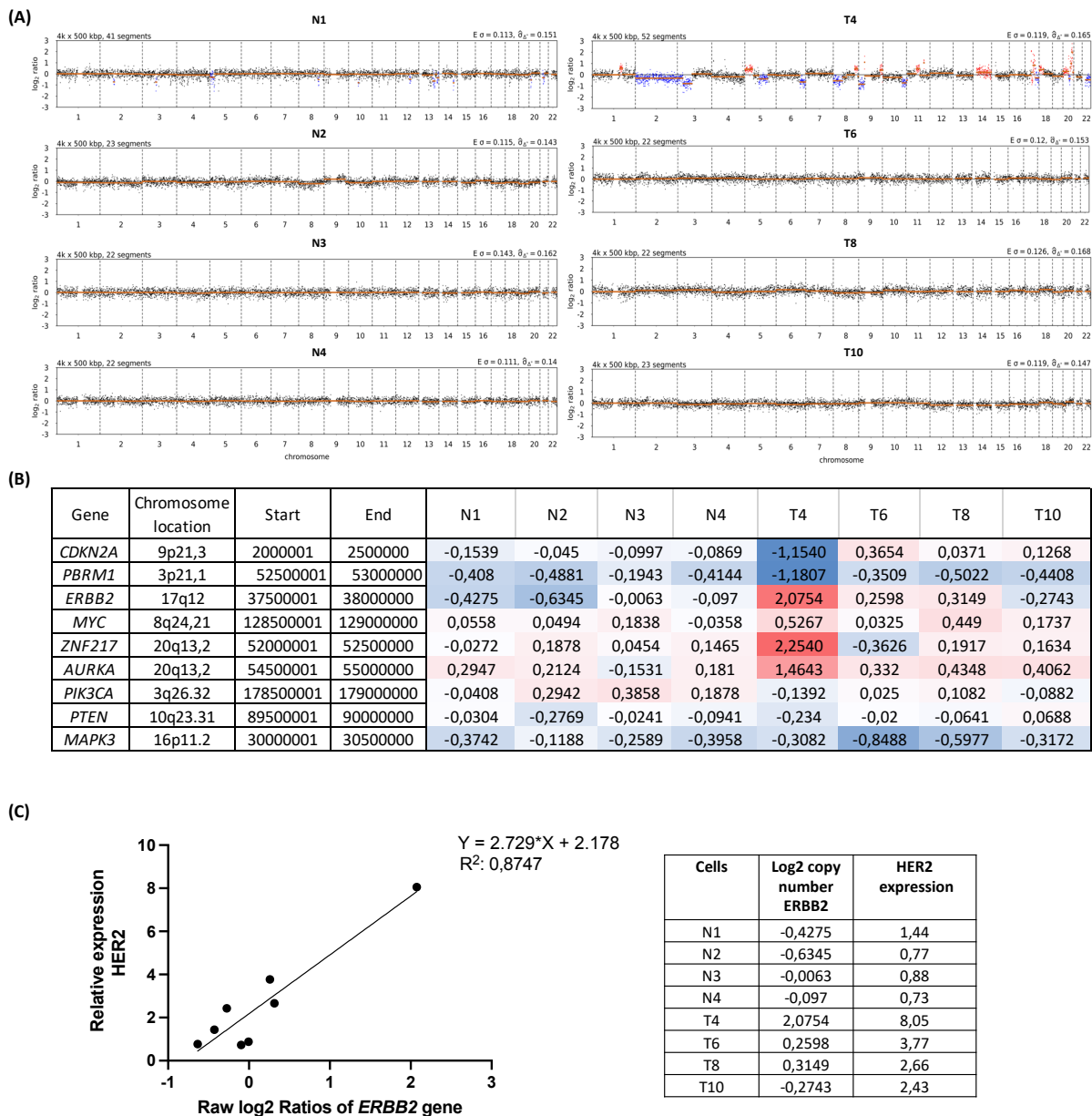


Figure 52. CNV analysis and correlation with HER2 protein expression. (A) Genomic profiles of normal cells (N1-N4) and putative DCCs (T4, T6, T8, T10) are presented. A histogram depicting genomic gains (red) and losses (blue) is shown for one DCC (T4). (B) Raw log2 ratios of genes relevant for breast cancer extracted from CNV output data table indicating the number of chromosomes, bin start and end positions (500 kb each). Chromosome loss, showing in blue, identified for *CDKN2A* and *PBRM1* and gains were identified for *ERBB2*, *MYC*, *AKT3*, *ZNF217* and *AURKA* genes indicated in red. (C) Scatter plot illustrating the linear correlation between HER2 protein expression measured using

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qPCR and *ERBB2* gene amplification by CNV, accompanied by a table displaying the corresponding values.

