Chapter 13

DNA-PAINT Super-Resolution Imaging for Nucleic Acid Nanostructures

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Abstract

Far-field super-resolution fluorescence microscopy has allowed observation of biomolecular and synthetic nanoscale systems with features on the nanometre scale, with chemical specificity and multiplexing capability. DNA-PAINT (DNA-based point accumulation for imaging in nanoscale topography) is a super-resolution method that exploits programmable transient hybridization between short oligonucleotide strands, and allows multiplexed, single-molecule, single-label visualization with down to ~5–10 nm resolution. DNA-PAINT provides a method for structural characterisation of nucleic acid nanostructures with high spatial resolution and single-strand visibility.

Key words DNA-PAINT, Super-resolution imaging, Fluorescence microscopy

1 Introduction

1.1 Overview of DNA-PAINT Super-Resolution Method

Super-resolution fluorescence microscopy has provided an important tool for biologists and nanoscientists, to study single-molecule conformations and dynamics in nanoscale biomolecular and synthetic systems [1, 2]. Recent advances in methods such as STED, SIM, PALM, STORM, and PAINT have allowed optical interrogation of subcellular and nanoscale structures with down to 10–20 nm resolution, allowing direct visualization and quantitation of material defect centers, synthetic nanoscale structures and patterns, cytoskeletal, vesicle and membrane features, single-molecule diffusion and intracellular transport, and so on. In this chapter we discuss a particular implementation of super-resolution microscopy, DNA-PAINT (DNA-based point accumulation for imaging in nanoscale topography), that is particularly suited for single-molecule observation of nucleic acid nanostructures, and detail the design principles, experimental methods, and data analysis procedures for performing experiments with DNA-PAINT.

Yonggang Ke and Pengfei Wang (eds.), *3D DNA Nanostructure: Methods and Protocols*, Methods in Molecular Biology, vol. 1500, DOI 10.1007/978-1-4939-6454-3_13, © Springer Science+Business Media New York 2017

Traditional characterization methods for observation of nucleic acid nanostructures, in a single-molecule fashion, have mostly relied on atomic force microscopy (AFM) and electron microscopy (EM). While providing immensely useful information regarding nanostructure assembly quality and integral structural features, these methods have their limitations. Compared with AFM, EM provides an efficient method for observation of a large sample area, but it operates under non-biological conditions, and does not allow visualization of single nucleic acid strand components due to lack of contrast, and therefore is not sufficient for interrogation of features such as assembly defects. On top of that, both methods lack chemical (strand) specificity and are limited to single-channel images output of electron density and surface topography graphs, respectively.

DNA-PAINT fluorescence microscopy provides an alternative method for interrogation of nucleic acid nanostructures, that allows single-strand visibility and multiplexed detection with high specificity, and operates in biocompatible environment. DNA-PAINT method falls into the family of stochastic localization microscopy (also called single-molecule localisation microscopy, or SMLM) of the recent fluorescence super-resolution microscopy methods. In brief, super-resolution visualization is achieved by temporally separating nearby target fluorescence emission via stochastic switching of each target between a fluorescence on-state and an off-state, and determining their respective positions with sub-diffraction-limit precision [3]. These methods include PALM (photo-activated localization microscopy) [4, 5], STORM (stochastic optical reconstruction microscopy) [6, 7], and PAINT (point accumulation for imaging in nanoscale topography) [8] and their many variants, and mostly differ from each other in the way that stochastic single-molecule switching is achieved.

Methods based on the PAINT principle rely on diffusion and stochastic transient binding of a fluorophore-conjugated affinity probe that is specific to the imaging target. When bound to the target, the fluorophore transiently stays and produces an apparent bright blinking spot on the recorded camera frame. The relative brightness (or blinking signal-to-noise ratio) of the spot is determined by the accumulated photon emission from the binding relative to background generated from the unbound, freely diffusing probes, and can be dramatically enhanced by placing the sample under total internal reflection (TIR) illumination setup. After the first introduction of the PAINT principle [8], several variants were published with different affinity probes (including DNA-PAINT, uPAINT, BALM, *see* refs. [9–11]). Jungmann et al. [9] first noticed that transient binding between short oligonucleotide strands could be used as such affinity probes to produce blinking patterns suitable for super-resolution imaging (DNA-PAINT). In detail, a short oligonucleotide strand (the "docking strand") is labelled on the

molecular target of interest, and a complementary sequence (the "imager strand") is labelled with a fluorophore and used as the affinity probe (Fig. 1a). The method has quickly found application in studying nucleic acid nanostructure conformations and defects, single-molecule binding kinetics and detection of nucleic acid substrates [12, 13].

The method was further developed by Jungmann et al. [14] for multiplexed cellular imaging, Jungmann et al. [15] to quantitative target counting, and Dai et al. [16] to ultra-high-resolution discrete molecular imaging (DMI). In detail, Jungmann et al. [14] generalized the original method to multiplexed, 3D, cellular imaging with orthogonal DNA binding sequences, astigmatism-based 3D point spread function (PSF) detection, and antibody-conjugated DNA strands as target probes. Jungmann et al. [15] developed the method for producing quantitative target counting in a resolution-limited image area. Dai et al. [16] further developed the method to 5 nm resolution, multiplexed single-target imaging via the discrete molecular imaging framework and stringent stage drift correction control. Figure 1b–d illustrates the multiplexing capability, single-target detectability, and high-resolution imaging with DNA-PAINT. In this chapter, we focus on the application of DNA-PAINT as a method for interrogating the self-assembly and molecular arrangements in nucleic acid nanostructures, and discuss the principles for obtaining high-resolution, high-quality images on these nanostructures.

