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Impact of Docking Strand Design on Spatial Resolution in DNA-Points Accumulation for Imaging in Nanoscale Topography

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ABSTRACT

DNA points accumulation for imaging in nanoscale topography (DNA-PAINT) has become a widely adopted single-molecule localization microscopy (SMLM) technique owing to its high spatial resolution, versatile labeling strategies, and theoretically unlimited multiplexing capability. Recent developments in repetitive docking strand designs have enabled faster image acquisition by increasing the number of potential binding motifs per target. However, the effect of such architectural modifications on effective spatial resolution remains largely unexplored. Here, we systematically quantify how repetitive docking strands influence localization distributions and effective resolution using the well-defined geometry of the trimeric proliferating cell nuclear antigen (PCNA) as a model system. Whereas classical single-motif docking strands resolve the expected ~6 nm spacing between PCNA subunits with high precision, repetitive docking motifs produce broadened localization distributions, despite comparable localization precision. Our results suggest that spatial blurring arises from a combination of variable binding site geometry, rotational flexibility of elongated multivalent DNA docking sequences, as well as the dynamic behavior of imager strands. This study provides a quantitative framework for understanding how docking strand architecture determines resolution limits in DNA-PAINT and underscores the need to balance multiplexing and imaging speed with structural fidelity. Our results thus offer guidance for the rational design of docking strands for high-precision DNA-PAINT imaging of protein complexes.

1 | Introduction

Super-resolution fluorescence microscopy (SRM) has revolutionized our ability to visualize molecular architectures below the diffraction limit. Among the most precise and widely accessible implementations, DNA points accumulation for imaging in nanoscale topography (DNA-PAINT) achieves sub-10-nm spatial resolution by exploiting the stochastic and transient hybridization of fluorescently labeled imager strands to complementary docking sequences attached to target molecules [1–3]. A key strength of DNA-PAINT lies in its conceptual and experimental

simplicity: it delivers nanometer-scale resolution on a conventional widefield microscope without requiring complex illumination schemes or photoswitchable dyes. Consequently, DNA-PAINT has become one of the most robust single-molecule localization microscopy (SMLM) techniques currently available. Beyond 2D imaging, DNA-PAINT has been successfully extended to 3D SMLM modalities, such as metal-induced energy transfer (MIET-PAINT) and confocal implementations that achieve axial nanometer precision [4, 5]. These advances, which combine DNA nanotechnology with tailored optical readouts, have established DNA-PAINT as

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a cornerstone technique for quantitative mapping of nanoscale molecular architectures in complex biological systems.

Recent developments have aimed to accelerate imaging and enhance multiplexing by employing repetitive docking strand designs that enable high-frequency binding and orthogonal sequence combinations [2, 3, 6]. While these architectures markedly improve throughput, their influence on effective spatial resolution remains underexplored. Specifically, it is unclear how repetitive docking motifs affect the positional fidelity of localizations in densely labeled molecular assemblies. Previous studies have shown discrepancies between localization precision and true spatial resolution in super-resolution microscopy, particularly at distances below 10 nm [7–9], often arising from photophysical dye interactions, photoswitching synchronization, or geometric complexity in multifluorophore labeling schemes [6, 7, 10, 11]. However, the consequences of docking strand architecture, especially in repetitive DNA-PAINT sequences, on the achievable spatial resolution have not been quantitatively examined.

To address this question, we employed proliferating cell nuclear antigen (PCNA), a trimeric protein complex with a defined ~6 nm subunit spacing, as a molecular reference structure [12]. Labeling was achieved via genetic code expansion and bioorthogonal click chemistry, as these approaches have been shown to enable site-specific, stoichiometric, and minimally perturbing conjugation of fluorophores to protein targets, making them ideally suited for quantitative benchmarking of nanoscale architectures [13, 14]. Binding kinetics such as on- and off-times have recently been shown to vary substantially with the local molecular environment, differing between DNA origami references and protein targets due to steric and electrostatic constraints [12, 15]. Consequently, calibration solely on DNA origami may introduce bias, underscoring the relevance of protein-based nanorulers like the PCNA “PicoRuler” for benchmarking resolution and binding kinetics under near-physiological conditions. In this study, we compare classical single-motif docking strands (5′–3′: TCC TCC T, 1 × R1) with repetitive multi-motif sequences (5′–3′: TCC TCC TCC TCC TCC TCC T, 5 × R1) designed to accelerate DNA-PAINT acquisition. We analyze photon statistics, localization precision and effective spatial resolution to elucidate how sequence architecture governs achievable image fidelity. Our results show that repetitive docking motifs, while maintaining high localization precision per binding event, induce spatial blurring of localization patterns at sub-10-nm scales. This finding emphasizes that achieving quantitative nanometer-scale resolution in DNA-PAINT requires rigorous control of parameters such as docking strand architecture, binding kinetics and labeling density. We outline practical criteria for optimizing DNA-PAINT measurements toward a balanced trade-off between imaging speed, multiplexing capacity as well as spatial accuracy and discuss emerging challenges as the method approaches its fundamental resolution limits.

2 | Results and Discussion

2.1 | Trimeric PCNA Model for Benchmarking Sub-10 nm Resolution

To investigate how DNA-PAINT docking strand architecture affects spatial resolution, we employed proliferating cell nuclear antigen (PCNA) as a geometric benchmark. PCNA (S186Norb

K110I mutant) forms a well-defined trimeric ring with click-sites spaced approximately 6 nm apart, making it an ideal protein-based imaging calibration optical nanoruler (PicoRuler) for sub-10 nm resolution imaging. Two DNA-PAINT labeling strategies were compared: the classical single-motif (1 × R1) docking strand used in standard DNA-PAINT measurements, featuring a single 7-nt binding motif with a theoretical total linearized length of ~2.5 nm, and a recently developed repetitive 5 × R1 design comprising five tandem repeats (19 nt total), yielding a theoretical total linearized length of ~6.5 nm. In both docking strand types, the fluorophore was positioned at the 3′-end of the imager strand, directed towards the protein target. For the 5 × R1 sequence, this geometry predicts a theoretical localization spread of up to 4 nm due to variable binding positions along the docking strand. In addition, to quantitatively model geometric variability, we applied a coarse-grained worm-like-chain (WLC) analysis of docking strand flexibility under imaging buffer conditions (1 × PBS + 500 mM NaCl, pH 7.4), providing an estimation of ssDNA elasticity. For the short 1 × R1 docking strand, which consists of 7 nt plus a rigid C5 linker (~1.2 nm) between the MeTet anchor and the ssDNA, the expected fluorophore displacement relative to the protein is dominated by the linker and short ssDNA segment. Under our high-salt conditions (1 × PBS + 500 mM NaCl), standard worm-like-chain estimates for ssDNA [16, 17] predict a 3D RMS end-to-end distance of ~3–4 nm and a corresponding 2D projection in the imaging plane of ~2–3 nm. For the 5 × R1 strand (19 nt plus the same C5 linker), the effective contour length increases by roughly a factor of three, which leads to an expected 2D spread of ~4–5 nm.