Additionally, mean fluorescence intensities (MFI) of EpCAM (Cy3) and CK (FITC) were measured in the four putative DCCs and compared to background bone marrow (BM) cells, which served as the baseline. The analysis revealed elevated expression of both markers in the candidate DCCs relative to the baseline, supporting their epithelial origin. However, the expression patterns varied among the cells. The first cell, characterized by an aberrant CNV profile, exhibited the highest CK and lowest EpCAM expression, with EpCAM still surpassing the baseline average. In contrast, the remaining three cells displayed lower CK expression and higher EpCAM expression, both distinctly elevated compared to the baseline average (Figure 53).

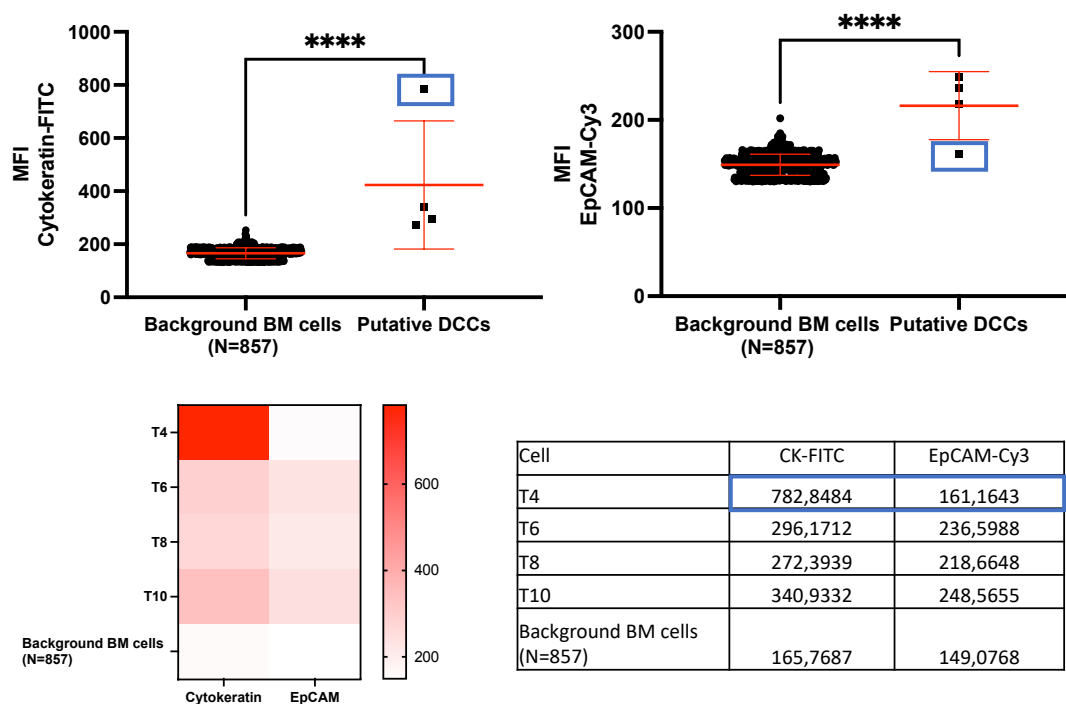


Figure 53. ImageJ analysis of HER2 positive breast cancer patient. MFI of CK-FITC and EpCAM-Cy3 measured in 4 putative DCCs and compared to the background BM cells. The heatmap provides a visual representation of the relative expression levels of each marker across the putative DCCs, while the table presents the corresponding numerical values for each marker in each cell.

