High-speed 10-plex DNA-PAINT with a larger sequence repertoire

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Abstract

DNA-Points Accumulation for Imaging in Nanoscale Topography (DNA-PAINT) enables multiplexed super-resolution imaging of biological samples. We expand the repertoire of speed-optimized DNA sequences for DNA-PAINT imaging to facilitate the molecular-resolution imaging of up to ten targets in a sequential manner. These imager sequences showcase a range of binding kinetics, providing the flexibility to select optimal sequences for specific applications. We demonstrate the application of twelve speed-optimized imager sequences for multiplexed super-resolution imaging of the microtubular network and Nuclear Lamins in the cellular context. We anticipate that the ability to image as many as ten targets using the simple Exchange-PAINT strategy at accelerated speed will drive its further adoption for diverse cellular imaging applications.

Introduction

The past couple of decades have seen tremendous development in a variety of super-resolution imaging techniques.[1-5] These technologies are gaining popularity as they enable us to gain greater insights into the spatial distribution of biomolecules at nanometer resolution in intact cells. Single-Molecule Localization Microscopy (SMLM) is one of the super-resolution modalities that relies on blinking of individual fluorophores by switching them between bright and dark states to computationally generate a spatial distribution map of target molecules by localization of individual binding events.[2-4] DNA-PAINT is one such SMLM modality that achieves the “blinking” by taking the advantage of the stochastic, transient hybridization between two short complementary single stranded DNA.[4,6]

DNA-PAINT is a versatile technology that enables multiplexed imaging by encoding the target species identity in to DNA docking strand.[7] One caveat of DNA-PAINT imaging is that the acquisition times of data is enormous, requiring up to several hours for each imaging round.[8] In fact, the binding kinetics of initial DNA sequences used for DNA-PAINT, called P-sequences, were rather slow,[4] thus making the process of acquire meaningful data is time consuming. The P-sequences were composed of all four nucleotides (A, T, G, and C),[9] probably leading to the formation of weak secondary structures, thus displaying slower binding kinetics.

Recently, there has been an improvement in the speed of DNA-PAINT imaging by clever design of imager sequences.[10,11] These imager sequences are made up of two non-complementary nucleotides arranged in repetitive blocks to build a concatenated sequence.[12] This arrangement of the imager sequence prevents any canonical secondary structure formation, thus improving the rate of binding. Additionally, the repetitive pattern allows for overlapped concatenation which provides greater number of docking sites for a given imager without the cost of linear length increase, thus further improving the rate of binding (Supplementary Figure 1a). Together, these optimized sequences result in an order of magnitude faster imaging compared to P-sequences.

Currently, there are only six speed-optimized sequences reported, allowing for six-plex super-resolution imaging. To increase the multiplexing capability, recent studies have utilized secondary DNA probes[13] or adapter probes[14] for conceptually unlimited multiplexing, showcasing their applicability for 30-plex[13] and 13-plex[14] cellular imaging. However, implementation of these strategies required rather complex strand hybridization and dissociation schemes. Additionally, secondary probe sequences need to be carefully designed and experimentally verified to ensure optimal hybridization and dissociation kinetics while minimizing the non-specific binding before their implementation. Consequently, there is a constant search for enhancing the multiplexing capabilities of speed-optimized DNA-PAINT with minimal difficulty for its broader applicability.

Recently, an astonishing sub-nanometer resolution was achieved using a variant of the DNA-PAINT technique called resolution enhancement by sequential imaging (RESI).[15] In RESI, the target molecule is imaged over four rounds of DNA-PAINT, and the localization clusters from each round are individually fitted and rendered together to improve resolution. However, due to the limited availability of speed-optimized sequences, RESI was restricted to single-target imaging, underscoring the need for more imager sequences.

In this report, we lay out the design principles for generating all possible imager sequences constituting non-complementary nucleotides for faster imaging. In total, we report 12 optimized speed sequences of which ten can be used for multiplexed DNA-PAINT imaging in cellular settings. These speed-optimized DNA-PAINT sequences enable us to perform multiplexed super-resolution imaging of as many as ten targets in sequential exchange rounds without adopting any complicated strategies.

Results

We started searching for all possible imager-docking strand combinations that are orthogonal to each other comprising of non-complementary nucleotides. Two key rules that were followed in this search are that the imager strand must not comprise nucleotides that are complementary, and that they must form units that can be overlapped and repeated to form longer docking strands[12] (Supplementary Figure 1b). By applying these rules, we observe that there are two di-nucleotide units, four tri-nucleotide units, and six tetra-nucleotide units that satisfy these rules in the possible sequence combination space (Figure 1a and Supplementary Figure 1c). We reserved the blocks containing cytosines (C) and Adenine (A) or Thymine (T) for the docking strands, and guanines (G) and Adenine (A) or Thymine (T) for the imagers as a convention. As these sequences preclude secondary structures within them, a high fraction of encounters with cognate complementary strands would result in duplex formation, hence showcasing faster association rates. On the contrary, any secondary structure in the sequences will have reduced probability for contact and hybridization, hence slower association rates. Concatenation of the blocks provide additional improvements in terms of binding rates as this increases the number of ways the