The degree of labeling (DOL) was determined by spectral deconvolution, separating nucleic acid and protein contributions (Figure S1). Accurate determination of docking density is crucial for interpreting binding event frequency and labeling efficiency in different docking designs. For the 1 × R1 sequence, the DOL was ~1 for the single labeled (monomeric condition) and ~3 for the fully labeled trimeric PCNA. For the repetitive 5 × R1 design, the single labeled sample exhibited a DOL of ~1, comparable to 1 × R1, whereas the fully labeled PCNA reached only ~2. Identical labeling protocols for both constructs indicate that the reduced DOL of repetitive docking strands originates from intrinsic sequence properties rather than methodological differences.

The lower labeling efficiency observed for longer, repetitive docking strands likely reflects steric hindrance and electrostatic repulsion, which reduce conjugation efficiency and accessibility. In addition to strand design, factors such as linker flexibility and fluorophore positioning relative to the protein surface also influence localization accuracy. Variations in linker length and orientation can introduce additional spatial uncertainty beyond the hybridization site. A more detailed polymer-physics treatment would need to account for additional factors such as steric constraints at the PCNA surface, transient interactions between dye and protein, and partial duplex formation along the 5 × R1 strand during imager binding, all of which further increase the effective blur but are difficult to constrain quantitatively. Imaging conditions were adjusted individually for each strand type: 2.5 nM imager concentration for 1 × R1 and 0.5 nM for 5 × R1, accounting for the fivefold increase in binding motifs per docking strand. Additional experiments with 2.5 nM 5 × R1 imager resulted in aberrant binding behavior, likely due to simultaneous multi-site binding, underscoring the need for concentration optimization

when comparing such systems (Figure S2). All imaging was performed using identical imager sequences labeled with Cy3B under buffer conditions containing triplet-state quenchers and enzymatic oxygen scavengers for photostabilization. Minor variations in buffer composition, ionic strength, and pH can substantially influence hybridization kinetics and duplex stability. Therefore, standardized buffer formulations and temperature control are critical for reproducible DNA-PAINT measurements across laboratories.

2.2 | Quantitative Comparison of Single Motif Docking Designs

We first present representative reconstructions of mono- and trimeric PCNA for both docking designs (Figure 1), then quantify localization-cloud geometry and apparent inter-site distances (Figure 2), and finally compare binding kinetics and photon statistics across conditions (Figure 3 and Table 1). Quantitative analysis of single labeled PCNA systems revealed distinct relationships between photon yield, binding kinetics and spatial resolution (Figure 3 and Table 1). For the statistics shown in Figure 3 and summarized in Table 1, all detectable PCNA particles in the field of view were included in the analysis, irrespective of their labeling state, so that the reported values represent the mean number of binding events per PCNA particle. The classical single-motif single labeled system ($1 \times R1$, 2.5 nM imager) exhibited mean ($\pm$ SD) on-times of ~ 287 (± 160) ms and a photon flux of ~ 31.1 (± 7.6) photons ms^{-1} , yielding an average photon budget of $\sim 7,600$ photons per binding event (Figure 3 and Table 1). Despite a relatively low number of detected events per PCNA ($\sim 29 \pm 15$), the photon yield ensured high localization precision (2.0 ± 0.3 nm; Table 2), allowing clear optical resolution of the trimeric PCNA structure with a peak-to-peak distance of $\sim 7.9 \pm 1.5$ nm (Figures 1 and 2b).

Samples with a single label site were analyzed for excessive broadening to characterize imager behavior at isolated docking sites without confounding effects from simultaneous binding to multiple docking strands. We determined the convex hull area for each cluster with a given localization count. In Figure 2a,b the areas are plotted as function of localization count and compared to the simulated convex hull areas as expected for localization-precision-limited clusters [19]. For expectation values we assumed a localization precision of 3 nm as was measured on the same data sets by a nearest-neighbour (NeNA) approach following established procedures for precision estimation in single-molecule localization data [20]. The ratios between observed and expected areas of 1.6 ± 0.7 (mean $\pm$ SD) (Table 2) reveal modest broadening beyond precision-limited expectations. NeNA analysis (~ 3 nm precision) excludes significant drift, indicating this arises from design-intrinsic flexibility (C5-linker length, ssDNA pivoting) and minor anchoring-point variability at the PCNA surface, rather than sample heterogeneity.

Increasing the labeling density to triply labeled PCNA ($3 \times 1 \times R1$, 2.5 nM imager) modestly increased the mean photon flux to 33.4 (± 8.4) photons ms^{-1} , approximately doubled the number of detected events per trimer (29 (± 15) vs. 61 (± 45)) and slightly enhanced the photon budget ($\sim 8,980$ photons per event) without compromising localization precision (Tables 1 and 2). The increase in photon flux, as well as the mean on-times for triply labeled $1 \times R1$ likely arises from occasional overlapping or re-binding events on different docking strands within the same trimer, which cannot always be temporally resolved at the given frame rate and imager strand concentration. This effect could be mitigated by lowering the imager concentration and is expected to become more prominent with increasing docking site density and imager strand concentration. Despite local crowding effects, structural fidelity remained intact, and the PCNA trimer was clearly resolved (Figure 1). These trends were consistent across independent