Discussion

5. Discussion

In this thesis, we successfully developed and optimized a reliable method for isolating bone marrow-derived DCCs from archived cytopins, demonstrating that high-quality genomes can be extracted from densely loaded cytopins. Additionally, we showed that key proteins, including CK and EpCAM, can be reliably detected from archived cytopins, establishing the potential for long-term biomarker analysis. The optimized methods applied to samples from DCIS patients yielded promising results, demonstrating its reliability and effectiveness. Additionally, we established a combined genomic and proteomic (G&P) profiling approach for single-cell analysis. This innovative method was successfully showcased by applying it to a HER2-positive breast cancer patient. The results highlighted its capacity to link genomic alterations with activated signaling protein expression, providing a deeper understanding of the molecular mechanisms driving cancer progression.

5.1. Isolation of DCCs from archived cytopins

In 2006, a consensus protocol was published for the standardized detection of DCCs in bone marrow from patients with primary breast cancer. This protocol outlines procedures for slide preparation and the detailed analysis of 2 million bone marrow cells per patient [115]. Since DCCs are so rare, most investigators therefore prepared cytopins at high cellular density to increase the number of cells screened at minimum cost [116]. Previously, our lab developed a protocol for isolating DCCs from archived cytopins that combined laser microdissection with manual micromanipulation (LCAM) using PALM microscopy [96]. This protocol was standardized by incorporating background staining and dark dyes to minimize laser-induced damage to DNA during the dissection process. However, in this study we aimed to improve both identification and isolation of single cells by using the MMI microscope. Adjusting the laser parameters was a crucial aspect of optimizing the performance of the MMI for laser microdissection. By adjusting the laser parameters to improve precision and speed, and evaluating their effects of laser energy and heat on genomic profiles, we were able to obtain high-quality and balanced CNV profiles from normal cells. The greatest advantages of the MMI system lies in its ability to precisely relocate and isolate target cells, making it particularly effective for combining IF and polymer staining. The application of IF staining further improved the identification and isolation of tumor cells,

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including EpCAM⁺ or CK⁺, double EpCAM⁺/CK⁺ DCCs, and CD3⁺ T cells from a single slide. Such advancements facilitate more comprehensive analyses of cell populations while maximizing the utility of limited sample material. Since we and others previously found that in ductal carcinoma in situ about 13-20% of patients harbor CK⁺ cells, bona fide DCCs in bone marrow [5, 7], we aimed to apply this method to detect, isolate and characterize such very rare cells. We successfully applied a standardized protocol to isolate DCCs from archived bone marrow cytopspins of 4 patients with DCIS. Out of the four patients analyzed, we succeeded in isolating a subset of cells and a subsequent CNV profiling to assess chromosomal alterations of one patient. The CNV analysis revealed genomic instability across DCCs, with heterogeneous profiles and balanced profiles for CD3⁺ T cells. The most frequent chromosomal aberrations observed were losses on chromosomes 4, 8, 13, and 14, which are among the commonly identified chromosomal alterations in DCIS patients [117]. By utilizing a standardized protocol for processing archived cytopspins, we successfully showcased the feasibility of this approach for isolating rare tumor cells in a clinically relevant setting. Given that thousands of archived bone marrow cytopspins are stored and suitable for single-cell genomic analysis, along with the availability of long-term patient follow-up data, the established protocol can be efficiently used for isolating single cells from these slides in the future.

5.2. Development of G&P method

However, before mining the archived samples, we strived for developing another method that enables assessment of multiple proteins in single cells. So far, protein expression of DCC was limited to one or two antigens in addition to the DCC detection markers [118, 119]. Multi-omics methodologies, which enable simultaneous profiling of multiple levels of cell function regulation, provide a more integrated view, revealing the dynamic interactions among gene regulation, expression patterns, and protein function within single cells. Although techniques like CITE-seq, REAP-seq, and Mission Bio's Tapestry Platform enabled single-cell multi-omics analysis, most are optimized for large cell populations and lack the sensitivity needed to profile rare cell types such as DCCs and CTCs. In this study, we successfully established a protocol for parallel genotype and phenotype analysis of DCCs, facilitating a deeper understanding of the molecular signatures. This dual-pathway approach provided an

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integrated view of genotype and phenotype, enabling characterization of cellular diversity and functional states within rare cancer cells.