imager can bind on the docking strand successfully. Here, we propose that di-, tri- or tetra-nucleotide blocks can be used for generating as many as 12 sequences without compromising the speed and orthogonality of the imagers (Figure 1a and 1b) of which six sequences were reported earlier[12]. We note that increasing the block length to five-nucleotides will result in cross-binding between imagers of different block lengths with a one-nucleotide or a two-nucleotide mismatch, leading to no gain in imager numbers while maintaining strict orthogonality (Supplementary figure 1d).

To test the applicability of all the speed sequences for DNA-PAINT super-resolution imaging for their multiplexing capabilities, we utilized unique DNA nanostructures carrying docking strands for each of the imager sequences. For visual identification and testing the resolution, we designed origami nanostructures carrying docking strands in a 4×3 grid pattern where each strand is separated from its neighbor by 20 nm. We immobilized the origami structures in a single microfluidic channel and imaged them in 12 rounds using Exchange-PAINT modality[6] (Figure 1c). As expected, all 12 species of origami nanostructures were clearly visible in the merged data (Figure 1d). While majority of the imagers showed clear specific binding on their target docking strands, two sets of imagers (R1 and R9, R2 and R10) displayed unexpected cross-binding events (Figure 1e), emphasizing the tolerance for certain DNA mismatches to form detectable duplexes with just five-nucleotide complementarity. Interestingly, R1 imager binds to the R9 docking strand but not vice versa. Because of this cross-binding, only one imager from each of these pairs can be used for multiplexed imaging. Together, Exchange-PAINT imaging of “12-color” data shows that we can achieve multiplexing capabilities of as many as ten targets at high-speed and high-resolution (Figure 1d, 1e and Supplementary Figure 2).

We next performed kinetic analysis of all the twelve imager-docking strands. For this, we utilized DNA nanostructures decorated with docking strands in a rectangular pattern to recognize their position on the surface. To study kinetics of a given docking strand, we placed it in the center of the same DNA nanostructure (Supplementary Figure 3a). We performed two color DNA-PAINT imaging to obtain the pattern of the nanostructure and kinetics of the docking strand (Supplementary Figure 3b). Our kinetic analysis shows that these imagers

display a range of binding times from few hundred milli-seconds to over a second (Figure 1f and Supplementary Figure 3c). This versatile imager pool with varying binding times enables us to utilize imager strands based on the required properties for a given imaging application. For instance, imagers binding one or two frames will always result in compromised localization precision because of the partial binding over a given acquisition frame. Because R8-R12 imager sequences display binding times longer than five hundred milliseconds, they are expected to provide better localization precision than R1-R7 which show an average binding time of less than three hundred milliseconds (Figure 1f).

We next wanted to perform multiplexed DNA-PAINT super-resolution imaging in cellular settings using all 12 imager-docking strands. As a proof-of-principle application, we chose the microtubular network in HeLa cells as our target structure. We labelled the microtubular network with primary antibody against alpha-tubulin. The primary antibody was then labeled with secondary antibody that is conjugated with all 12 docking strands in a single pot reaction providing homogenous tagging with all docking strands (Figure 2a). We performed 12-color imaging in exchanging rounds of DNA-PAINT in a sequential manner. Each imager data shows the expected pattern for microtubule structure, thus showcasing their applicability in cellular settings in a multiplexed super-resolution imaging (Figure 2b). We note that the entire 12-color Exchange-PAINT imaging was only requiring below four hours.

Additionally, we performed DNA-PAINT imaging of nuclear Lamin structure using each of the imagers in separate samples of HeLa cells. For this, we labeled the Nuclear Lamin with primary antibody and DNA docking strand conjugated secondary nanobody. All the imagers showed the expected super-resolution data for the Nuclear Lamin structure encompassing the nuclear periphery (Supplementary Figure 4). Line density profile for the number of localizations showed a good signal to noise ratio across most imagers (Supplementary Figure 5). Unexpectedly, the R3 imager showed non-specific signal appearing like the nucleolar structure entailing it unfit to image nuclear targets (Supplementary Figure 4 and 5). This data demonstrates that we can perform at least nine-color imaging of nuclear targets with an Exchange-PAINT based approach with the repertoire of speed sequences.