1.2 General Principle for High-Quality and High-Resolution Imaging

In general, the imaging quality and resolution of SMLM microscopy method are determined by a few factors (or technical requirements, *see* Fig. 2) [16]: (1) high localization precision (of single-molecule blinking events), (2) high target signal-to-noise ratio (target SNR in the super-resolved image),[1] (3) low fraction of false localizations (from double-blinking events), and (4) accurate microscope stage drift compensation. Depending on the application, these requirements may be weakened without affecting the imaging results, but all of these requirements are critical for obtaining molecular-resolution single-target imaging, as can be demonstrated by considering a densely packed grid pattern, simulated under different imaging conditions.

The detailed technical discussions of these principles and their characterisation can be found in Dai et al. [16], and are beyond the scope of this method chapter. But it is important to understand the effects of these factors and how they can be achieved in experiments, especially, (1′) high localization precision can be obtained from collecting a large number of photons from single-molecule localization, (2′) high target SNR can be achieved by collecting a large

[1] Not to be confused with blinking SNR, which is calculated in a single recorded frame.

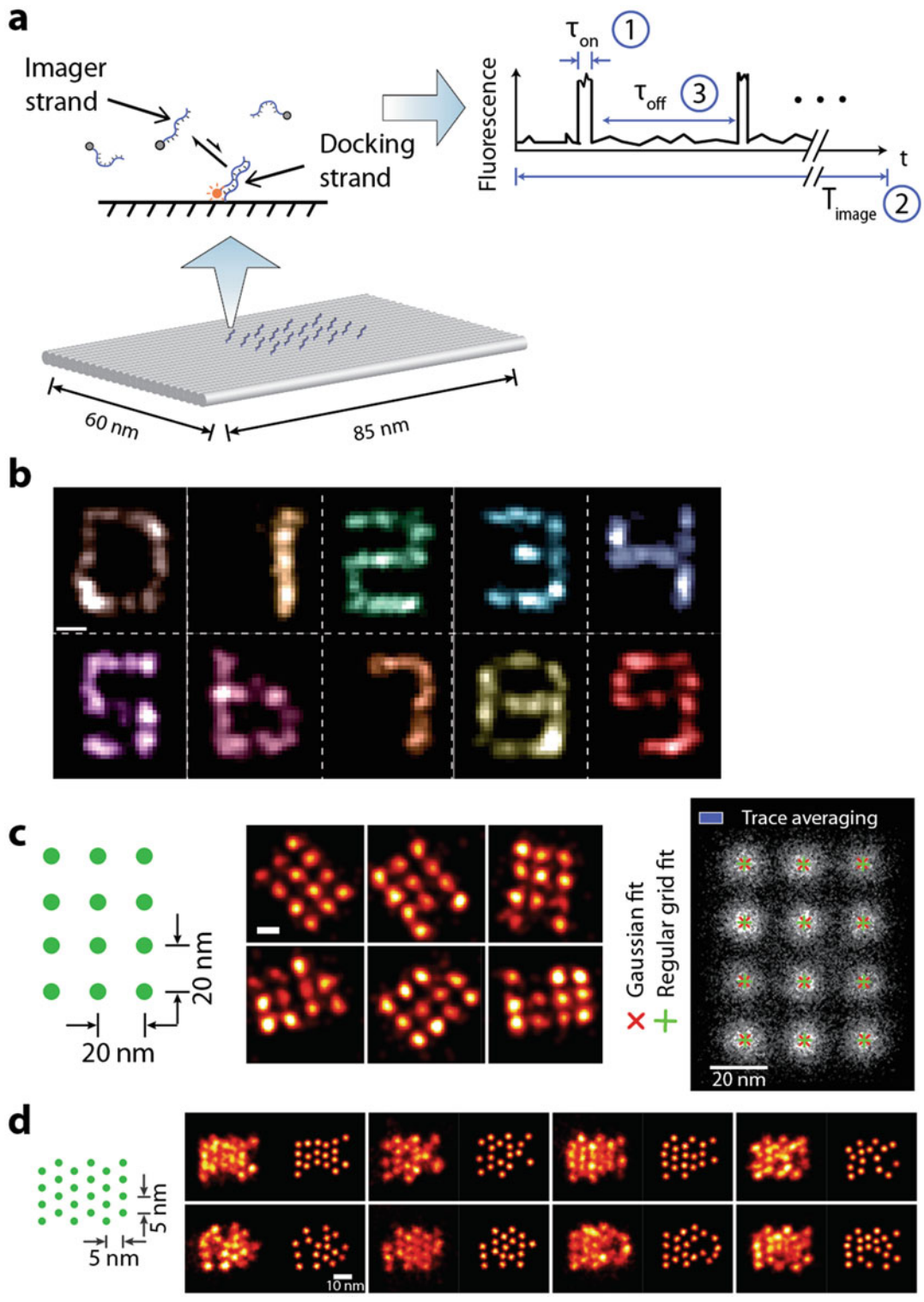


Fig. 1 Principle and examples of DNA-PAINT. (**a**) Illustration of DNA-PAINT principle: transient binding between a docking strand and dye-conjugated imager strands (**top left**) generates a single-molecule blinking time trace (**top right**), illustrated on a synthetic DNA origami nanostructure, where each cylinder represents a DNA

