Fluorogenic DNA-PAINT for faster, low-background super-resolution imaging

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DNA-based points accumulation for imaging in nanoscale topography (DNA-PAINT) is a powerful super-resolution microscopy method that can acquire high-fidelity images at nanometer resolution. It suffers, however, from high background and slow imaging speed, both of which can be attributed to the presence of unbound fluorophores in solution. Here we present two-color fluorogenic DNA-PAINT, which uses improved imager probe and docking strand designs to solve these problems. These self-quenching single-stranded DNA probes are conjugated with a fluorophore and quencher at the terminals, which permits an increase in fluorescence by up to 57-fold upon binding and unquenching. In addition, the engineering of base pair mismatches between the fluorogenic imager probes and docking strands allowed us to achieve both high fluorogenicity and the fast binding kinetics required for fast imaging. We demonstrate a 26-fold increase in imaging speed over regular DNA-PAINT and show that our new implementation enables three-dimensional super-resolution DNA-PAINT imaging without optical sectioning.

Single-molecule localization microscopy has become an invaluable tool in biological research, revealing subcellular structure and function at ten or more times the resolution of conventional fluorescence microscopy. Among single-molecule localization microscopy techniques, DNA-based points accumulation for imaging in nanoscale topography (DNA-PAINT) microscopy is becoming increasingly popular because of the high-quality images that can be obtained with relative ease. DNA-PAINT, in contrast to the other fluorescence microscopy methods, relies on the detection of single fluorophores instead of aggregates. The main reason for this popularity is that DNA-PAINT is a robust technique, not limited to imaging regions within a few hundred nanometers of the coverslip and therefore easily adjustable (Supplementary Note 2).

There are several advantages to this approach: (1) binding-based blinking frequency is proportional to the imager probe concentration and is therefore easily adjustable (Supplementary Note 2); (2) fluorophores and buffers are not limited to those suitable for photophysical blinking and can be chosen to allow for maximum brightness and thereby best localization precision. (3) DNA-PAINT is robust against photobleaching as bleached probes are replenished from the large reservoir of probes in solution; (4) sequential multiplex imaging is possible with the use of multiple pairs of orthogonal imager probes and docking strands; and (5) DNA-PAINT can take advantage of the powerful toolbox available for in vitro DNA technology, for example, creating libraries of fluorescence in situ hybridization probes for OligoDNA-PAINT, using photoreactive nucleosides for Action-PAINT or labeling via click-chemistry reactions.

However, the use of transient chemical binding-based switching is also responsible for the greatest weakness of DNA-PAINT: unbound imager probes can contribute massive amounts of background fluorescence, which drown out the signal peaks of individual bound probe molecules. To minimize background, early DNA-PAINT realizations therefore relied on low probe concentrations and the use of total internal reflection fluorescence (TIRF) to minimize illumination volume. Consequently, DNA-PAINT was limited to imaging regions within a few hundred nanometers of the coverslip. Using a spinning-disk confocal microscope, regions deeper in a cell could be imaged but at the cost of a compromise in resolution, caused by lower detected photon numbers per blinking event. The constraint on probe concentration to typically less than 5 nM also limits the number of blinking events per second, leading to slow imaging speeds. This problem can be mitigated to some extent by increasing the rate constant of an imager probe binding to a docking strand ($k_{on}$), as recently demonstrated. While these publications showed super-resolution images acquired in minutes instead of hours, they still required TIRF to keep background low.

None of these approaches, however, solve another background-related problem: at high camera frame rates, as required for fast imaging, exposures are so short that unbound molecules are no longer blurred by diffusion. These molecules will appear as local fluctuations in the background and, at worst, erroneous binding events.