Discussion

While DNA-PAINT is a versatile super-resolution technique that enables both molecular resolution and multiplexing, the sparsely characterized imager-docking strand sequences limit its applications at full potential. Two recent studies showcased theoretically unlimited multiplexed cellular imaging using adapter DNA strands in combination with a few speed optimized DNA sequences.[13,14] Implementation of the rather complicated adapter strand strategy was prompted by the limited availability of only six speed-optimized imager strands, highlighting the need for increasing the number of imager strands. With the newly introduced additional speed imager strands, now a 10-plex imaging can readily be performed in a simple Exchange-PAINT super-resolution imaging. The enhanced multiplexing also means that adapter strand strategy for "unlimited" multiplexing can be done in batches of ten instead of six targets per labeling round which would drastically reduce the time, and the number of adapter exchange rounds required for multiplexing. In addition, the increased repertoire of speed-optimized imager-docking strand sequences will facilitate multi-color sub-nanometer resolution imaging with RESI.[15] With the advent of the new sequences that make 10-plex imaging more accessible with no increase in method complexity, the newer imager sequences will find a broader applications in the field of spatial proteomics and interactome building in additional to other allied fields including biomedical sciences.

Acknowledgements

We acknowledge Vinay Bhardwaj for helping with purifying anti-rabbit IgG secondary nanobody. A.B. acknowledges the support from the Prime Minister's Research Fellowship (PMRF), Ministry of Education, Government of India. We greatly acknowledge Sarit Agasti for allowing us to use their laboratory facilities and S. Kalita for help with HPLC purification of DNA imager strands.

Funding Sources

We also acknowledge Department of Science and Technology, Ministry of Science and Technology, India DST-FIST Program funded Central Facility, Department of Biochemistry, IISc. This work has been supported in part by a DBT/Wellcome India Alliance intermediate

fellowship (IA/I/21/2/505928) and the Department of Biotechnology (BT/PR40186/BTIS/137/3/2020). We greatly acknowledge the support from Max-Planck Institute of Immunology and Epigenetics Freiburg, Germany in terms of partner group to Mahipal Ganji lab.

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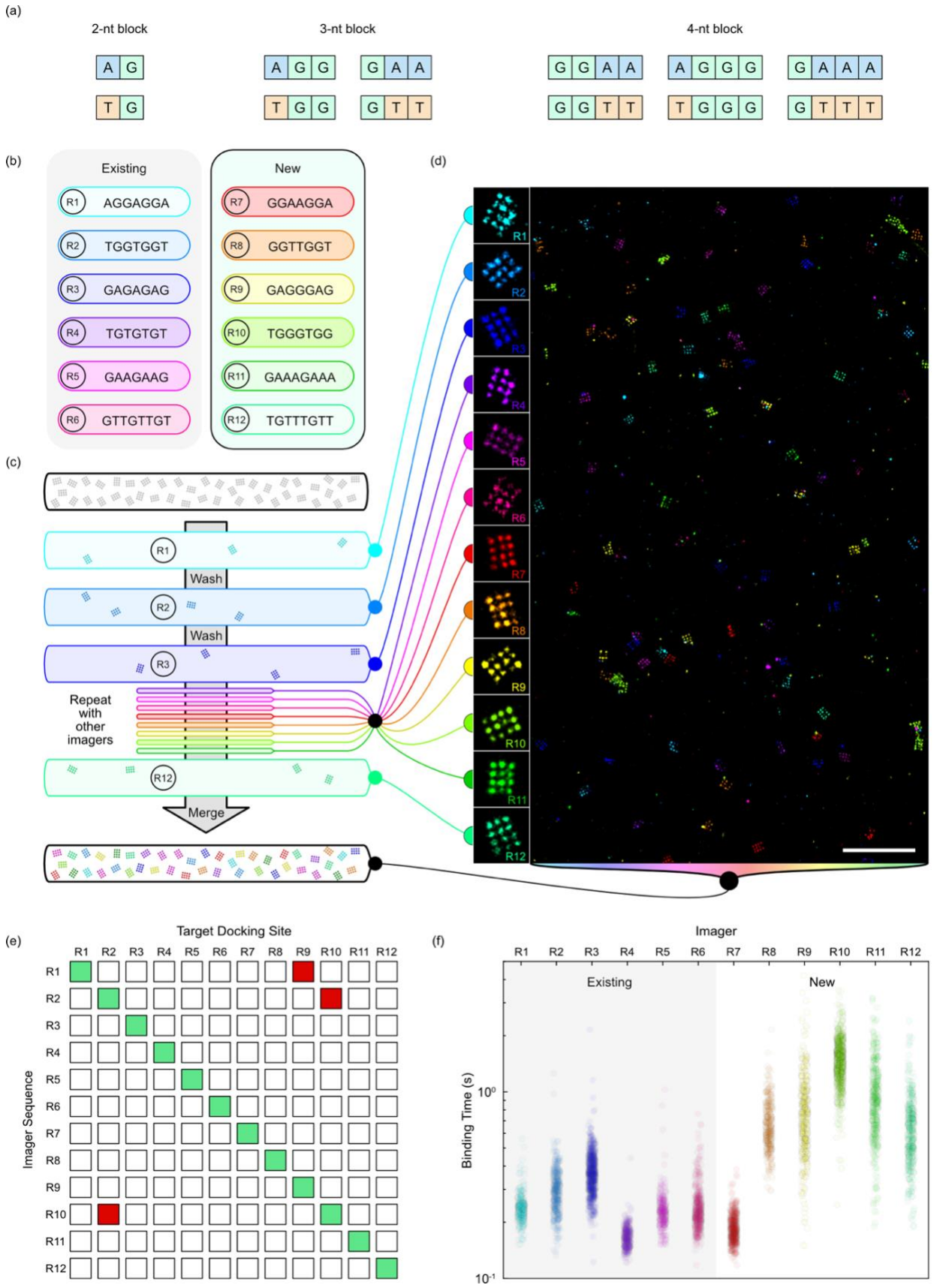
(a)
2-nt block
3-nt block
4-nt block
A G
T G
A G G
T G G
G A A
G T T
G G A A
G G T T
A G G G
T G G G
G A A A
G T T T
(b)
Existing
R1 AGGAGGA
R2 TGGTGGT
R3 GAGAGAG
R4 TGTGTGT
R5 GAAGAAG
R6 GTTGTTGT
New
R7 GGAAGGA
R8 GGTTGGT
R9 GAGGGAG
R10 TGGGTGG
R11 GAAAGAAA
R12 TGTTTGTT
(c)
R1
Wash
R2
Wash
R3
Repeat with other imagers
R12
Merge
(d)
R1
R2
R3
R4
R5
R6
R7
R8
R9
R10
R11
R12
(e)
Target Docking Site
Imager Sequence
R1 R2 R3 R4 R5 R6 R7 R8 R9 R10 R11 R12
(f)
Imager
Existing
New
Binding Time (s)
10^0
10^{-1}