number of blinking events from individual single targets, and (3′) low fraction of false localizations can be achieved by using a low blinking on-off duty cycle. These properties can be controlled by tuning the single-molecule blinking kinetics of the affinity probes (in our case, the oligonucleotide probe), and will be referred to in the design and experimental method sections below.

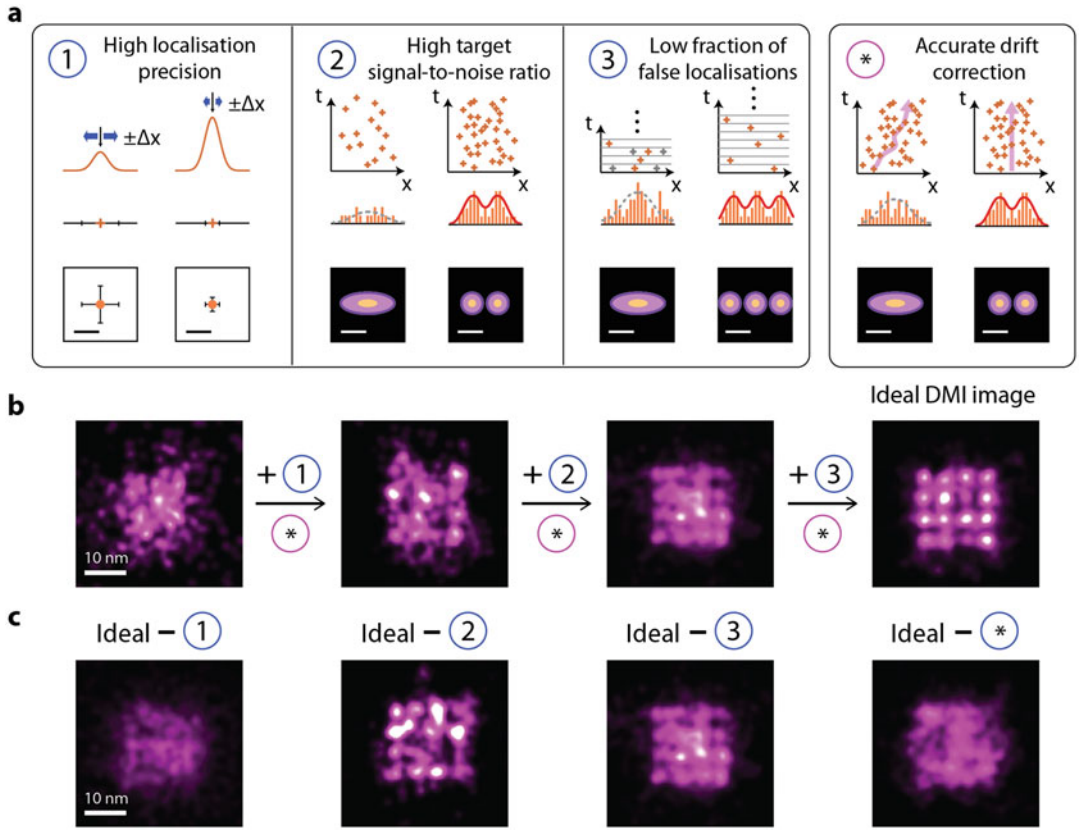


Fig. 2 Technical requirements for achieving discrete molecular imaging. (**a**) Each panel outlines one technical requirement, and depicts schematically the effect on imaging quality before (**left** column) and after (**right** column) the requirement is satisfied. (**b**–**c**) Simulations of imaging effects of the technical requirements for a 4×4 regular array structure, under increasingly better imaging conditions without stage drift (**b**), or under non-ideal imaging conditions with one of the four requirements unsatisfied (**c**). Figure adapted and reprinted with permission from Dai et al. [16]

Fig. 1 (continued) double helix (**bottom**). (**b**) Pseudo-colored, 10-round multiplexed exchange-PAINT images of ten different origamis displaying digits 0–9 in one sample. Image obtained using only one fluorophore (Cy3b) through ten imaging-washing cycles. Scale bar: 25 nm. (**c**) Design schematics of a 3×4 square grid with 20 nm point-to-point spacing on a DNA origami nanostructure (**left**), representative DNA-PAINT super-resolution images of the 20 nm grid structure (**middle**), and single-particle average (**N** = 700) for structural studies. (**d**) Design schematic of triangular grid with 5 nm spacing (**left**) and discrete molecular imaging (DMI, **right**). For each representative single-molecule image, the fluorescent super-resolution image (**left**) and the automatically fitted image (**right**) are shown. Figure adapted and reprinted with permission from Jungmann et al. and Dai et al. [14, 16]

1.3 Design of DNA-PAINT Imaging Probes

The binding and blinking kinetics of the imaging probe determines the single-molecule blinking events at the imaging target that are critical for obtaining high-quality super-resolution images, as mentioned above. In brief, imaging probe need to be designed with an appropriate binding strength (free energy) and high binding specificity, and with a photostable fluorophore, to allow for a large number of photons collected (principle 1′ above). Since a photostable fluorophore (such as cy3b, atto655, or atto647n dyes) could usually be predetermined and is suitable for a range of imaging probes and applications, the process of designing imaging probe generally consists of designing an appropriate probe binding sequence (the docking and imager strands) and deciding on target anchoring sites.