5.2.1. Proof of concept and protocol development

The primary aim was to demonstrate that the G&P assay can effectively quantify HER2 expression levels, an oncogene overexpressed in 15-20% of human breast cancers [120], as well as its phosphorylated form, which provides deeper insights into HER2 signaling pathways and functional activity [121]. To achieve this, we selected three breast cancer cell lines including MDA-MB-231 (HER2 low), MDA-MB-453 (HER2 medium) and BT474 (HER2 high). We successfully quantified the HER2 expression of cells from each line at the single-cell level, revealing variations in protein expression. Notably, the assay also showed a strong correlation between the quantity of HER2 DNA-tagged mAb and the fluorescence intensity measured by FACS. These results primarily showed the potential of the G&P assay to accurately quantify protein expression and capture variations in HER2 expression across different cell populations.

While the G&P quantification method for cell lines demonstrated considerable success in accurately measuring HER2 expression, an unexpected finding emerged during the analysis of PBMCs, HER2 negative control cells, that unexpectedly were found to display positive Tag-reads suggesting HER2 expression. First, we considered the possibility of passive carryover of the DNA-tagged antibodies which resulted in amplification of any residual or unbound DNA tag. Second, we hypothesized that the discrepancy could be due to a quantification error caused by differences in cellular ploidy between the tested cell types. The higher DNA content in polyploid cells could influence the relative abundance of genomic DNA and the DNA tag, leading to discrepancies in the quantification of HER2 expression when compared to diploid cells. To address these issues experimentally, we implemented two key strategies: First, we optimized blocking conditions using dextran sulfate which reduced background noise caused by non-specific binding of the DNA tag to PBMCs. Second, we tackled the issue of ploidy-induced variability by normalizing the qPCR readout using an exogenous DNA tag spike-in. By adding a defined quantity of the spike-in oligonucleotide to each isolated cell, we were able to normalize the qPCR

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measurement independently of the cellular DNA abundance, mitigating the impact of ploidy differences on the final readout. Overall, these adjustments enabled us to ensure an accurate quantification of HER2 expression.

Successful quantification of HER2 expression across different breast cancer cell lines established the foundation for comparing the findings with the CNV analysis of the *ERBB2* gene. To achieve this, cell lines (MDA-MB-231, MDA-MB-453 and BT474) as well as PBMCs (negative control) were quantified for HER2 protein expression and assessed for CNV analysis of the *ERBB2* gene. This analysis, revealed aberrant genomic profiles in all three tested cancer cell lines. On the other hand, PBMC showed balanced CNV profiles with no detectable chromosomal aberrations indicating that our workflow enabled specific and unbiased detection of CNVs in single cells. Additionally, to correlate CNV data of *ERBB2* gene with HER2 protein expression three approaches were used. In the first approach, qPCR results were correlated with the average Log2 values derived from the CNV pipeline, which included a broader genomic region around the *ERBB2* locus. The second approach correlated qPCR data with raw Log2 values for the *ERBB2* locus and adjacent ± 1 Mb genomic regions. The third approach used the raw Log2 values for the *ERBB2* locus, providing a more targeted analysis. The first two approaches showed weaker correlations due to the inclusion of surrounding regions, where copy number losses particularly in the MDA-MB-453 cell line weakened the signal from the *ERBB2* gene gain. As a result, the overall CNV appeared lower and less indicative of the *ERBB2* gene amplification. However, the third approach, focusing specifically on the *ERBB2* locus, provided the strongest and most consistent correlation between CNV data and HER2 protein expression levels. These findings demonstrated the utility of the G&P assay as a robust method for linking genomic alterations to proteomic assessment at the single-cell level.

For an exploitation of the approach by using far more antibodies than conventional IF studies could include, efficient DNA-tagging of antibodies is crucial. Antibody-oligo conjugation is a critical aspect, both in terms of cost-effectiveness and sensitive quantification. We initially utilized the commercial service provided by Biolegend for HER2 antibody-oligo conjugation, and then aimed to test two distinct methods for other target proteins: the LASIC and the maleimide method. The two approaches differed in their chemistry and were found to display varying levels of efficiency. The LASIC