Figure 1: Full repertoire of imager strands for high-speed DNA-PAINT and their binding kinetics. (a) All possible building blocks for constructing imager strands. (b) The sequences of all 12 imager strands. (c) Scheme for 12-color multiplexed imaging using Exchange-PAINT. (d) Merged 12-color DNA-PAINT data of DNA origami nanostructures. (e) Chart showing orthogonality and cross-binding of imagers. Red squares depict unexpected cross-binding. Green squares depict specific binding. (f) Scatter plot showing average of individual binding times per binding site of each docking strand. In (b) and (f), 'existing' refers to imagers reported earlier.[12] (Scale: (d) 500nm)

Figure 2:

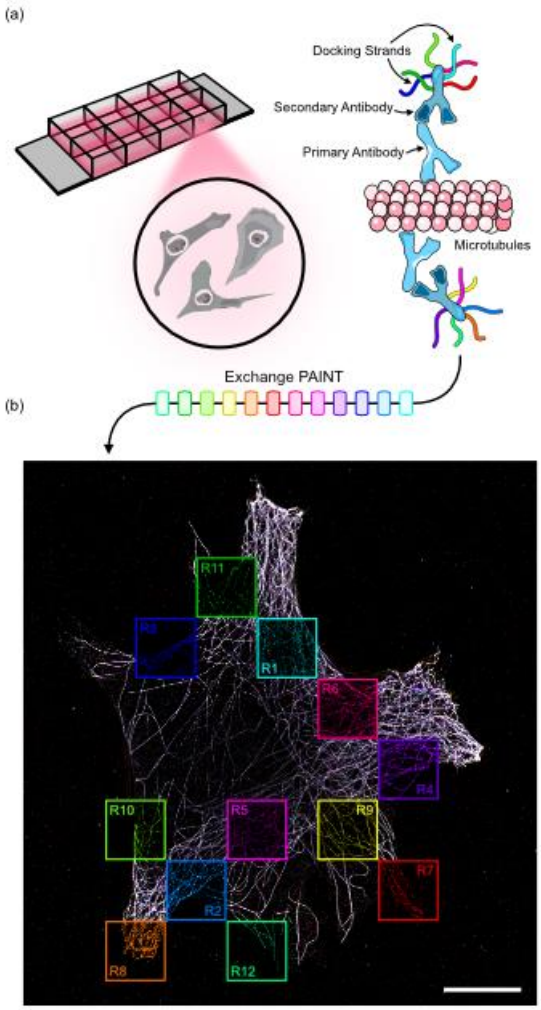

Figure 2: Multiplexed imaging using Exchange-PAINT of microtubules. (a) Schematic diagram showing HeLa cells grown in an eight-well chamber where microtubules were stained with anti-alpha-tubulin primary antibody. The secondary antibody was conjugated with all 12 docking strands (R1 to R12) in a single pot for homogenous labeling of microtubules for 12 rounds of Exchange-PAINT imaging. (b) Super-resolved DNA-PAINT data merging all 12 rounds of imaging. Regions highlighted in colored rectangle show data from specific imager strand and the remaining region encompass the merge of all colors.