The principle for designing sequences with appropriate binding strength is to aim at a characteristic binding on-time that would both give the highest blinking SNR, and to match the recording frame time. The single-strand diffusion time scale (the time it takes a single-stranded oligonucleotide to diffuse out of a diffraction-limited area on the surface) and probe binding on-time are both buffer dependent. Under typical buffer conditions for nucleic acid nanostructures (10 mM Mg2+) the single-strand diffusion time is around 30–50 ms, and it is recommended to design probes with on-time between 0.5 and 1 s. Nucleic acid binding energy calculation packages such as NUPACK [17] could be used to help design sequences with desired binding strength. Probes with 8–9 nt in length, 50% GC content, and no secondary structure typically give a binding free energy of −10 to −11 kcal/mol (at 25 °C, 10 mM Mg2+, 50 mM Na+), and achieve an optimized binding on-time, as well as allowing enough coding space for multiplexed imaging applications.

After the binding sequence is decided, the docking strand is typically designed by extending the target nucleic acid strand (such as a staple in DNA origami, a strand in DNA tile or brick-based structure) with a short linker (one or two thymine bases) and the docking sequence. It is recommended to extend the target strand from the 3′-end, since current synthetic nucleic acid chemistry using phosphoramidite extends in the 3′-to-5′ direction, and therefore 3′ extensions has a lower truncation percentage. In conjunction, it is recommended to label the fluorophore on the 3′-end of the imager strand. Other factors that need to be considered include potential secondary structures on the docking strand, especially between the docking sequence and any unprotected single-stranded sequence nearby. If the docking sequence is to be placed within a loop, steric hindrance would also play a role, and a spacer size of at least 8 nt (such as 8 thymine bases, on each side) is recommended to maximally expose the docking sequence and preserve the binding on-time (data not shown).

1.4 Design of Nucleic Acid Structure Surface Fixation

Nucleic acid nanostructures will need to be fixed to the glass surface for microscopy imaging. Biotin-streptavidin-biotin linkage is a recommended method that is both easy to implement and provide clean and stable surface fixation. There are two strategies for biotin anchoring to the nanostructure, either direct conjugation of biotin (with a linker of 3–6 thymine bases) to a subset of the strands in the nanostructure before the assembly or indirect labeling via a common, stable handle sequence (typically 15 nt or longer), which results in a longer linker spacing and thus less stable attachment, but provides a significantly more economic option. For stable attachment, at least six biotin anchors per nanostructure on a flat (non-twisted) surface, and separated at least 10 nm from each other, are recommended (data not shown).

2 Materials

2.1 Chemicals

For DNA nanostructure synthesis, all unmodified and biotin-modified DNA oligonucleotides can be ordered from IDT (Integrated DNA Technologies). For DNA-PAINT imaging probes, fluorophore-conjugated oligonucleotide used as DNA-PAINT imaging probes could be either purchased commercially (Bio-Synthesis Inc.) or conjugated in-house (see protocol below). All imaging buffers should be prepared with ultrapure water and filtered with 0.22 μm filter before use. Biotin-conjugated BSA, streptavidin, oxygen-scavenging buffers, DNA imager strands, and synthetic DNA nanostructures should be stored at −20 °C.

2.2 Buffers for Nanostructure Surface Fixation

1. Immobilization buffer: 10 mM Tris–HCl, 100 mM NaCl, 0.1% (v/v) Tween 20, pH 8.0.
2. Biotinylated BSA solution: 1.0 mg/ml solution of biotin-conjugated BSA (A8549, Sigma Aldrich) in immobilization buffer.
3. Streptavidin solution: 0.5 mg/ml solution of streptavidin (S-888, Invitrogen) in immobilization buffer.

2.3 DNA-PAINT Imaging Buffers

DNA-PAINT imaging can be performed in a few different buffer choices, depending on application and requirements on imaging quality. The *imaging solution* is prepared by diluting the imager strand to desired concentration (typically between ~1–20 nM) in the *imaging buffer* of choice. The optimal imager concentration depends on the detailed properties of the sample, especially the density of DNA-PAINT imaging targets. Without prior knowledge, it is recommended to start imaging with ~5 nM, and adjust iteratively to optimize the imaging quality depending on the results.

1. Imaging buffer B: For regular imaging of DNA and RNA nanostructures.

   Buffer components: 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 % Tween 20, pH 8.0. Depending on application, $MgCl_2$ concentration can be adjusted to be compatible with nanostructure stability or to tune DNA-PAINT blinking kinetics.