To address both background-related issues requires a reduction in the molecular brightness of the diffusing unbound probes. Recently, two groups introduced Förster resonance energy-transfer (FRET)-based DNA-PAINT to achieve this effect. Donor and acceptor fluorophores were conjugated to separate imager probes; only when they coincidentally bind to the same docking strand will donor excitation lead to acceptor emission (Fig. 1a, top right). Unfortunately, the demonstrated localization precision was substantially worse than that for regular DNA-PAINT, failing the ‘gold standard’ of resolving the hollow center of labeled microtubules. This reduction in image quality can be attributed to FRET DNA-PAINT being fundamentally limited by the trade-off between maximizing energy-transfer efficiency for bright blinking events and minimizing excitation and emission cross-talk to reduce background.

High background fluorescence therefore remains a substantial problem in DNA-PAINT and severely limits its application: it has not yet been possible to image whole cells in three dimensions using

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DNA-PAINT without optical sectioning. Even with optical sectioning, compromises are made between quality and speed, requiring either imaging for hours at low probe concentrations to acquire high-quality DNA-PAINT images or imaging for minutes at high probe concentrations at the risk of undersampling, resolution loss, the introduction of artifacts from too many molecules blinking at once and high, inhomogeneous background.

In this study, we improved upon current designs in imager probes and docking strands to develop fluorogenic DNA-PAINT. Using these new probes, we demonstrate a 26-fold increase in imaging speed. Three-dimensional (3D) super-resolution images of microtubules and mitochondria acquired throughout whole mammalian cells show that fluorogenic DNA-PAINT does not require optical sectioning. We further present the development of two orthogonal sets of imager probes and docking strands, which allowed us to perform simultaneous two-color fluorogenic DNA-PAINT imaging.

Results
Imager probe and docking strand design. Our fluorogenic DNA-PAINT implementation is based on modifications in the design of the imager probe and the docking strand so that the probe is dark when in solution and bright when bound to a docking strand, while still achieving the fast binding kinetics required for fast image acquisition (Supplementary Note 3). This development enables us to perform high-quality DNA-PAINT imaging without compulsory optical sectioning. It also allows us to substantially increase the probe concentration and record blinking events at 100 frames per second (fps) with negligible compromises in localization precision.

Our goal was to implement fluorogenic DNA-PAINT without sacrificing the many benefits of regular DNA-PAINT. To maintain resilience against photodamage, any photosensitive component should reside solely in the probes so that any damaged probes can be quickly replaced. Furthermore, a single probe system would be desirable to keep binding kinetics simple and avoid the need to titrate probe ratios.

At first glance, molecular beacons (Fig. 1a, bottom left) might serve this purpose as they are highly fluorogenic17. However, their fluorogenicity is maximized by sacrificing binding kinetics, which makes them incompatible with DNA-PAINT. They have a slow binding on rate as they are stabilized with a stem loop in their unbound ssDNA state (2.4% ± 0.8%; two-sided two-sample t-test of normalized fluorescence, t statistic = 37, P < 0.001, n unbound = 8). On binding to their complementary docking strand, its fluorescence increases 57-fold, compared with a 2.4-fold increase for the regular probe (56.6 ± 20.3 versus 2.4 ± 0.6; two-sided two-sample unequal variance t-test, t statistic = 6, P = 0.002, n unbound = 8). Averaged statistics are presented as mean ± s.d.

![Fig. 1 | Imager probes for DNA-PAINT. a. Comparison of different DNA-PAINT imager probe and docking strand systems. Top left, imager probe for regular DNA-PAINT is always fluorescent and contributes to high background. Top right, in FRET DNA-PAINT, acceptor fluorescence is only observed when both donor- and acceptor-conjugated probes are bound to the docking strand (energy transfer is indicated by blue arrows). Bottom left, regular molecular beacons are fluorogenic, but their binding on and off rates are too slow for DNA-PAINT. Bottom right, our DNA-PAINT probes are fluorogenic and have fast binding kinetics owing to the absence of a stem secondary structure and mismatches between the probe and docking sequences (shown in red). b. Comparison of fluorescence between a regular imager probe (Cy3B) and our fluorogenic imager probe (Cy3B with BHQ2; imager probe A) in solution. In their unbound ssDNA state, the fluorogenic probe is less than 2.5% as bright as the regular probe (2.4% ± 0.8%; two-sided two-sample t-test of normalized fluorescence, t statistic = 37, P < 0.001, n unbound = 8). On binding to their complementary docking strand, its fluorescence increases 57-fold, compared with a 2.4-fold increase for the regular probe (56.6 ± 20.3 versus 2.4 ± 0.6; two-sided two-sample unequal variance t-test, t statistic = 6, P = 0.002, n unbound = 8). Averaged statistics are presented as mean ± s.d.](image1)