Methods

Buffers

10× Folding Buffer: 500 mM Tris-Cl pH 8.0 (Tris-Base, Sigma #77861; HCl, Fisher Scientific #29507), 125 mM $MgCl_2$ (EMPLURA #105833) and 2 mM EDTA (SRL #35888).

Buffer A: 10 mM Tris-Cl pH 8.0 and 100 mM NaCl (SRL #3205)

Buffer A+: 10 mM Tris-Cl pH 8.0, 100 mM NaCl and 0.05% v/v Tween 20 (Sigma #P9416-100ML)

Buffer I+: 50 mM Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.05% v/v Tween 20.

Lysis Buffer: 50 mM Tris-Cl pH 7.5, 300 mM NaCl, 10 mM Imidazole (SRL #61510) and 5 mM DTT (SRL #84834).

Elution Buffer: 50 mM Tris-Cl Ph 7.5, 150 mM NaCl, 500 mM Imidazole and 10% Glycerol (SRL #77453)

Maleimide Labelling Buffer: 100 mM Sodium Phosphate (NaH_2PO_4 SRL #59443, Na_2HPO_4 Merck #61795105001730) pH 7.5, 150 mM NaCl and 250 mM Sucrose (EMPLURA #1.94953.0521)

Low Salt Buffer: 1× PBS (SRL #78529).

High Salt Buffer: 1× PBS with 1 M NaCl.

PEM Buffer: 80 mm PIPES (SRL #49159), 5 mm EGTA (SRL #62858), 2 mm $MgCl_2$, pH 6.8.

Cytoskeleton Extraction Buffer: 0.25% Triton X-100 (Sigma #T8787-250ML), 0.1% Glutaraldehyde (EMS #16020) in PEM, pre-heated to 37 °C.

Cytoskeleton Fixation Buffer: 0.25% Triton X-100, 0.5% Glutaraldehyde in PEM, pre-heated to 37 °C.

PFA Fixation Buffer: 4% Paraformaldehyde (EMS # 15710) in 1× PBS pre-heated to 37 °C.

Quenching Buffer: 0.1 mg/ml of $NaBH_4$ (Sigma #213462-25G) in 1× PBS.

Blocking Buffer: 3% w/v BSA (Sigma #A4503-50G), 0.05 mg/ml Salmon Sperm DNA (Invitrogen #15632011) and 0.25% v/v Triton X-100 in 1× PBS.

Antibody Incubation Buffer: 3% w/v BSA, 0.05 mg/ml Salmon Sperm DNA, 0.02% v/v Tween-20 and 1 mM EDTA in 1× PBS.

Buffer C: 1× PBS supplemented with 500 mM NaCl.

Flow Cell Preparation

Slides and coverslips for microscopic imaging of DNA origami nanostructures were prepared as previously described.[16] Slides (VWR #631-1550) were drilled with five pairs of holes using a diamond head drill bit (Meisinger #801-009-HP) using a drill gun (DigitalCraft #LRUXOR) mounted on a drill stand (Dremel #220-01 Workstation™) under a shallow water bath. Slides were then washed thoroughly using 5% v/v dish washing detergent to remove any trace stains and contaminants. The slides were then thoroughly rinsed with Milli-Q® water followed by 70% ethanol and once again with Milli-Q®. Coverslips (Marienfeld #0107222) were cleaned similarly prior to use. Flow cells were assembled using double sided tape (3M Scotch #136D MDEU) and the open ends were sealed with quick setting epoxy (Araldite Klear). These flow cells were used for all experiments where DNA origami nanostructures were used.

For multiplexed origami imaging, the flow cells were attached with tubing and syringes for ease in buffer exchange.

DNA Origami Nanostructure Folding

Picasso Design[9] was used to get all the staple sequences (Supplementary Table 1 and 2). M13mp18 ssDNA (Bayou Biolabs #P107) was used as the scaffold ssDNA for folding all the DNA origami nanostructures. Oligonucleotide staples containing biotin at 5'-end (Supplementary Table 3) were used to anchor the DNA origami nanostructures onto the surface. A 30 µl of 1× folding buffer folding reactions were set in PCR Tubes (BioRad #TFI0201) with 10 nM of scaffold DNA, 100 nM of biotin staples, 100 nM of blank staples, 1 µM of staples with docking strands. The mix was then heated up to 80 °C and held for five minutes before

a stepwise cooling of 0.1 °C every five seconds till 4 °C in a thermocycler (Applied Biosystems™ ProFlex™ PCR System #4484073).

The folded DNA origami nanostructures were purified from free staples using concentrator centrifugal spin filters (Sartorius Vivaspin® 500 #VS0132). The filters were equilibrated at 3000 ×g for 5 minutes with 500 µl of Milli-Q® water followed by 500 µl of 1× folding buffer. Following this, the origami mix was applied on the column along with 470 µl of 1× folding buffer and spun at 3000 ×g till the volume is reduced to about 50 µl. A 450 µl of 1× folding buffer was then added, and the process was repeated three times to remove the unincorporated staples. The origami structures were then recovered and stored at -20 °C for later use.