2. Imaging buffer TP: With oxygen-scavenging and redox system, for high-performance imaging of DNA and RNA nanostructures (*see* **Note 1**).

   Buffer components: 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 % Tween 20, 10 nM PCD (protocatechuate-3,4-dioxygenase), 2.5 mM PCA (protocatechuic acid), 1 mM trolox, pH 8.0.

   2.1. Prepare 100× PCD stock solution (1 μM) in 50% glycerol, 50 mM KCl, 1 mM EDTA, and 100 mM Tris–HCl, pH 8.0; prepare 40× PCA stock solution (100 mM) in ultrapure water and adjust pH to 9.0 with NaOH; prepare 100× trolox stock solution (100 mM) by first dissolving in 100 % methanol (430 μl per 100 mg of trolox) and bring the volume up with ultrapure water; adjust pH to 9.0 with NaOH.

   2.2. Prepare buffer TP from buffer B and concentrated stock solutions for each additional component from above. Add 100 μl of 2× imaging buffer B; add 2 μl of 100× trolox, 5 μl of 40× PCA, and 2 μl of 100× PCD, and appropriate volume of imager strand solution; and bring the total volume up to 200 μl. Pipette up and down gently to mix well, and incubate for 10 min before use (*see* **Note 2**).

3 Methods

3.1 Fluorophore-Labeled Oligonucleotide as DNA-PAINT Imager Strands

For commercially ordered imager strands, resuspend the sample in ultrapure water to 100 μM and store at −20 °C.

For in-house production of imager strands, follow the protocol below. Order 3′ (or 5′) amino-modified DNA oligonucleotide from IDT, and NHS-ester-modified organic fluorophores from GE (cy3b NHS ester) or Sigma Aldrich (atto655 NHS ester and atto647n NHS ester).

1. Conjugation

   1.1. Prepare 3′ (or 5′) amino-modified DNA oligonucleotide in ultrapure water at 1 mM concentration, prepare NHS ester-modified fluorophore (e.g., cy3b, atto655,

atto647n) in DMSO at 20 mg/ml, and prepare 1 M NaHCO3 buffer at pH 8.0.

1.2. Prepare conjugation reaction solution by adding 20 µl amino-modified DNA solution, 2 µl NaHCO3 buffer, and 4 µl fluorophore NHS-ester solution in order, and gently vortex to mix.

1.3. Incubate the conjugation solution on shaker and protected from light for 2 h (room temperature).

2. Column purification.

2.1. Pre-wash size-exclusion column (GE NAP-5) with 2 ml ultrapure water four times.

2.2. Bring total volume of conjugation solution to 100 µl and add to column, and add more ultrapure water (~3 ml) to finish collection. Collect every three droplets to a separate well on a microplate.

2.3. Determine peak position and width with fluorescence plate reader (optional), and combine and collect the wells with brightest signals (typically 3–4 wells close to the beginning).

2.4. Freeze the sample with liquid nitrogen, cover with aluminum foil, and lyophilize overnight.

3. HPLC purification.

3.1. Prepare HPLC buffer A (0.1 M TEAA, 5% ACN) and HPLC buffer B (0.1 M TEAA, 50% ACN), and resuspend lyophilized sample in 0.1 M TEAA to 50 µl total volume.

3.2. Pre-wash C18 2.5 µm column (Xterra MS C18, Waters) and equilibrate with buffer A.

3.3. Set HPLC pumping rate to 1 ml/min, and set linear buffer gradient from 100% HPLC buffer A to 50%:50% HPLC buffer A and B over 30 min. Monitor the elution with absorption at 260 nm (for DNA) and appropriate absorption peak for the fluorophore of interest (e.g., 559 nm for cy3b, 663 nm for atto655 and 644 nm for atto647n), and collect purification product from the sample peak where absorption can be seen at both channels (typically this is the maximum peak at both channels). Collection can be performed either with peak-based automatic collection or by collecting all products in a microplate and manually combining the sample wells.

3.4. Freeze the sample with liquid nitrogen, cover with aluminum foil, and lyophilize overnight.

4. Quantification of conjugation product.

4.1. Resuspend the sample with 30 µl of ultrapure water.

4.2. Measure conjugated imager concentration by absorption at the fluorophore's maximum absorption peak.

4.3. Optionally, determine the labelling efficiency by measuring the oligonucleotide concentration at 260 nm absorption. Typically a labeling efficiency of >90 % is observed.

4.4. Resuspend the sample in ultrapure water to 100 μM and store at −20 °C.

3.2 Sample Preparation for Imaging Nanostructures

1. DNA-PAINT imaging of synthetic nucleic acid nanostructures are carried out in custom-built microscopy flow chamber either on a glass microslide, or on an Ibidi chamber (Ibidi GmbH) for multiplexed imaging with buffer exchange.

   1a. Making flow chamber on a glass microslide.

      1a.1. Clean glass slide and cover slip surfaces with isopropanol.

      1a.2. Build flow chamber by putting down two parallel stripes of double-sided tape on the slide, spaced apart slightly narrower than the width of the cover slip, and then placing the cover slip on the top to form a channel between the cover slip and the glass slide.

      1a.3. Press down the cover slip on both sides to make the edges watertight. The finished flow chamber contains a volume of about 20 μl.