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method involved covalent attachment of the oYo-link DNA oligonucleotide to the antibody. While this method proved to be effective across the antibodies tested, its efficiency appeared to vary based on the specific immunoglobulin (Ig) used. Variability was observed both between antibodies derived from different species and among subclasses from the same species, with rabbit IgG demonstrating the best conjugation efficiency. Nevertheless, all antibodies tested using this method displayed moderate to high crosslinking efficiency, as confirmed by SDS-PAGE analysis, and were successfully established into the G&P staining. On the other hand, the maleimide method, which relies on partial reduction of disulfide bonds in the IgG antibodies and subsequent attachment of maleimide-modified DNA oligonucleotides, yielded low or undetectable levels of conjugation. The conjugation efficiency, assessed by flow cytometry, SDS-PAGE, and CODEX staining, revealed the lack of successful conjugation. One significant challenge with this method was the use of a 143 bp oligonucleotide (designed for the G&P assay), whereas CODEX studies, for which the maleimide method is mostly used, typically employ shorter 20–25 bp oligonucleotides. The 143 bp oligo was significantly longer than the standard 20–25 bp oligo. This increased length may lead to spatial interference or steric hindrance during conjugation, making it more difficult for the oligo to bind to the intended conjugation site on the antibody. Additionally, the larger oligonucleotide is more prone to aggregation, further reducing the number of functional binding events. [122]. Therefore, we hypothesized that the lower efficiency observed with the CODEX method was due to steric hindrance, particularly when targeting disulfide bonds near the antibody's internal structure, which may be more prone to interference with larger oligonucleotides. In contrast, the LASIC method appeared to be more efficient as it targets exposed lysine residues on the antibody surface.

5.2.2. Validation of the G&P assay

Having overcome the technical hurdles of the G&P assay we validated its robustness and versatility by evaluating its ability to measure cellular and molecular responses to HER2-targeted therapies using lapatinib, a small-molecule HER2/EGFR tyrosine kinase inhibitor [123, 124]. Lapatinib inhibits HER2 phosphorylation, effectively disrupting key downstream signaling pathways such as PI3K/AKT/mTOR and RAS/RAF/MAPK, which are essential for promoting cell survival and proliferation in

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HER2-positive cancers [125]. We targeted two key HER2 phosphorylation sites, Tyr1248 and Tyr1221/1222, known to play critical roles in HER2 signaling [100], with Tyr1248 being particularly significant as an independent prognostic marker for poor breast cancer outcomes [109]. Consequently, we included assessment of this phosphorylation site in the G&P assay. We treated BT474 (HER2 amplified/overexpressing-lapatinib sensitive) and MDA-MB-468 (HER2/pHER2 negative lapatinib insensitive) cell lines with varying concentrations of lapatinib to evaluate its dose-dependent effects on cell growth and apoptosis. This approach helped identifying an optimal concentration that sufficiently inhibited HER2 signaling while minimizing extensive cell death, ensuring that the observed changes in HER2 phosphorylation reflected the drug's mechanism. We first showed by IF staining that lapatinib significantly inhibited phosphorylation at both Tyr1248 and Tyr1221/1222 in BT474 cell line, without affecting total HER2 expression. Also, G&P analysis demonstrated a marked reduction in phosphorylated HER2 at Tyr1248 in lapatinib-treated cells compared to controls. In contrast, the HER2-inhibition insensitive MDA-MB-468 cell line showed no detectable changes in either total HER2 or phosphorylated HER2, reinforcing the assay's sensitivity and specificity. This highlighted the method's ability to accurately reflect molecular changes induced by targeted therapies. Moreover, assessing phosphorylated HER2 provided a more precise representation of HER2 signaling and functional activity. These findings highlighted the G&P assay's utility not only in quantifying protein expression but also in analyzing pathway activation, particularly in the context of targeted therapies like lapatinib.

The first application of the G&P assay to clinical samples tested its sensitivity and reliability for detecting and quantifying CK expression in bone marrow-derived DCCs from breast cancer patients. CK expression was compared between MCF7 cells (positive control for CK expression), bone marrow-derived DCCs (CK⁺), and normal cells (CK⁻). The method showed a significant difference in CK expression between MCF7 cells and normal cells. While CK expression in DCCs showed a measurable 3.1-fold increase compared to normal cells, this difference did not reach statistical significance, likely due to low number of cells and the overall lower expression of CK by DCC compared to MCF7 cells. We addressed this point by application of a threshold, defined as the mean value of normal cells plus three times the standard deviation (3xSD), as it is regularly done in clinical assays such as FISH analyses for

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HER2 amplification [65]. This threshold minimized the false positive detections and enabled a more precise evaluation of the assay's sensitivity (94%) and specificity (100%). Additionally, a comparison between the G&P CK quantification and ImageJ-based CK-IF quantification revealed a high correlation of the results.