Imager Fluorophore Conjugation and Purification

Labeling of DNA strands with fluorophore for preparing imager strands were done by following the published protocol.[16] The imager sequences were purchased from Merck carrying amine modification at their 3'-end (Supplementary Table 4) and dissolved in Milli-Q® water.

Fluorophores were dissolved in DMSO (Sigma #D8418) and stored at -20 °C at the below mentioned concentrations. Cy3B-MonoNHS-Ester (Cytiva #PA63101) was dissolved at 13 mM concentration. Atto647N-MonoNHS-Ester (Sigma #18373-1MG-F) was dissolved at 11.8 mM concentration.

A 15 nanomoles of DNA (15 µl of DNA stored at 1 mM stock) in 1× PBS and 0.1 M $NaHCO_3$ (Sigma #S5761) was used for labeling in each reaction. The labeling was done by using five-fold excess of Cy3B-MonoNHS-Ester or Atto647N-MonoNHS-Ester. The reaction was then left overnight on a shaker at 4 °C.

The incubation mix was then purified to separate the free fluorophore and the unconjugated DNA oligonucleotides from the conjugated product using reverse phase (Phenomenex #00B-4442-E0 Clarity® 5 µm Oligo-RP, LC Column 50 × 4.6 mm, Ea) HPLC (Agilent Technologies). The conjugated product was then lyophilized and the dissolved in Milli-Q® and then stored at -20 °C for future use.

Sample Preparation for DNA Origami Nanostructure Imaging

Microfluidic flow cells were created as mentioned previously.[9] In Brief, the channels were first incubated with 10 to 15 µl of 1 mg/ml Biotinylated Bovine Serum Albumin (Sigma #A8549) diluted in Buffer A. This was followed by a thorough wash of the channel with Buffer A+. A 10 to 15 µl of 0.2 mg/ml of Neutravidin (Sigma #31000) in Buffer A was then introduced and incubated for a minute. Channels were then washed with Buffer A+ thoroughly. Buffer I+ was used for washing the channel prior to its introduction. Origami nanostructures were then diluted appropriately in Buffer I+ and then incubated on the flow cell for 20 minutes. Thorough washing with 600 – 800 µl of Buffer I+ was performed to remove any unbound DNA origami structures.

Flow cells were incubated with Gold Nanoparticles (Sigma #753688-25ML) at a dilution of 1:10 in buffer I+ for 10 minutes and then washed with 600 – 800 µl of Buffer I+ prior to imaging.

Antibody Conjugation

Secondary antibody against rat IgG (Jacksonn #712-005-150) was purchased. The antibodies were buffer exchanged to 1× PBS and concentrated to over 1.5 mg/ml using centrifugal concentrators as mentioned above. Spins were performed at maximum recommended protocol of 12000× g. This ensures better conjugation between the crosslinker and the antibody. DBCO-PEG4-NHS Ester crosslinker (Jena Biosciences #CLK-A134-10) stock solution was stored at a concentration of 15.39 mM. A 25-fold excess of the crosslinker was added to around 100 µg of secondary antibodies and incubated at ambient temperature for 2 hours with mild shaking. Excess crosslinkers were removed by buffer exchange using the same centrifugal concentrators till the crosslinker concentration becomes at least 100 folds lower that the antibody concentration. Conjugation ratio of the crosslinker with antibody is tested using a NanoPhotometer® (Implen NP80). This reaction resulted in a conjugation ratio of around nine cross-linkers per Antibody.

All 12 unique docking strands with azide modification was pooled at equimolar concentration prior to adding them to the antibody mix to ensure homogenous labelling. A 15× excess of azide modified DNA docking strands altogether (Supplementary Table 5) was

added to the cross-linker conjugated antibodies and incubated overnight at 4 °C under mild shaking. Excess docking strands was then removed using the centrifugal filters as mentioned above. The antibodies were stored at 4 °C until further use.

Nanobody Purification and Conjugation

Nanobodies were purified and conjugated as stated earlier.[17] Plasmid (pTP1183) containing Anti-Rabbit IgG Fc nanobody sequence with a single cysteine (AddGene #104163) originally deposited by Dirk Görlich Lab was purchased. Plasmid were isolated from *E. coli* colonies grown on Luria Bertani Agar and in Luria Bertani Broth supplemented with 50 µg/ml of Kanamycin (Sigma #60615) using Qiagen Plasmid Extraction Mini-Prep Kit (Qiagen # 27106) using manufacturer's protocol. The plasmids were then used to transform *E. coli* BL21 cells and grown on Luria Bertani Agar with 50 µg/ml of Kanamycin. Single colonies were picked for inoculating 25 ml of 2×YT (HiMedia #M1251-500G) primary culture with 50 µg/ml of Kanamycin at 28 °C overnight at 180 rpm. This culture was then added to 250 ml of 2×YT secondary culture at 25 °C at 180 rpm. The culture was then induced after one hour with 0.2 mM IPTG and incubated at 25 °C at 180 rpm for four and a half hours. Cells were then harvested on a Beckman Coulter centrifuge with the JA10 rotor at 8000 rpm at 4 °C. The pellets were stored at -80 °C.