   1b. Making flow chamber for multiplexed exchange-PAINT imaging on an Ibidi chamber.

      1b.1. Clean a large (24 × 60 mm) glass cover slip with isopropanol.

      1b.2. Open an Ibidi sticky slide (sticky slide VI$^{0.4}$), remove the protective cover, and position the cover slip on the sticky glue, with the cleaned surface inside (facing the chamber).

      1b.3. Press down the cover slip along the boundary of the flow chambers, make sure the chamber is well sealed and watertight (the finished flow chamber contains a volume of about 40 μl in the middle part).

      1b.4. Clean the chamber by placing it inside a UVO cleaner (Jetlight 42) with the lid open, for 5 min.

      1b.5. Rinse flow chamber with 100 μl isopropanol twice, followed by 100 μl ultrapure water twice.

      1b.6. Pre-wash both inlet and outlet tubing with 100 μl isopropanol twice.

      1b.7. Rinse flow chamber with 100 μl deionized water twice, followed by 100 μl imaging buffer B twice.

2. Surface immobilization of biotin-labeled nanostructure samples via biotin-streptavidin-biotin linkage. The following protocol is given for microslide-based flow chamber; for Ibidi flow chamber, replace all volumes with 40 μl (instead of 20 μl).
    2.1. Dilute biotin-labeled nanostructures in imaging buffer B to desired concentration. Recommended final concentration is 0.1–0.2 nM. For high imaging quality, the optimal concentration will depend on the sample of interest, especially the density of DNA-PAINT imaging targets on the sample structure.
    2.2. Rinse flow chamber with 20 μl immobilization buffer twice. Avoid introducing bubbles into the chamber (*see* **Note 3**).
    2.3. Flow in 20 μl of 1 mg/ml biotinylated BSA solution, and incubate for 2 min.
    2.4. Rinse flow chamber with 20 μl immobilization buffer twice.
    2.5. Flow in 20 μl of 0.5 mg/ml streptavidin solution, and incubate for 2 min.
    2.6. Rinse flow chamber with 20 μl immobilization buffer twice.
    2.7. Rinse flow chamber with 20 μl imaging buffer B twice.
    2.8. Flow in 20 μl of biotin-labeled DNA origami sample solution (~0.5 nM, in buffer B), and incubate for 2 min.
    2.9. Rinse flow chamber with 20 μl buffer B twice.
    2.10. Flow in 20 μl DNA-PAINT imaging solution twice.
    2.11. Place the sample on the microscope under live mode (see below), and check the density of blinking spots on the camera. If the density of blinking spots is too high (such that the point spread function (PSF) of neighboring blinking spots are close to each other or almost overlapping), or the number of blinking spots is too low (minimum ~50 blinking events in each camera frame), go back to change **step 2.8** and adjust the nanostructure concentration appropriately such that a desired density of blinking spots could be achieved (*see* **Note 4**).
    2.12. (Optional, for multiplexed DNA-PAINT imaging with Ibidi flow chamber only) Connect both inlet and outlet tubings to the Ibidi chamber, and connect outlet to a syringe (5 ml volume). Rinsing and buffer exchange from here onward are performed by feeding the solution from the inlet and pulling on the syringe from the outlet end.

3.3 Single-Molecule DNA-PAINT Super-Resolution Microscopy

The following steps require a well-adjusted microscope, comparable to the setup described in Dai et al. [16]. DNA-PAINT super-resolution fluorescence microscopy of nanostructures is typically performed with total internal reflection (TIR) illumination. Automatic focus lock systems (such as the perfect focus system from Nikon, and the definite focus system from Zeiss) are necessary for maintaining focus over an extended imaging time. Both CCD and CMOS based cameras are suitable for DNA-PAINT imaging.

1. Optimization of imaging conditions and super-resolution movie acquisition.
    1.1. Place the microslide or Ibidi flow chamber with nanostructures in DNA-PAINT imaging solution on the microscope stage.
    1.2. Turn on the appropriate excitation laser and set the excitation intensity to ~0.5 kW/cm^2. Select appropriate filter modules or cubes for the laser. Set the camera exposure time to ~200 ms, and turn on live mode.
    1.3. Adjust the microscope focus (coarsely) to the surface with immobilized nanostructures. When focused, bright, diffraction-limited blinking spots of DNA-PAINT imager strands should become visible.
    1.4. Adjust the TIR illumination angle to maximize surface illumination while suppressing transmission; in particular, maximize the signal-to-noise ratio between the pixel intensities of the bright blinking spots and the illumination background.
    1.5. Adjust camera exposure time in the range of ~100–500 ms, such that each blinking event ideally lasts ~2–5 imaging frames. For EMCCD cameras, do not use electron multiplying gain if possible, as this would result in a lowered imaging quality.
    1.6. Adjust the microscope focus (finely) to the bright blinking spots; in particular, maximize the pixel intensity of the central (brightest) pixels. When well focused, a sharp, and potentially pixelated image of the point spread function (PSF) could usually be seen for each of the blinking spots.
    1.7. Start camera acquisition of ~5000–20,000 frames. The optimal length of acquisition depends on the sample properties and desired imaging quality. In particular, longer acquisition allows better target signal-to-noise ratio (tSNR) and higher overall imaging quality.
2. (Optional) Multiplexed DNA-PAINT imaging with buffer exchange. For every additional imaging channel, perform

imaging buffer exchange followed by another super-resolution movie acquisition as in the previous step.