DNA-PAINT in TIRF (imager probe A concentration = 250 nM, frame rate = 100 Hz), measuring a diameter of 60.5 ± 2.0 nm and displaying ~9% multi-emitter artifacts (Supplementary Fig. 2). Scale bar, 50 nm.

Fast fluorogenic DNA-PAINT imaging of DNA origami nanostructures. a. Top and front schematic view of the DNA origami nanostructure, with 48 docking strands attached to the 62-nm ring. O.D., outer diameter. b. Average of 20 origami rings imaged with fast fluorogenic DNA-PAINT in TIRF (imager probe A concentration = 250 nM, frame rate = 100 Hz), measuring a diameter of 60.5 ± 2.0 nm and displaying ~9% multi-emitter artifacts (Supplementary Fig. 2). Scale bar, 50 nm.

c. Examples of individual rings at different time points. Scale bars, 100 nm.

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about 15 or more bases (0.34 nm per base along the helical axis versus a Förster radius between the quencher and fluorophore of ~6 nm)\(^1\). However, this conflicts with our need to achieve high off rates for fast DNA-PAINT imaging, which demands ten or less complementary base pairs between the imager probe and docking strand. We solved this dilemma by designing docking strand sequences with internal mismatches against a long 15-base imager probe, which destabilize the binding for a faster off rate (Extended Data Fig. 2). The second probe that we designed in this manner was further optimized by selecting an effective contact quencher to further mitigate the need for a long probe (imager probe B; ATTO 643 with Iowa Black FQ). Additionally, the probes were designed without a self-complementary sequence (or ‘stem’ in molecular beacons) to avoid stabilizing the free imager probes in their quenched form\(^1\).

The biggest advantage of using DNA for PAINT is that oligonucleotides are highly programmable, and their secondary structures, based on complementary base pairing, are predictable. To select suitable docking strands, we employed computational screening based on a number of predicted parameters (Biopython\(^{10}\) and NUPACK\(^{12}\); Supplementary Note 3 and Supplementary Table 3) including melting temperature and low probability of self-dimerization. Unfortunately, it is difficult to predict unquenching efficiency based on the sequence of the docking strand as it depends not only on the specific fluorophore and quencher but also on the method by which and location at which they are conjugated onto the oligonucleotide, proximal nucleobases and the 3D structure of the double-stranded DNA complex formed, which is unlikely to be a perfect double helix due to the mismatches. Ultimately, computational screening generated a short list of oligonucleotides that we individually screened for suitable kinetics and brightness under optimized DNA-PAINT imaging conditions.

We first designed an imager probe conjugated with the dye Cy3B and the quencher BHQ2 (imager probe A) and quantified its fluorogenicity in solution. In its freely diffusing unbound state, our probe is less than 2.5% as bright as a regular DNA-PAINT probe (conjugated with Cy3B but no quencher; Fig. 1b), providing evidence that, even in the absence of a stem, ssDNA can be sufficiently dynamic and flexible to provide strong quenching in the unbound state. On binding with its fully complementary sequence, we observed a 57-fold increase in fluorescence. Similar to previous reports, we also measured a weak fluorogenic effect of a twofold increase for the regular probe\(^2\). Overall, our new imager probe was 24 times more fluorogenic than the regular DNA-PAINT probe.