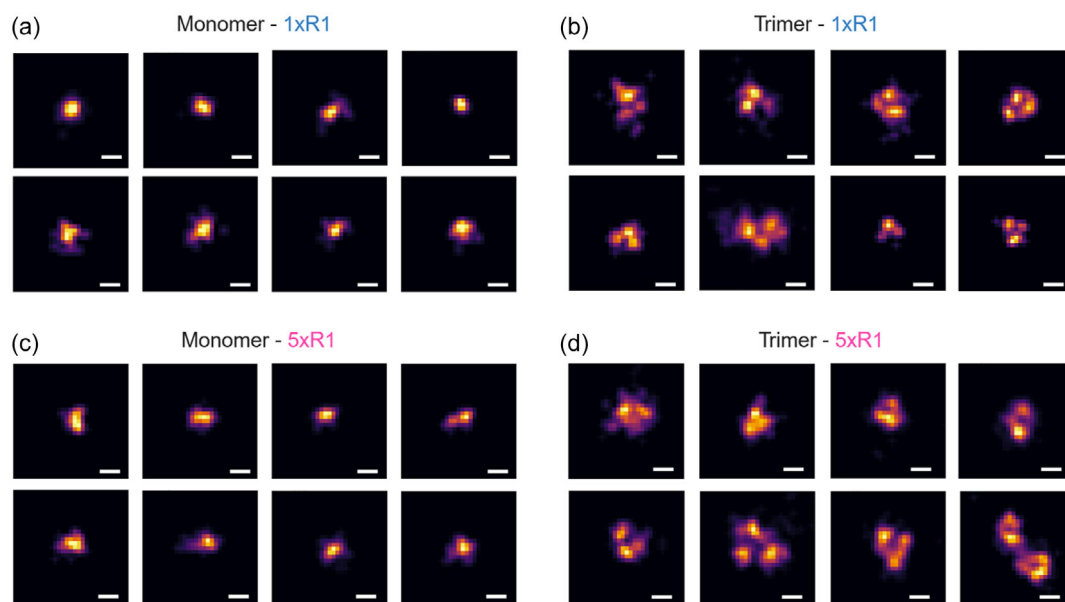


FIGURE 1 | Representative DNA-PAINT reconstructions of PCNA labeled with mono- and multivalent docking strands measured on a single-molecule surface. (a,b) Monomeric and trimeric PCNA labeled via click chemistry using single-motif $1 \times R1$ docking strands with 2.5 nM imager strands. (c,d) Corresponding labeling with multivalent $5 \times R1$ docking strands using 0.5 nM of imager strands. In both cases, the trimeric arrangement of PCNA is clearly resolved, demonstrating the suitability of both docking strand designs for subdiffraction imaging of defined multimeric protein assemblies. Scale bars: 10 nm.

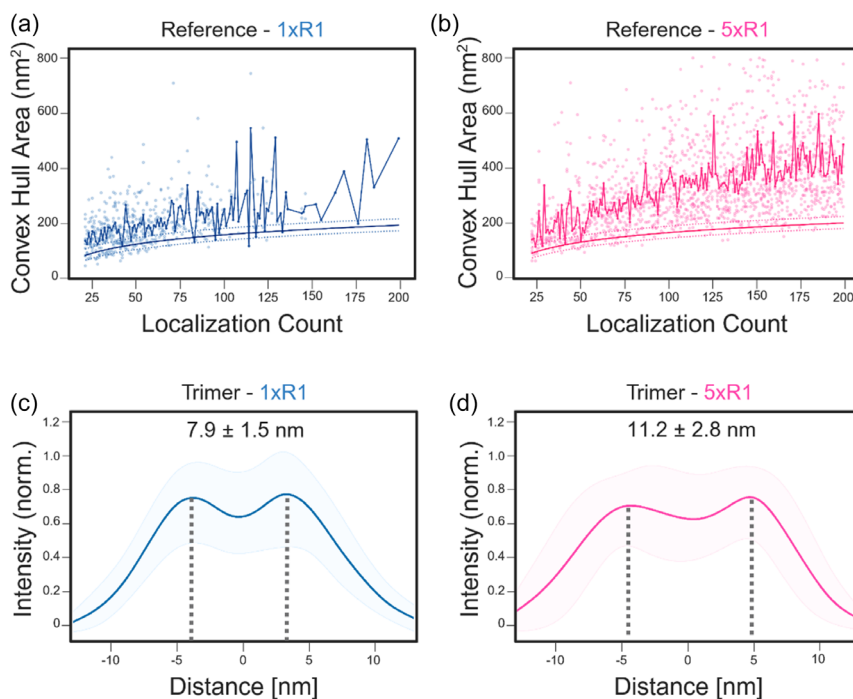


FIGURE 2 | Quantitative assessment of spatial resolution and inter-site separation in DNA-PAINT imaging of PCNA. (a) Convex hull cluster areas as function of localization count per cluster for 1 × R1 (a) and 5 × R1 (b) from $n = 540$ for 1 × R1 and $n = 1582$ for 5 × R1. Colored dots represent the individual cluster values; blue connected dots indicate mean values for each localization count; the solid line shows the mean expectation value for a localization precision of 3 nm with dotted lines indicating the standard deviation of expectation values. (c,d) Line profiles through 20 picked reconstructed PCNA trimers labeled with 1 × R1 (c) and 5 × R1 (d) docking strands, showing mean peak-to-peak distances of 7.9 ± 1.5 nm and 11.2 ± 2.8 nm, respectively. Shaded regions indicate standard deviations.