2.1. Remove previous imaging buffer by flowing in 400 μl of imaging buffer B and incubate for 2 min.

2.2. Introduce new imaging solution by flowing in 200 μl of imaging solution into the chamber and incubate for 2 min.

2.3. Adjust microscope and camera settings as necessary, and acquire another super-resolution movie following the procedures described in the previous step.

3.4 Super-Resolution Imaging Data Processing

Super-resolution imaging data processing was generally performed in two steps: (1) spot detection, localization, and filtering and (2) drift correction and super-resolution rendering. We offer a data processing and analysis software suite with graphical user interface (from Dai et al. [16]) for an integrated, easy-to-use data processing. In the following we provide an outline of the key steps and principles of each step in the data analysis, accompanied with instructions in using the custom software for each step.

1. Super-resolution spot detection and single-emitter localization:

   1.1a. Spot detection is performed by the principles of background subtraction and Gaussian smoothing, with Gaussian filter size tuned to match the single-molecule blinking PSF size to produce the best sensitivity and specificity for DNA-PAINT super-resolution movies. Single-emitter localization is performed with one of many 2D Gaussian fitting routines, such as a maximum likelihood estimation algorithm from Smith et al. [18].

   1.1b. In the DNA-PAINT image analysis software, click "Spot detection and localisation" to open the parameter dialogue box. Enter the acquisition parameters for the movie (movie size, camera pixel size, count to photon conversion factor), and use ~0.25 × [dye emission maximum wavelength] as the PSF fitting standard deviation.

2. Super-resolution image rendering and drift correction:

   2.1a. Super-resolution image rendering is performed by transforming a list of single-molecule localized positions to a 2D histogram with each localization smoothed by a Gaussian function.

   2.1b. With the DNA-PAINT image analysis software, click "Load" to open the trace file generated from the previous step. Depending on the desired structural separation, set the sub-pixel resolution to [camera pixel size]/20, such that each display pixel is 20 nm in size, and adjust the display brightness.

2.2a. Determine maximally supported imaging resolution from distance between adjacent-frame localizations (DAFL) analysis (*see* **Note 5**). In DAFL analysis, all pairs of localizations from adjacent imaging frames and spatially close to each other are collected and their 2D distance pooled and their distribution plotted as a histogram. The histogram is fit to a theoretical distribution function to determine the maximally supported imaging resolution.

2.2b. With the DNA-PAINT image analysis software, open "Data quality" window, click "Analyse connectivity," and wait until the analysis is finished and then click "DAFL analysis." An automatic fitting to the distribution will be performed and the supported resolution will be displayed. Close the window and set the Gaussian smoothing standard deviation to be $1/2.35\times$[maximal supported resolution].

2.3a. (Optional, if the drift traces of all nanostructures are overlapping and not separable) Perform DAFL-based drift correction by collecting all pairs of localizations from adjacent imaging frames and close to each other as in the previous step. For every imaging frame, calculate the drift vector by computing the average vector offset from all pairs of localizations that spans the previous and the current frame, and use the vector cumulative sum as the image drift correction trace.

2.3b. (Optional, as above) With the DNA-PAINT image analysis software, open "Data quality" window, click "DAFL drift correction," wait until the analysis is finished, click "Use as drift correction," and close the window. In the main window, click "Apply" to apply the drift correction.

2.4a. Correct for imaging drift by selecting an isolated nanostructure as the drift marker. Extract its blinking time trace over time, and smooth over its time trace as the imaging drift correction.

2.4b. With the DNA-PAINT image analysis software, click "Select ROI" to select an isolated nanostructure, and click "Analyse trace" to open trace display window. Enter an appropriate time window (recommended ~500–2000) for the smoothing, click "Calculate," and wait until calculation finishes. Click "Use as drift correction," close the window, and click "Apply" in the main window to apply the drift correction.

2.5a. Correct for imaging drift by selecting a pool of isolated nanostructures simultaneously, and calculate the photon count-weighted average of their time traces as the imaging drift correction.

2.5b. With the DNA-PAINT image analysis software, first set the sub-pixel resolution to be ~1/3–1/4 of the structure size, and set a lower intensity threshold to convert the super-resolved image to a binary image mask by setting the "Min density" to ~3–5. Click "Select regions" to open another window, and use the structure size filter to select for structures of the correct size range (typically in the range of ~5–50). Use a combination of binary operations to remove unwanted noise and structures that are very close together, typically by performing a series of 2–3× dilation, area filtering, and 2–3× erosion in order. Click "Accept" and then "Process," wait until calculation is finished, and then click "Apply" in the main window to apply drift correction.

3.5 Super-Resolution Image Quality Analysis

1.1a. Photon count, localization precision, and image resolution: Photon count is directly reported from single-emitter localization algorithm (*see* **Note 6**). Localization precision for individual localizations can be determined from two methods, fitting reported uncertainty (FRU) or distance between adjacent-frame localizations (DAFL), *see* **Note 5**. FRU is typically reported by the single-emitter localization algorithm, such as the reported Cramer-Rao lower bound (CRLB), and DAFL resolution is measured same as in **step 2.2a** above.