The pellets were resuspended in lysis buffer and incubated on ice for 30 minutes prior to 1 hour sonication. The lysate was centrifuged at 30000 rpm on the above-mentioned equipment for 40 minutes at 4 °C. The supernatant was passed through a gravity-based Ni-NTA Affinity Purification Column (GBioscience 786-940). The column was washed thrice with an increasing concentration of imidazole in the lysis buffer (20 mM, 40 mM, and 80 mM). The protein was then eluted with elution buffer.

Buffer was exchanged to maleimide labelling buffer using Cytiva HiPrep 26/10 Desalting Column (#71-5004-28-EG) on a BioRad NGC System. For tag cleavage, bdNEDP1 protease was added to the desalted eluant at a final concentration of 300 nM and incubated overnight at 4 °C. The tag and the protease were separated from the cleaved nanobodies using a gravity-based Ni-NTA affinity chromatography as stated earlier. The tag and the protease bind

to the column and the nanobody is collected in the flow through. The single cysteines are then reduced using 10 mM DTT and 1 mM EDTA overnight at 4 °C. Desalting was repeated as stated above to remove the reducing agent that would hinder the reaction of crosslinker attachment.

DBCO-PEG4-Maleimide (Jena Bioscience #CLK-A108P-10) crosslinker in DMSO was stored at a concentration of 59.2 mM. A five-fold molar excess of the crosslinker was added to the nanobodies an incubated overnight at 4 °C. Excess crosslinker was then removed using centrifugal filters (Merck Millipore Amicon Filter #UFC501096) using the manufacturer's protocol. A three-fold molar excess of DNA with Azide modification was added to 100 µg of the nanobody carrying the crosslinker and incubated overnight at 4 °C for attaching DNA to the nanobodies.

Separation of labelled nanobodies from unlabeled nanobodies is essential to maximize labelling efficiency of the antibodies with nanobodies carrying docking strands. This separation was done using anion exchange column Resource Q (Cytiva #17117701). The column was equilibrated using low salt buffer. The nanobody-DNA mix was diluted using 0.5× PBS to a volume of 250 µl and then injected onto the column. This was followed by a 5 ml wash with low salt buffer. Unlabeled nanobodies don't bind to the column and come out in the flow through or washes. Low flow rate of 1 ml/minute was used with an increasing salt gradient of up to 50% high salt buffer over 20 ml. The DNA labelled nanobody elutes first followed by the free DNA molecules. The fraction containing labelled nanobody is then buffer exchanged to 1× PBS using Amicon centrifugal filters and aliquoted. The nanobodies are either flash frozen in liquid N_2 and stored at -80 °C for long-term storage or stored at 4 °C for up to 1 month.

Cell Culture and Staining

HeLa Kyoto cells (A kind gift from the Maharana's lab) were cultured in Dulbecco's Modified Eagle Medium (DMEM Gibco #11995081) supplemented with 10% Fetal Bovine Serum (FBS Gibco #10270106) and 100 U/ml of Penicillin and 100 µg/ml of Streptomycin (Gibco #15140122) at 37 °C with 5% CO_2. Cells were split when they reached 70% confluency. For

experiments, cells were trypsinized with 0.05% Trypsin-EDTA (Gibco #25300054) for five minutes. Cells were then resuspended in DMEM, centrifuged at 300× g at room temperature for 4 minutes and supernatant was discarded. The pellet was then resuspended in 1 ml of DMEM. Cells were counted on a Countess™ 3 Automated Cell Counter (Thermofischer). Cells were then seeded on cover glass bottom dishes (CellVis #C8SB-1.5H) at 4000 cells per well. Cells were grown overnight as mentioned above.

For microtubule fixation, after 12 to 16 hours, media was removed from the wells and the cells were pre-extracted using prewarmed cytoskeletal extraction buffer for 45 to 90 seconds. This buffer was then replaced by prewarmed cytoskeletal fixation buffer, and the cells were allowed to cross-link for 10 minutes at 37 °C. Cells were then quenched using freshly prepared quenching buffer for seven minutes. Cells were then rinsed thrice with 1× PBS and stored at 4 °C until further use.

For Nuclear Lamin fixation, after 12 to 16 hours, media was removed from the well and the cells were fixed with prewarmed PFA fixation buffer for 10 minutes at 37 °C. Cells were then rinsed thrice with 1× PBS and stored at 4 °C until further use.