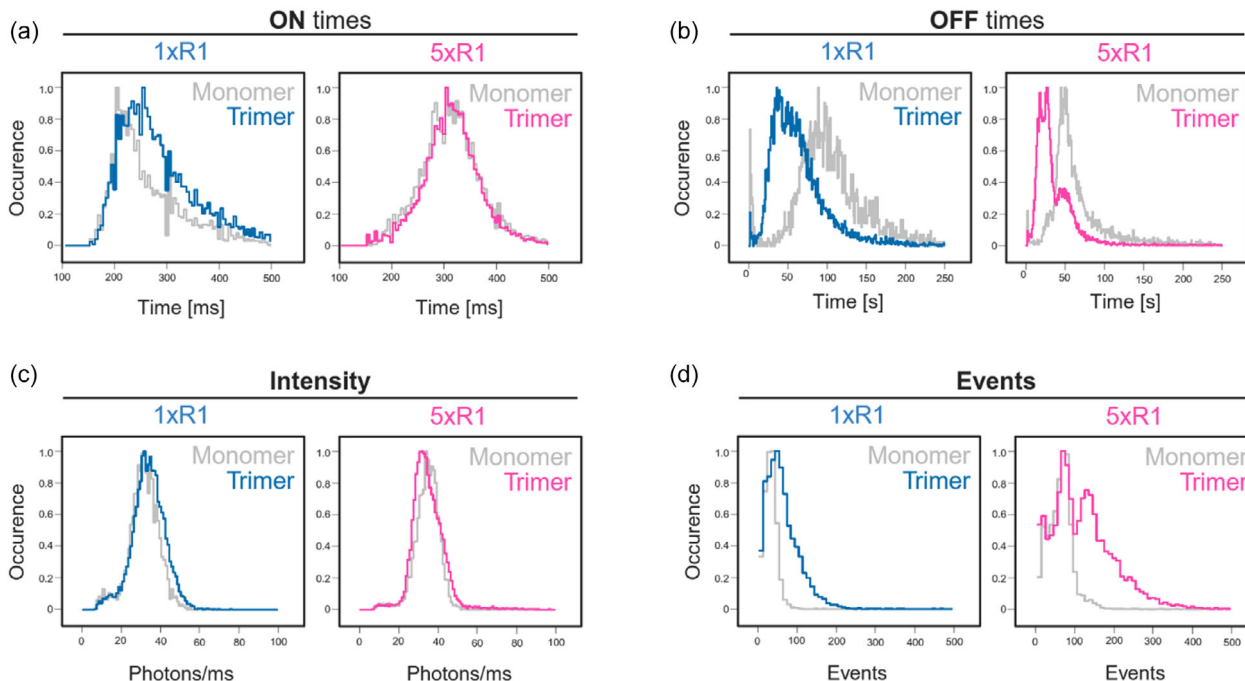


FIGURE 3 | Photophysical statistics of DNA-PAINT imaging of PCNA with single- and multivalent docking strands ($n = 3000$ – $10\,000$ particles). (a) ON-time distributions (ms). (b) OFF-time distributions (s). (c) Intensity distributions (photons ms^{−1}). (d) Distributions of the number of binding events per molecule. In all panels, blue denotes 1 × R1 and magenta denotes 5 × R1; gray shows monomer-like PCNA, and the colored trace shows trimer-like, fully clicked PCNA. Occurrence is normalized to the respective maximum. Corresponding data values are shown in Table 1. All data were acquired under identical acquisition settings using 2.5 nM and 0.5 nM of imager strands for 1 × R1 and 5 × R1, respectively.

TABLE 1 | Photophysical parameters of DNA-PAINT measurements comparing monovalent ($1 \times R1$) and multivalent ($5 \times R1$) docking sequences. Mean $\pm$ SD values are based on $N_{\text{exp}} = 3$ independent experiments per condition; for each experiment ~ 3000 – $10,000$ PCNA particles were analyzed under identical acquisition conditions (~ 0.2 kW/cm²). On- and off-times refer to dwell and dark times of individual binding events; intensity is reported in photons per millisecond. Statistical comparisons of mean intensities between conditions were performed using an independent samples *t*-test implemented in OriginPro. All reported differences discussed in the text were significant ($p < 0.001$ unless stated otherwise).

Sample	ON-time, ms Mean $\pm$ SD/ median	OFF-time, s Mean $\pm$ SD/ median	Intensity, ph/ms Mean $\pm$ SD/ median	Events Mean $\pm$ SD/ median
1x R1 single labeled (2.5 nM)	287 $\pm$ 160/243	110 $\pm$ 61/99	31.1 $\pm$ 7.6/31.4	29 $\pm$ 15/29
1x R1 3x labeled (2.5 nM)	300 $\pm$ 134/268	67 $\pm$ 51/56	33.4 $\pm$ 8.4/33.5	61 $\pm$ 45/51
5x R1 single labeled (0.5 nM)	329 $\pm$ 129/312	70 $\pm$ 53/55	33.9 $\pm$ 6.4/34.4	59 $\pm$ 34/59
5x R1 3x labeled (0.5 nM)	324 $\pm$ 103/314	47 $\pm$ 71/29	34.4 $\pm$ 8.2/33.5	119 $\pm$ 86/107
5x R1 single labeled (2.5 nM)	314 $\pm$ 116/304	29 $\pm$ 56/14	34.3 $\pm$ 7.9/34.2	205 $\pm$ 142/204
5x R1 3x labeled (2.5 nM)	280 $\pm$ 76/275	27 $\pm$ 63/10	35.9 $\pm$ 8.1/35.3	328 $\pm$ 251/284

TABLE 2 | Localization precision and relative convex hull ratios for different docking strands ($1 \times R1$ and $5 \times R1$) and labeling densities (single and triple labeled PCNA). Localization precision per frame was calculated according to [18], including pixel size, photons and background signal. Values represent mean $\pm$ SD over $N_{\text{exp}} = 3$ independent experiments; within each experiment 20,000–500,000 localizations were analyzed per condition. Relative convex hull ratios are provided as mean $\pm$ SD with $n = 540$ ($1 \times R1$) and $n = 1582$ ($5 \times R1$) particles in total (Figure 2a,b).

Sample	Mean $\pm$ SD	Median
1x R1 single labeled (2.5 nM)	2.0 $\pm$ 0.3	2.0
1x R1 3x labeled (2.5 nM)	2.1 $\pm$ 0.4	2.1
5x R1 single labeled (0.5 nM)	1.9 $\pm$ 0.3	1.9
5x R1 3x labeled (0.5 nM)	1.9 $\pm$ 0.4	1.9
1x R1 single labeled (2.5 nM)	1.59 $\pm$ 0.70	1.45
5x R1 single labeled (0.5 nM)	2.07 $\pm$ 0.90	1.84

experiments, and statistical analysis on averages confirmed that the observed differences in intensity and event numbers are significant.

2.3 | Repetitive Docking Strands Alter Binding Dynamics and Apparent Resolution

The repetitive $5 \times R1$ single labeled sample (0.5 nM imager) showed prolonged mean on-times of 329 (± 129) ms and a slightly higher photon flux of 33.9 (± 6.4) photons ms^{−1}, resulting in a higher photon budget of $\sim 10,700$ photons, about 40% greater than the average photon budget of $\sim 7,600$ photons per binding event for the single labeled $1 \times R1$ system (Figure 3 and Table 1). The higher event counts observed for the single labeled $5 \times R1$ (59 $\pm$ 34) compared with single labeled $1 \times R1$ (29 $\pm$ 15) are consistent with the multivalent design of the $5 \times R1$ docking strand, which offers five adjacent binding motifs and thereby facilitates frequent binding or re-association events even at reduced imager strand concentration.

In addition, the relative convex hull ratios increased from 1.6 ± 0.7 for $1 \times R1$ to 2.1 ± 0.9 for $5 \times R1$ reflecting increased positional

spread and a reduced effective resolution (Figure 2a,b and Table 2). As control, the measurement of NeNA localization precisions revealed temporally stable 3 nm for both samples indicating negligible drift in the setup and no differences in the focusing depth (Figure S4).