Signaling pathways play a critical role in breast cancer progression, influencing tumor growth, survival, metastasis and therapy resistance. [7]. Key signaling pathways implicated in breast cancer include the estrogen receptor (ER) pathway, HER2 signaling pathway, phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway, mitogen-activated protein kinase (MAPK) pathway, and the cyclin D1/cyclin-dependent kinase 4/6/retinoblastoma protein (cyclin D1/CDK4/6/RB1) pathway [7, 126]. HER2 signaling activates multiple downstream pathways, including PI3K/AKT, MAPK, and JAK/STAT, driving proliferation and survival [127]. The PI3K/AKT pathway, can also be hyperactivated by PIK3CA mutations or loss of regulatory proteins like PTEN and INPP4B, contributing to uncontrolled growth and resistance to ER and HER2-targeted therapies [128, 129]. MAPK pathway activation, frequently associated with ER signaling and HER2 overexpression, contributes to chemotherapy resistance and poorer disease-free survival [130, 131]. The importance of downstream effectors such as pAKT and pERK1/2 has been well established, with studies showing that their coexpression in primary tumors is predictive of shorter disease-free survival in HER2-positive early-stage breast cancer patients [132]. Given the complexity of these pathways and their roles in tumor growth, metastasis, and resistance to treatment, we were prompted to apply the G&P assay to assess HER2 expression and the phosphorylation status of key downstream markers, including pHER2 (Tyr1248), pERK1/2 (Thr202/Tyr204), pAKT (Ser473), and pSTAT3 (Tyr705), all of which are critical for understanding the activation of these pathways. To achieve this, IF staining was established for pERK1/2, pAKT and pSTAT3 antibodies which were then successfully conjugated to tag oligos and included into the G&P assay. Multiplex phenotyping was initially validated by testing a combination of DNA-tagged antibodies across various breast cancer cell lines.

To compare signaling pathway dynamics across distinct tumor types, we selected luminal B/HER2 overexpressing cell line BT474 and two TNBC subtypes, MDA-MB-

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468 (mesenchymal stem-like phenotype) and MDA-MB-231 (basal like). IF staining was used to identify cancer cells via CK and EpCAM protein expression, while qPCR was employed for quantitative measurements of HER2 and key signaling pathway markers. The protein expression patterns measured using G&P aligned closely with genetic alterations reported in the literature: BT474, exhibited markedly high expression of HER2 and pHER2, consistent with *ERBB2* gene amplification in this cell line. This amplification leads to HER2 overexpression, driving autophosphorylation at Tyr1248, a key site that activates downstream signaling pathways. Elevated levels of pAKT observed in BT474 can be attributed to both HER2 overexpression and PIK3CA gain-of-function mutations in this cell line [133], which result in the constitutive activation of the PI3K/AKT/mTOR pathway, promoting tumor growth and survival. The elevated expression of pERK in BT474 further highlights the cross-activation of the MAPK pathway by HER2 signaling. pSTAT3 expression in BT474 was observed at medium levels, a finding supported by reports suggesting crosstalk between HER2 and JAK/STAT pathways [134]. The MDA-MB-468 cell line, displayed increased expression of pERK and pAKT despite low HER2 expression. These findings aligned with the cell line's known genetic profile, which includes EGFR overexpression and PTEN loss-of-function mutations [135]. EGFR overexpression activates the MAPK and PI3K/AKT pathways, driving tumor growth and proliferation. PTEN loss further amplifies PI3K/AKT signaling by removing the inhibitory effect on PI3K, leading to uncontrolled activation of this pathway. The absence of HER2 signaling in MDA-MB-468 emphasizes the heterogeneity within TNBC, where alternative pathways, such as EGFR-mediated signaling, become dominant drivers of malignancy. Intermediate pSTAT3 expression in this cell line may be attributed to cytokine signaling or pathway crosstalk, as described in the literature, particularly between the PI3K/AKT and JAK/STAT pathways [136]. MDA-MB-231, exhibited elevated levels of pERK. These findings may be correlate with BRAF and KRAS gain-of-function mutations [137], which are known to hyperactivate the MAPK pathway. The low-intermediate pSTAT3 expression observed in this cell line suggested a crosstalk between the MAPK and JAK/STAT pathways [136]. By employing the G&P assay, we quantitatively analyzed protein expression levels linked to key signaling pathways, reinforcing the interplay between genetic mutations and protein-level changes and subsequent tumor progression.