**Increased imaging speed.** We characterized the performance of the Cy3B–BHQ2 probe with its partially mismatched docking strand by imaging DNA origami nanostructures. DNA origami technology allows for the design of custom structures with precise definition in the position and number of docking strands. The chosen ring-shaped origami structure was originally designed for building simplified nuclear pore mimics\(^2\) and has an outer diameter of 62 nm (Fig. 2a). Here we extended 48 docking strands from the exterior of this DNA ring. This relatively low density of docking strands (48 targets per µm\(^2\)) compared to typical antibody labels (hundreds of targets per µm\(^2\)) in cells makes DNA-PAINT imaging very inefficient unless the imager probe concentration is high enough to lead to substantial binding frequencies. It therefore represents an excellent test of the performance of fluorogenic DNA-PAINT under conditions in which the background is high. The ring shape also provides an easy and visual means of assessing the achievable resolution as well as blinking artifacts. Imaging with a fluorogenic imager probe concentration of 250 nM, around 50 times higher than that for regular DNA-PAINT, and a camera frame rate of 100 Hz with TIRF illumination, we could resolve the rings within 1 min (Fig. 2c). The ring diameter was measured to be marginally less than the expected size (60.5 ± 2.0 nm (mean ± s.d.), \(n_{\text{res}} = 20\); two-sided one-sample \(t\)-test against 62 nm, \(t\) statistic = 3.31, \(P = 0.004\); Shapiro–Wilk test for normality, \(W\) statistic = 0.948, \(P = 0.340\)). Fourier ring correlation analysis yielded an excellent resolution of 17.8 nm (Methods). The hollow centers of the rings could be easily resolved in individual rings as well as in an average of 20 rings (Fig. 2b), confirming that the frequency of multi-emitter artifacts is low (~9%; Supplementary Fig. 2).

For quantification of imaging speed and resolution with a biological sample that features a large number of docking strands per diffraction-limited area, we compared two-dimensional (2D) DNA-PAINT TIRF images of immunolabeled microtubules in COS-7 cells using a regular imager probe and our fluorogenic probe (Fig. 3). Under these high-density labeling conditions, the limiting factor is the off rate, as multi-emitter artifacts need to be avoided (Supplementary Fig. 1f). Regular DNA-PAINT imaging was performed at a frame rate of 4 Hz (expected off rate, ~1 s\(^{-1}\)) with a probe concentration of 0.1 nM, similar to parameters previously used for high-quality DNA-PAINT imaging\(^3\), whereas, with our fluorogenic imager probe, imaging was performed at 100 Hz (off rate, ~50 s\(^{-1}\)) and a probe concentration of 20 nM. With both higher on and off rates for our fluorogenic probe, approximately 26 times more blinking events per second could be observed than with the regular DNA-PAINT probe (4.3 versus 0.2 blinks µm\(^{-1}\) s\(^{-1}\) of microtubules; Fig. 3b).

**Fig. 3 | Comparison of regular and fluorogenic DNA-PAINT imaging.** a. Qualitative comparison of regular versus fluorogenic DNA-PAINT TIRF imaging of fixed microtubules (regular, probe concentration = 100 pM, frame rate = 4 Hz; fluorogenic, imager probe A concentration = 20 nM, frame rate = 100 Hz). Scale bars, 200 nm. b. Quantitative comparison showing that blinking data are acquired 25.8 times faster with the fluorogenic probe (quantified by the number of blinks per length of microtubule per unit of time; regular, 0.17 ± 0.04 µm\(^{-1}\) s\(^{-1}\), \(n_{\text{res}} = 8\); fast fluorogenic, 4.30 ± 0.49 µm\(^{-1}\) s\(^{-1}\), \(n_{\text{res}} = 10\); two-sided Mann–Whitney U-test, \(U\) statistic = 0, \(P = 0.0004\); ROI, region of interest). Averaged statistics are presented as mean ± s.d.
This improvement was less than anticipated based on the 200-fold increase in probe concentration, which suggests that the additional bases of the fluorogenic probe may have reduced $k_{ON}$. Nonetheless, achieving 1,000 blinking events per $\mu$m of microtubule required only $\sim$4 min of imaging with the fluorogenic probe compared to $\sim$1.4 h with the regular probe. With fluorogenic DNA-PAINT, 2D projections of hollow microtubules were easily observable after 3 min (Fig. 3a). By contrast, regular DNA-PAINT required hours of imaging to obtain similar image quality.