In fully labeled $5 \times R1$ PCNA, the average number of binding events further increased to ~ 119 (± 86) events per trimer, but the photon budget per event remained stable ($\sim 10,520$ photons) (Figure 3 and Table 1). Longer docking sequences and extended on-times suggest hindered dissociation dynamics, potentially arising from transient interactions between adjacent binding motifs or fluorophore–DNA contacts within extended docking regions. In addition, fast re-association of imagers within the local volume defined by several closely spaced docking motifs may further prolong apparent dwell times and distribute emission over multiple binding positions, thereby contributing to the observed positional blurring. The binding behavior of the $5 \times R1$ docking strand also aligns well with the estimated DOL (~ 2.1) of the fully labeled PCNA- $5 \times R1$. Analysis of dark times and the number of detected events per protein indicates a substantial fraction of singly labeled PCNA, showing similar kinetic parameters as the single labeled sample, a smaller population of doubly labeled PCNA and only a minor fraction of fully labeled PCNA (Figures 3 and S1). While doubly labeled samples exhibit intermediate binding frequencies consistent with progressive crowding, their unambiguous identification is challenging due to spatial overlap of adjacent docking sites, justifying our focus on fully labeled species for quantitative resolution benchmarking. For downstream analyses, particularly for quantifying trimer spot distances, only clearly resolved, triply labeled PCNA- $5 \times R1$ and $1 \times R1$ trimers were selected. Due to the lower labeling efficiency of the long multivalent docking strand, such fully labeled species were rare. To ensure comparability across datasets, randomly picked resolved trimeric species were likewise selected from fully labeled single-motif samples. Despite similar localization precision (1.9 ± 0.4 nm; Table 2), the apparent resolution was reduced and the reconstructed localization pattern exhibited noticeable smearing (Figure 2d). Although the $5 \times R1$ construct yields a higher number of binding events per PCNA trimer than the $1 \times R1$ design (Figure 3, Table 1), this statistical advantage does not translate into improved spatial accuracy. Conceptually, approaches that exploit repeated binding events to refine the

emitter position, demonstrate that an increased number of localizations can in principle enhance the determination of the localization centroid when the emitter position is fixed relative to the label and linkage error is minimal [6]. In our case, however, the effective resolution is dominated by systematic geometric uncertainty introduced by the longer and more flexible $5 \times R1$ docking architecture. The increased convex hull areas and broader line profiles, together with the larger apparent peak-to-peak distances (Figure 2, Table 2), indicate that localizations are distributed over an extended accessible volume defined by multiple tandem binding motifs rather than being constrained by localization precision alone. Under these conditions, additional localizations primarily refine the centroid of this broadened volume, while the anchor position remains obscured by the intrinsic linkage error of the multivalent docking strand. This positional blurring originates from geometric variability among tandem binding motifs, which distribute localization coordinates across a ~ 4 nm range. The resulting localization distribution displayed peak-to-peak distances of $\sim 11.2 \pm 2.8$ nm, i.e. about 40% larger than in the single-motif single labeled case (Figure 2c). The WLC predictions are consistent with the experimentally observed broadening of localization clouds (Figure 2 and Table 2), confirming that the increase in blur for $5 \times R1$ is of the magnitude expected from the extended and more flexible docking architecture. A fully quantitative description would require accounting for protein surface constraints, dye-DNA-protein interactions, and partial duplex formation along the $5 \times R1$ strand during imager binding, all of which contribute additional blurring beyond the simple WLC model but are difficult to constrain precisely.

At higher imager concentrations, the likelihood of temporally overlapping binding events increases across all samples and is most pronounced for repetitive constructs, where the higher event rates per target lead to partially overlapping localization clouds and a potential bias in event quantification. These effects arise from concentration-dependent multi-emitter events rather than from an intrinsic limitation of the $5 \times$ design and can, in practice, be mitigated by reducing the imager concentration or by applying post-processing filters based on spot intensity and local clustering of localizations [9, 16].

2.4 | Mechanistic Origins of Apparent Resolution Loss in Repetitive Docking Strands

In the following, we distinguish between several interrelated but conceptually different contributions to the effective resolution, including geometric linkage error and docking strand flexibility, loss of resolution due to overlapping or simultaneous binding events and the influence of sampling statistics and total imaging time, while noting that the underlying localization precision remains comparable between $1 \times R1$ and $5 \times R1$. Overall, our data demonstrate that while speed-optimized repetitive docking strand designs significantly enhance imaging throughput, they compromise effective resolution through positional blurring. In the case of $5 \times R1$, several factors contribute to this loss of effective resolution. First, the increased number of docking motifs per protein increases the likelihood of overlapping or double-binding events on different sites within the same trimer, which can manifest as prolonged on-times and elevated photon budgets on the level of individual localization traces. These temporally overlapping or spatially adjacent binding events can

result in apparent localization centroids representing an averaged position between true binding sites. However, since the underlying localization precision remains comparable across conditions, this averaging mainly leads to a modest broadening rather than a fundamental loss of precision. Second, localization precision and photon statistics. Minor differences in localization precision per event play a subordinate role, as NeNA-derived precisions remain at ~ 3 nm for both $1 \times R1$ and $5 \times R1$, indicating comparable photon statistics under our imaging conditions. Thus, the multiplexed $5 \times R1$ design does not adversely affect localization precision or photon yield and indeed benefits from longer binding events and higher photon rates. The observed loss in effective resolution therefore stems from geometric broadening of the accessible volume rather than from limitations in PSF fitting or photon statistics. Third, and most importantly, geometric linkage error from docking strand length and flexibility. The longer and more flexible docking strand introduces a geometric linkage error that broadens the accessible volume of the imager and thus the localization cloud, as captured by our WLC estimation. This broadening comprises two contributions: (i) thermal motion and bending of an individual flexible docking strand during a single binding event and (ii) binding at different positions along the multiple tandem motifs of the $5 \times R1$ sequence. This effect is quantitatively demonstrated in broadened localization clusters for single label sites and larger apparent inter-spot distances for fully labeled PCNA (Figure 2, Table 2). In contrast, classical single-motif DNA-PAINT, particularly at higher labeling densities, achieves superior spatial fidelity, albeit with slower acquisition rates.

2.5 | Design Implications and Practical Guidelines for DNA-PAINT Docking Strands

Consequently, the optimal docking strand architecture must balance imaging speed, photon yield, and positional precision according to the structural scale of interest, especially when resolving assemblies below 10 nm such as the trimeric PCNA complex. In addition, photo-induced depletion of docking sites can progressively reduce sampling over long acquisitions and the redundant binding domains of repetitive designs have been shown to mitigate such site loss and thereby stabilize event rates over time, which represents a practical advantage of multivalent docking strands in extended imaging experiments [21]. These practical advantages must be weighed against resolution trade-offs arising from the docking strand geometry itself.

Our findings highlight the trade-off between imaging throughput and spatial resolution in DNA-PAINT as a consequence of docking strand architecture. For short spatial distances, docking strand design becomes critical. While repetitive sequences such as $5 \times R1$ provide advantages in imaging speed, multiplexing and binding frequency, they impose inherent geometric ambiguity that limits their suitability for quantitative nanoscale measurements. The observed spatial blurring likely arises from a combination of distributed binding sites, a lower fraction of successfully labeled complexes in the fully labeled $5 \times R1$ samples and potential short-range re-binding or hopping between adjacent binding motifs within the flexible $5 \times R1$ docking region during a single apparent event, which effectively broadens the localization cloud (Figures 1, 2, S1, and S2).