1.1b. In the DNA-PAINT image analysis software, for FRU localization precision [18], click "Data quality," select "Localisation precision (nm)" from the drop-down menu, and click "Plot" to show the distribution of localization precision from all fitted localizations. The supported imaging resolution as reported by FRU is equal to 2.35 × [average localization precision]. The procedure for measuring DAFL imaging resolution analysis is the same as described in **step 2.2b** above.

1.2a. Number of blinking events and target signal-to-noise ratio (tSNR): These two parameters are analyzed on a target-by-target basis. Number of blinking events per target is analyzed by extracting the blinking time trace for a single imaging target and counting the number of localization bursts through time. Target SNR is calculated by locally plotting a projection histogram of two adjacent imaging targets and analyzing the fitted profile. Here, signal is calculated as the difference in the fitted peak and valley intensity, and noise is calculated as the statistical fluctuation of the fitted peak intensity, assuming Poisson statistics. To make results comparable, histogram bins are taken with width equal to 1/10 of the separation between the two targets.

1.2b. In the DNA-PAINT image analysis software, for the number of blinking events, first click "Select ROI" to select a single imaging target, and click "Analyse trace" to open the trace display window. Click "Analyse" to display the number of blinking events. For target SNR calculation, first click "Select ROI," select a region including two closely spaced imaging targets, or two clusters of imaging targets, click "Histogram fit," and open another window. In the new window, re-orient the display so that the two targets or clusters of interest align horizontally, and click "Select ROI" to select an area tightly enclosing all the localizations from the two imaging targets. Set the number of histogram bins to ~20 such that the bin spacing is equal to 1/10 of the target spacing and click "2-peak fit." The calculated target SNR values will be displayed.

1.3a. Blinking kinetics and fraction of false double-blinking localizations. Blinking kinetics are calculated either for a single imaging target, or a group of imaging target (such as all the imaging targets on a single synthetic nanostructure). Characteristic blinking on-time and off-time is separately and similarly calculated, by collecting the fluorescence on duration and off duration for all blinking events, plotting the histogram and fitting to expected distributions, and assuming independent stochastic process. Fraction of false double-blinking localizations is estimated by analyzing the photon count distribution of all localizations, and evaluating the proportion of localizations that has photon count higher than two standard deviation above average (*see* **Note 6**).

1.3b. In the DNA-PAINT image analysis software, for blinking kinetics analysis, first click "Select ROI" and select either one or a group of imaging targets, or an entire nanostructure. Click "Analyse trace" to open the trace display window, and click on "Analyse" to display the on-time and off-time histogram as well as the fitted results. For false double-blinking localizations, click "Data quality" and plot photon count distribution, similar to in **step 3.1b**. The estimated fraction of false localizations will be displayed.

4 Notes

1. Imaging buffer TP with oxygen-scavenging and redox systems helps increase dye brightness and photostability for cy3b and atto647n, but is not compatible with some other fluorophores such as atto655.

2. When preparing imaging buffer TP, mix by gently pipetting up and down, and avoid introduction of any air bubbles. An incubation of 10–20 min after preparation is recommended for the system to reach steady state before use. Sometimes an increase in the number of blinking spots per frame is observed at the beginning (~5–10 min) of the recorded image; this is usually due to incomplete buffer exchange when introducing the imaging solution. Flowing in an excess of imaging solution helps to mitigate the effect.
3. Avoid introducing bubbles into the flow chamber at any stage of the experiment. Air bubbles could not only adversely affect the imaging buffer environment, and also result in incomplete buffer exchange and introduce unwanted friction or blocking inside the Ibidi chambers.
4. During acquisition of super-resolution movies, it is important to maintain a suitable level of number of blinking spots per camera frame. This could be affected by several parameters, including the density of nanostructures, imager strand concentration, and the number of docking targets on the nanostructure sample. When designing the experiment, use an appropriate imager strand concentration to maintain a low false localization fraction, and then adjust nanostructure surface deposition density to maintain a suitable level of blinking spots per frame.
5. Determining the exact supported resolution of a super-resolution rendered image is generally difficult. The traditional method of measuring the full-width at half-maximum (FWHM) value of a single object (such as a labeled microtubule sample) is not ideal, and thus calculated resolution value does not always translate to the ability of reliable separation between two objects (spaced apart by the calculated distance). Here we provide two methods (FRU and DAFL) for estimating the maximal supported resolution. We note that both methods report an optimistic bound of the achievable resolution (i.e., the actual resolution in number is larger than either of those reported by the two methods). Between the two, FRU usually gives a more optimistic evaluation, while DAFL gives a more realistic estimation.
6. Depending on the detailed optical configuration of the microscope, sometimes illumination is not even throughout the entire field of view. Under such circumstances calculations of average photon count and estimated ratio false double-blinking localization will be inaccurate. To overcome the problem, cut out the central region of the super-resolution image (where illumination intensity is highest and the degree of uneven illumination is lowest) and perform photon count statistics calculation on the cut-out region.

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