Chemically cross-linked cells were blocked and permeabilized using blocking buffer for 1 hour. This was followed by antibody staining for the respective targets. For microtubular staining, anti-alpha-tubulin monoclonal antibody raised in rat (Invitrogen #MA1-80017) was used at a dilution of 1:200 in antibody incubation buffer, and the sample was incubated overnight at 4 °C. The sample was rinsed thrice using 1× PBS. Secondary antibody with all docking strands was added at a dilution of 1:200 to the sample in antibody incubation buffer and incubated for 45 minutes at ambient temperature. The sample was rinsed thrice with 1× PBS and was ready for DNA-PAINT imaging. For Nuclear Lamin staining, anti-Lamin B1 polyclonal antibody (Abcam #ab16048) was used at a dilution of 1:200 in antibody incubation buffer, and the sample was incubated overnight at 4 °C. The sample was rinsed thrice with 1× PBS. Secondary nanobody carrying a single docking strand was used in separate wells for staining the samples. The samples were stained with a 1:200 dilution of

secondary nanobodies in antibody incubation buffer for 45 minutes at ambient temperature. The sample was rinsed thrice with 1× PBS and was ready for DNA-PAINT imaging.

Samples were incubated with Gold Nanoparticles (Sigma #753688-25ML) at a dilution of 1:10 in buffer C for 10 minutes and then washed thrice with 1× PBS prior to imaging.

Microscopy and Imaging

All imaging was performed on a Nikon Ti2 eclipse microscope body mated to a motorized H-TIRF unit and a perfect focus system (PFS). Teledyne Photometrics PRIME BSI sCMOS camera or Hamamatsu Fusion BT sCMOS camera was used for capturing images. Illumination with the 561 nm laser or the 640 nm laser was achieved using the L6cc laser combiner from Oxxius Inc., France. Oil immersion high NA objective lens capable of Total Internal Reflection Fluorescence (TIRF) imaging from Nikon (Nikon #Apo SR TIRF 100×, 1.49 NA, oil immersion) was used in total internal reflection condition for all the imaging. The camera was set with 2×2 pixel binning along with cropping to effectively achieve a 66.56 × 66.56 μm^2 field of view. Each pixel size was 130 nm × 130 nm.

A 20× PCD (Protocatechuate 3,4-dioxygenase; Sigma #P8279-25UN) was prepared in 50 mM KCl, 1mM EDTA, and 100 mM Tris-Cl, pH 8.0, and 50% glycerol to a concentration of 6 μM. The stock PCD was then divided into 10 μl aliquots and stored at -20 °C for future use.

A 40× PCA (Protocatechuic Acid / 3,4-Dihydroxybenzoic acid; Sigma #37580-100G-F) was made by dissolving 154 mg of PCA in 8 ml of Milli-Q® and 3 M NaOH (SRL #96311) was added dropwise and stirred until PCD completely dissolved. Volume was made up to 10 ml and 10 μl aliquots were stored at -20 °C for future use.

An 100× Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma #238813-1G) was prepared by dissolving 100 mg of Trolox in 430 μl of methanol, 345 μl of 1 M NaOH and 3.2 ml of Milli-Q®. The solution was stored as 20 μl aliquots at -20 °C for future use.

For multiplexed origami nanostructure imaging was performed by Exchange-PAINT[6]. In brief, a 200 μl of imaging buffer was prepared in buffer I+ with a final concentration of 1× PCA, 1× PCD and 1× Trolox, along with imagers. After each imaging round, a 1 ml of buffer I+ was used

to wash the flow cell to remove the previous imager, and the next imager was introduced. This process was performed sequentially with all twelve imager sequences. Imaging was performed with the 561 nm laser.

For kinetics studies, 100 µl of imaging buffer was prepared as mentioned earlier. The imaging buffer was added into the flow cell and sealed prior to imaging. Imaging with the 640 nm laser was performed prior to 561 nm laser to minimize effect of photobleaching of Atto647N fluorophore by the higher energy light source.

For cellular imaging, 200 µl of imaging buffer was prepared in buffer C with a final concentration of 1× PCA, 1× PCD and 1× Trolox, along with imagers. For multiplexed cellular imaging, Exchange-PAINT was performed with all 12 imager sequences sequentially. Washes between each round were performed using at least 600 µl of 1× PBS thrice. Imaging was performed with the 561 nm laser.

Image Reconstruction and Kinetic Analysis

Image acquisition was performed using Nikon's proprietary software NIS Elements. Images were stored as *.nd2 files and read using Picasso Localize[9] to obtain the localization. Drift correction was performed using redundant cross-correlation (RCC) followed by drift correction using picked origami nanostructures or fiducial markers using Picasso Render[9]. In multiplexed imaging, multiple channels were aligned to each other using RCC followed by picked fiducial markers.

Kinetic analysis was performed using inbuilt functions in Picasso Render[9] where origamis showing signal for kinetic studies are picked individually and the properties of the picks are saved. The obtained files are then read using custom python codes and plotted.

For line density plots of nuclear Lamin, localizations from rectangular regions were extracted and a frequency density plot was made along the length of the picked region.