Some of these effects can, in principle, be mitigated experimentally by adjusting imager strand concentration or exposure time, which can reduce the incidence of overlapping binding events and improve photon statistics for a given structure. Lowering the imager concentration to suppress multi-site occupancy, however, inevitably slows down the occurrence of binding events over time and thus increases the total acquisition time required to reach a given localization density. In contrast, the geometric linkage error associated with docking strand length and flexibility is an intrinsic property of the labeling architecture and cannot be fully eliminated by tuning imaging parameters. In flexible multivalent architectures such as $5 \times R1$, these adjustments cannot compensate for the extended accessible volume set by docking strand geometry. Beyond these experimental adjustments, the intrinsic scaling of geometric blur with docking strand length sets fundamental limits.

Although this behavior does not constitute direct evidence for a distinct, long-lived trapped state, it represents a functional broadening mechanism that undermines the sub-10 nm resolution potential of DNA-PAINT. Consistent with a worm-like-chain description of the ssDNA docking region, the predicted projected blur increases to approximately 2.3 nm, 2.7 nm, and 4.3 nm for $2 \times R1$ (10 nt, $L \sim 6.5$ nm), $3 \times R1$ (13 nt, $L \sim 8.5$ nm), and $10 \times R1$ (34 nt, $L \sim 22.1$ nm), respectively. Achieving maximal spatial accuracy therefore requires monovalent architectures or shorter repetitive variants, but for a more detailed assessment, a system-specific threshold at which docking-strand-induced broadening of localization clouds becomes detrimental for achieving sub-10-nm resolution could be determined, for example by systematically increasing the repeat number ($1\times$, $2\times$, $3\times$, $4\times\dots R1$). Such geometric constraints interact with other imaging parameters in complex ways.

Maximal resolution depends on multiple interrelated parameters. High photon counts, obtained through longer binding times or optimized fluorophores, are essential for achieving high localization precision as long as the emitter position remains effectively fixed relative to its docking site. In densely labeled systems or multivalent architectures, however, prolonged apparent dwell times can partly arise from overlapping or rapidly re-associating binding events within a local cluster of docking sites. In such cases, the fluorescence signal is integrated over several nearby docking positions, so that additional photons no longer translate into higher spatial accuracy but instead contribute to a broadened localization cloud. Shorter binding times in combination with faster exposure rates can, in principle, reduce temporal overlap and still provide sufficient photons per event, and thus represent an effective handle to optimize temporal separation and photon yield. In flexible multivalent architectures such as $5 \times R1$, however, these adjustments cannot compensate for the geometric linkage error and extended accessible volume set by docking strand length and flexibility so that the effective resolution ultimately remains limited by docking-strand geometry rather than photon statistics alone. Increasing the exposure time can, in principle, further boost the photon yield per event, but at the same time raises the risk that multiple binding events on different docking strands are integrated within a single frame in densely labeled samples. Recent approaches such as RESI can improve precision by averaging repeated binding events but become unreliable when localization clouds broaden due to rotational flexibility or extended binding geometries [6]. Similarly,

techniques such as MINFLUX localize emission minima with sub-nanometer accuracy but still depend on stable binding events for sufficient photon statistics [22, 23]. The additional blur introduced by the $5 \times R1$ architecture, on the order of a few nanometers compared to $1 \times R1$, is particularly relevant for applications that aim to resolve sub-10 nm features, such as the PCNA trimer or other densely packed protein assemblies. In more conventional SRM experiments employing antibody-based labeling or other tags with linkage errors in the range of 10–20 nm, the incremental broadening from repetitive docking strands is often negligible relative to the existing labeling uncertainty. In such cases, the throughput advantages of multivalent designs can outweigh the loss in ultimate spatial fidelity, whereas for very high-resolution, minimally invasive labeling schemes, short single-motif docking strands remain the preferred option.

Furthermore, imager strand concentration must be optimized to balance binding frequency and signal overlap. Excessively high concentrations increase simultaneous binding, reducing localization precision through overlapping signals and apparent continuous occupancy. Careful titration of imager concentration is therefore essential to achieve optimal temporal resolution and quantitative accuracy.

In this work, we exclusively vary the docking strand architecture ($1 \times R1$ vs. $5 \times R1$) and keep the imager design constant; approaches based on modified imager strands (for example fluorogenic or self-quenching imagers) address a complementary aspect by allowing higher probe concentrations at similar binding kinetics [24–27]. For structures larger than 10 nm, optimized multivalent or two-dye-imager (TDI) strategies can offer high throughput and sufficient accuracy while significantly reducing acquisition time [24–27]. However, below the 10 nm threshold, even advanced approaches will face physical and photophysical limitations.

Thus, single-motif docking strand architectures such as $1 \times R1$ remain the optimal choice for applications requiring maximal spatial resolution and positional accuracy, whereas repetitive designs are more suitable for high-throughput or screening applications where imaging speed outweighs sub-nanometer precision. Here, parameters such as docking strand length, binding kinetics, and photon budget must be precisely balanced. Classical DNA-PAINT with short single-motif strands benefits from longer dwell times and higher photon counts, supporting superior localization precision. These architectures also minimize smearing effects from strand flexibility and enable higher labeling densities, which can be challenging for larger repetitive constructs.

3 | Conclusion

This study provides a quantitative framework to assess how DNA-PAINT docking strand architecture influences achievable spatial resolution. Using trimeric PCNA as a protein-based nanoruler, we show that repetitive docking motifs such as $5 \times R1$, while enabling faster image acquisition, introduce geometric variability that broadens localization distributions and degrades effective resolution. In contrast, classical single-motif strands maintain high positional fidelity and represent the optimal choice for imaging targets with sub-10 nm spacing. The observed trade-off between imaging speed and structural accuracy underscores the need for tailored docking strand designs

depending on experimental goals. Beyond benchmarking DNA-PAINT performance, these findings provide design principles applicable to diverse quantitative super-resolution approaches that rely on transient molecular interactions.