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5.2.3. Signaling pathway analyses in patient DCC

This showcase study was conducted to illustrate the application of the G&P assay to capture dynamic changes in signaling pathways and genomic profiles for the first time in patient DCC. A HER2-amplified breast cancer patient was selected due to the critical role of HER2 signaling in tumor progression and its well-established therapeutic relevance [138, 139]. Furthermore, HER2-amplified cancers are associated with distinct molecular features, including activation of downstream pathways like PI3K/AKT and MAPK which are critical for tumor growth and survival [139]. Bone marrow samples were collected at two clinical timepoints: Before (first timepoint) and after (second timepoint at surgery) administration of a neoadjuvant therapy. The DCCD was reported as 0.5 DCC per million mononuclear cells at the first timepoint and increased to 6 cells per million at the second timepoint. Bone marrow adhesion slides from both timepoints underwent multiplex G&P staining to identify CK/EpCAM-positive cells. These cells were further characterized for HER2 expression, alongside key downstream signaling markers. At the first timepoint, no CK/EpCAM-positive cells were detected. However, at the second timepoint, 14 CK⁺/EpCAM⁺ (putative DCCs) were isolated. Among these, 4 cells with high DNA quality were selected for G&P-based measurements. One of the putative DCC exhibited elevated expression levels of HER2, pHER2, pERK1/2, and pAKT, indicating the continued activation of HER2-driven signaling pathways despite treatment. This cell demonstrated an aberrant CNV pattern indicative of genomic instability and malignant origin. Notably, amplification of genes such as *ERBB2*, *MYC*, *ZNF217* and *AURKA* was observed, suggesting a potential mechanism for the continued activation of the HER2 pathway and downstream effectors, despite therapy. The ImageJ analysis revealed a very high CK and low EpCAM expression in this cell. Accordingly, high CK and HER2 expression highlighted the epithelial phenotype of this cell, reinforcing its oncogenic and treatment-resistant nature.

In contrast, the remaining three cells exhibited lower expression of HER2. Among these, one cell activated HER2 signaling, while the other two showed minimal HER2 activation but moderate expression of pERK, pAKT and pSTAT3 expression. This suggests that alternative survival and proliferation pathways were activated, even in the absence of significant HER2 overexpression. These cells displayed balanced CNV

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profiles, lacking the characteristic genomic aberrations typically associated with malignancy, which complicates their classification. ImageJ analysis of these three cells indicated lower expression of CK compared to the aberrant cell and medium EpCAM expression, suggesting that these cells may express a more plastic phenotype. Integrating these findings, we confirmed the oncogenic origin of one DCC, which displayed significant phenotypic and genomic alterations. The other three cells demonstrated activated signaling pathways but lacked definitive proof of malignancy due to the absence of genomic abnormalities. Therefore, they cannot be classified as either autochthonous bone marrow cells or disseminated cancer cells based on the currently available data. However, it should be noted that the absence of CNV are consistent with previous findings identifying CK positive cells in the bone marrow of breast cancer patients that lack chromosomal abnormalities [34]. In summary the presented case demonstrated the capability of the multiplex G&P assay to characterize the phenotypic and molecular heterogeneity of DCCs from a HER2-positive breast cancer patient. The findings emphasized the potential of the G&P assay for protein expression analysis, phosphorylation status assessment, and conducting genomic profiling.

6. Conclusion

To conclude, we established and optimized a method for isolating bone marrow-derived DCCs from archived cytopsins, offering improved reliability and efficiency compared to traditional techniques. The method demonstrated its potential in isolating high-quality genomic material, including from rare DCIS patient samples. This method can then be extended to integrate genomic and proteomic profiling in single-cell analysis, exemplified by a HER2-positive breast cancer case study. The fact that we identified a DCC with strong HER2 induced signaling after neoadjuvant therapy comprising anti-HER2 antibodies suggests that the protocol will open new ways into the study of therapy resistance in the adjuvant therapy setting. It underscores the power of this approach in linking genomic alterations to activated signaling pathways, providing valuable insights into the molecular mechanisms underlying cancer progression.

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