4 | Methods

4.1 | PCNA Sample Preparation

Generation of PCNA nanorulers for validating sub-10 nm spatial resolution in fluorescence imaging has been described previously [12]. Briefly, the clickable unnatural amino acid Norbornenyllysine (Norb-K) was incorporated into the K110I mutant of human Proliferating Cell Nuclear Antigen (PCNA, UniProt: P12004) by replacing Serine 186 via Genetic Code Expansion (S186Norb). The K110I mutation was introduced to prevent undesired inter-ring crosslinking of PCNA trimers during the crosslinking step before imaging (see below). Thus, *E. coli* C41(DE3) cells were co-transformed with suppression plasmid (pACYC NORS3T) [28] and expression plasmid (pRSET-A PCNA S186Norb K110I). Cultures were grown in 2XYT media with appropriate antibiotics (37°C, 200 rpm). When cell density (OD₆₀₀) reached 0.5, the medium was exchanged with a richer chemically defined media, BactoMedia (ThermoFisher), supplemented with 0.75% glycerol, 1 mM Norb-K, and antibiotics. After 30 min recovery, protein expression was induced with 0.3 mM IPTG at 27°C for 24 h. Protein purification was performed via Ni-NTA affinity chromatography, Strep-Trap purification, and size-exclusion chromatography as previously described [12]. The resulting trimeric PCNA complexes were verified by SDS-PAGE, analytical gel filtration, and spectroscopic quality control. Purified protein stocks were stored in clicking buffer (25 mM Tris buffer pH 7.5, 1 mM EDTA, 1 M NaCl) until further bioorthogonal coupling with DNA docking strands for DNA PAINT experiments. After clicking and determining the degree of labeling (DOL), the PCNA samples were crosslinked using 500 eq. BIS(NHS)PEG-9 in crosslinking buffer (20 mM HEPES pH 8.0, 150 mM NaCl) for 30 min at RT. Afterward, the samples were quenched with 50 mM Tris pH 8.0 for 15 min at RT prior diluting the crosslinked samples in appropriate buffers for imaging.

4.2 | PCNA Labeling with Docking Strands

For DNA-PAINT measurements the docking sequence motifs 1 x R1 (5'-3': TCC TCC T) and 5 x R1 (5'-3': TCC TCC TCC TCC TCC T), each modified at the 5' end with methyl-tetrazine (Me-Tet), were used (biomers.net). To prepare 3× clicked PCNA, PCNA (S186Norb K110I) (1.5 nmol) was reacted with 50 eq. of Me-Tet-docking strand in clicking buffer (25 mM Tris buffer, 1 mM EDTA, 1 M NaCl, pH 7.5) for 7 days to ensure complete ligation. The reaction mixture was then purified by SEC in Superdex 200 Increase 10/300 GL column (Cytiva) with 20 mM HEPES, 150 mM NaCl (pH 8.0) as elution buffer. Fractions containing PCNA were pooled and concentrated to 100 µL. For preparing 1× clicked PCNA, the reaction was performed with only 0.2 eq. of Me-Tet-docking strand to minimize the formation of multiply labeled species. Prior to mixing, the docking strand stock solution was pre-diluted in clicking buffer to ensure low local oligo concentration at the time of addition. Ligation and purification were carried out as described above.

4.3 | Estimation of the Degree of Labeling (DOL)

Steady-state absorption spectra were measured on a V-650 spectrophotometer (Jasco). Samples were measured in a 0.3 mm path-length fluorescence cuvette (Hellma, 105.251-QS) in crosslinking buffer (20 mM HEPES pH 8.0, 150 mM NaCl, pH 8.0). Protein concentration and degree of labeling (DOL) were calculated from the absorption spectra. To separate overlapping contributions from the protein and clicked DNA docking strands, peak deconvolution was performed. For this, an absorption spectrum of purified, unlabeled PCNA was recorded and used as a reference in the labeled samples. The measured absorbance spectrum of the DNA-conjugated PCNA was subsequently analyzed by peak deconvolution to quantitatively resolve the individual DNA and protein peaks and determine their relative contributions to the total absorbance signal. All deconvolution analyses were performed in OriginPro 2023 b using the Multiple Peak Fit tool with Gaussian peak fitting, enabling extraction of absorbance values for DOL calculation.

4.4 | Single Molecule Surface Preparation

The surfaces of 8-well chambered cover glass with high performance cover glass (Cellvis, C8-1.5H-N) were washed once with double distilled water (ddH₂O) and treated with 2% Hellmanex (Hellma) for 1 h. Afterwards, the treated chambers were washed 3× with ddH₂O, incubated with 1 M KOH (Fluka) for 20 min and rewashed twice with PBS and once with PBS containing 50 mM MgCl₂. Gold nanoparticles were used as fiducial markers. Therefore 200 nm gold nanoparticles (Nanopartz) were diluted 1:50 in PBS containing 50 mM MgCl₂ for 20 min. at RT. Afterward, the surface was washed thrice with PBS. The crosslinked PCNA samples were diluted to $\approx 1 \times 10^{-9}$ M in imaging buffer. Briefly, the prepared surfaces were incubated with the diluted protein samples for ≈ 20 s and washed 3× with imaging buffer.

4.5 | DNA-PAINT Imaging

Super-resolution imaging was performed on an inverted wide-field fluorescence microscope (IX-71, Olympus). For excitation of Cy3B imager strands (DNA-PAINT), a 561 nm diode laser (Genesis MX561-500 STM, Coherent) with irradiation intensity of ≈ 0.2 kW cm⁻² in combination with a clean-up filter (Laser Clean-up filter 561/14, Chroma) was used. All measurements were performed using circular polarized light by mounting a quarter-wave plate (Thorlabs) within the excitation path. The laser beam was focused onto the back focal plane of the oil-immersion objective (×60, NA 1.45, Olympus). For measurements, emission light was separated from the illumination light using a dichroic mirror (FF580-FDi01, Semrock) and spectrally filtered by a bandpass filter (BrightLineHC-600/50, Semrock). Images were recorded with an electron-multiplying CCD camera chip (iXon DU-897, Andor) using an EM gain of 200. Pixel size for data analysis was measured to 128 nm. For DNA-PAINT measurement, four movies of 36,000 images with an exposure time of 100 ms (frame rate 10 Hz) were recorded by total internal reflection fluorescence microscopy illumination, respectively. All DNA-PAINT experiments were performed with 2.5 (1 x R1) or 0.5 and 2.5×10^{-9} M (5 x R1) imager strand concentration

(5'-3': AGG AGG A, Eurofins), 3'-modified with Cy3B, in PBS-based buffer containing 500 mM NaCl, adjusted to pH 7.4. All imaging buffers included photostabilizing ingredients consisting of a PCA/PCD (3,4-dihydroxybenzoic acid/protocatechuate 3,4-dioxygenase from *Pseudomonas*) (Sigma-Aldrich) oxygen scavenger system and Trolox ((+/-)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (Sigma-Aldrich).²

4.6 | DNA-PAINT Analysis

All SMLM results were analyzed with rapidSTORM3.3, and the highly resolved pictures were reconstructed with ThunderSTORM [29, 30]. For analyzing the DNA-PAINT binding parameters of individual proteins, fluorescent spots containing more than 550 (DNA-PAINT) photons per frame were analyzed. The estimation of the number of localizations per labeled PCNA was calculated by using the tracking function (Kalman filter) of rapidSTORM3.3. Fluorescent spots were tracked over the whole image stack within a tracking radius of 70 nm and exported as tracked localization file. A custom written python script was used to calculate the number of frames between on-time events of the same fluorescent spot within the defined tracking radius (off-time) as well as the number of on-time events per tracked spot. The localization precisions were calculated according to Mortensen [18]. For this a total photon intensity was estimated by binning the successive frames for each extended binding event of imager strands. For showing resolved trimeric PCNA, ~20 structures were picked. These picked structures were also used to estimate mean inter-spot distances, which were determined using ImageJ by measuring the center-to-center spacing of resolved fluorescent peaks. The resulting distance distributions were fitted in OriginPro 2023b using Gaussian peak fits to extract characteristic mean and variance values for each dataset. Quantitative parameters were analyzed from $N=3$ independent experiments. Means $\pm$ SD were calculated and compared using independent t-tests (OriginPro 2023b). All key differences (photon flux, on-times, events, convex hull ratios) were highly significant ($p < 0.001$ – $p < 0.005$). To quantitatively model geometric variability of docking strands, we applied worm-like-chain (WLC) analysis under imaging buffer conditions (1×PBS + 500 mM NaCl, pH 7.4). Docking strands were approximated as semiflexible polymers with ssDNA contour length $L_{\text{ssDNA}} = n \times b$ (base spacing $b = 0.65$ nm/nt [16] and persistence length $l_p = 1.25$ nm at high ionic strength [17, 31]. The C5 linker between MeTet anchor and ssDNA 5'-end was treated as a rigid segment ($L_{\text{C5}} = 1.2$ nm, estimated from $L = 5 \times 0.126$ nm C–C bond length + torsional angle) added in quadrature to the WLC end-to-end distance:

$$\langle R^2 \rangle_{\text{total}} = 2 l_p * L_{\text{ssDNA}} * \left[1 - \left(\frac{l_p}{L_{\text{ssDNA}}} \right) * \left(1 - e^{-\frac{L_{\text{ssDNA}}}{l_p}} \right) \right] + (L_{\text{C5}})^2$$

For 1×R1 (TCC TCC T, 7 nt, $L_{\text{ssDNA}} = 4.55$ nm) this yields r_{3D} , RMS = 3.09 nm. For 5×R1 (TCC TCC TCC TCC TCC T, 19 nt, $L_{\text{ssDNA}} = 12.35$ nm) r_{3D} , RMS = 5.31 nm. The 2D-projected blur in the imaging plane is r_{2D} , RMS = r_{3D} , RMS / $\sqrt{3} = \sim 2.52$ nm (1×R1) and ~ 4.34 nm (5×R1).

To estimate the localization spread from repetitive sequences, we used python scripts based on locan (version 0.21.0) and the scientific python stack [32]. Localizations with a photon intensity

between 2000 and 6000 photons per frame were selected. A linear drift correction based on an iterative closest point algorithm was applied. Clusters were then identified by DBSCAN (minPoints = 3 and epsilon = 20 according to and selected for localization counts (between 20 and 200), temporal variation of binding events (standard deviation of frame number to be larger than 500), and circularity computed from the inertia moments of each localization cluster (less than 0.8) [19]. From the selected clusters the convex hull area as function of localization counts per cluster was computed. As control for z-drift, the nearest-neighbor-based localization precision was computed as implemented in locan [20].

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Representative raw DNA-PAINT image stacks supporting the findings of this study have been deposited in the Open Science Framework (OSF) and are available at <https://doi.org/10.17605/OSF.IO/4TN58>. These datasets include raw TIFF movies for monomeric and trimeric PCNA labeled with 1×R1 and 5×R1 docking strands. Additional processed data, localization files, and analysis scripts are available from the corresponding authors upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. The authors have cited additional references within the Supporting Information [30, 31]. **Supporting Fig. S1:** Absorbance-based characterization of PCNA ($\epsilon_{280} = 27,390 \text{ M} \cdot \text{cm}^{-1}$ per monomer) labeled with single- and multivalent docking strands ($\epsilon_{260} = 57,600 \text{ M} \cdot \text{cm}^{-1}$ and $\epsilon_{260} = 154,400 \text{ M} \cdot \text{cm}^{-1}$). (a,b) UV-vis absorbance spectra of monomeric (a) and trimeric (b) PCNA labeled with single-motif $1 \times \text{R1}$ docking strands. (c,d) Corresponding spectra of monomeric (c) and trimeric (d) PCNA labeled with multivalent $5 \times \text{R1}$ docking strands. Black, blue, and red traces represent total absorbance, DNA and protein contributions, respectively. Spectra were measured in crosslinking buffer (20 mM HEPES pH 8.0, 150 mM NaCl) using a 0.3 mm path-length cuvette. Differences in absorbance ratios at 260 nm and 280 nm were used to estimate the degree of labeling (DOL). **Supporting Fig. S2:** Representative DNA-PAINT reconstructions of PCNA labeled with $1 \times \text{R1}$ and $5 \times \text{R1}$ docking strands measured on a single-molecule surface. (a,b) Representative single-molecule reconstructions of monomeric (a) and trimeric (b) PCNA labeled with single-motif $1 \times \text{R1}$ docking strands using 2.5 nM of imager strands. (c,d) Corresponding reconstructions for monomeric (c) and trimeric (d) PCNA labeled with multivalent $5 \times \text{R1}$ docking strands using 0.5 nM of imager strands. Both designs enable the visualization of the trimeric ring structure at sub 10 nm resolution. Scale bars: 10 nm. **Supporting Fig. S3:** Photophysical characterization of PCNA labeled with $5 \times \text{R1}$ docking strands at 2.5 nM imager concentration. (a) ON-time distributions (ms). (b) OFF-time distributions (s). (c) Intensity distributions (photons ms^{-2}). (d) Number of binding events per molecule. Gray traces represent monomeric PCNA, and magenta traces correspond to trimeric PCNA. All histograms are normalized to their maxima and were obtained under identical imaging conditions at $\approx 0.2 \text{ kW cm}^{-2}$ excitation. **Supporting Fig. S4:** Differences in the x- and y-coordinates of near-by localization positions in successive frames as used to determine NeNA localization precision. Position deltas are plotted as function of recording frame averaged by a sliding window of 2000 frames. (a) data for a measurement with the $1 \times$ docking strand. (b) data for a measurement with the $5 \times$ docking strand.