**Three-dimensional super resolution without optical sectioning.** We next tested whether the fluorogenic properties of our new probe would be strong enough to replace the background suppression provided by optical sectioning through TIRF illumination and thereby allow 3D imaging of volumes thicker than a few hundred nanometers. We first imaged microtubules using regular widefield illumination and astigmatic detection for 3D localization. We were able to image with our fluorogenic probe at a concentration of 10 nM and at a camera frame rate of 100 Hz to acquire a high-quality 3D imaging.
super-resolution image of the microtubule network (3D Fourier shell correlation (FSC) resolution = 41.1 nm) in only 10 min (Fig. 4a–c and Extended Data Fig. 3). We halved the imager probe concentration compared to the concentration in 2D experiments to compensate for the larger size of the blinks caused by the astigmatic detection and thereby avoid an increase in multi-emitter artifacts. The obtained localization precision (Extended Data Fig. 3d; peak of 1.7 nm for xy and 4.5 nm for z) was comparable to that reported for regular DNA-PAINT. The hollow centers of microtubules can be seen in the microtubule cross-section or in the 30-nm-thick z slice as they pass through the plane (Fig. 4b and Supplementary Video 1).

Having eliminated the need for optical sectioning, we were also able to perform fast fluorogenic DNA-PAINT under widefield illumination on a 4Pi-single-molecule switching (SMS) microscope.
that interferometrically combines the fluorescence collected by two opposing objectives for improved axial localization precision \(^{24,25}\). We imaged mitochondria throughout the cell (Fig. 4d,e; four overlapping imaging volumes spanning ~2 μm; immunolabeled for the outer membrane protein OMP25). Regular DNA-PAINT is incompatible with 4P-SMS imaging owing to its lack of optical sectioning, whereas fluorogenic DNA-PAINT allows for 4P-SMS imaging at high sampling density.

Simultaneous multicolor imaging. To expand fluorogenic DNA-PAINT to multicolor imaging, we developed a second set of an imager probe and docking strand that are orthogonal to our first set in both emission spectrum (ATTO 643 with Iowa Black FQ) and binding affinity (Fig. 5a). In contrast to previous multiplexed DNA-PAINT approaches that are performed sequentially\(^{27}\), we imaged the two imager probes simultaneously in separate color channels, thereby avoiding any loss of speed compared to single-color imaging. We demonstrated this with dual-color imaging of the endoplasmic reticulum and mitochondria in U-2 OS cells in 10-min recordings at high localization density without optical sectioning (Fig. 5b–f and Extended Data Figs. 4–6). The apparent absence of cross-talk between the two probes (Fig. 5e,f) was quantitatively confirmed by both a pixel-dependent colocalization analysis based on correlation of pixel intensity (Fig. 5b, \(r = 0.18\)) and a point-dependent analysis based on correlation of neighbor distribution (coordinate-based colocalization\(^{26}\), \(C = -0.1\); Extended Data Fig. 5a–c).

Discussion

Our current implementation of fluorogenic DNA-PAINT with partially mismatched docking strands also opens the way for other multiplex imaging schemes that we have not explored in this study. Because docking strands are not fully complementary to the imager, there are potentially many suitable docking strands for any imager. On one hand, this introduces potential for cross-specific binding, which we have demonstrated to be avoidable with proper sequence design (Fig. 5). On the other hand, a single imager can be used to simultaneously image multiple targets, each with a different docking strand. Discriminating target identity can potentially be achieved, for example, by encoding docking strands with different binding kinetics, a form of kinetics fingerprinting\(^{27}\).

While in this study we demonstrate imaging at 100 fps and generating 2D and 3D super-resolution images with excellent localization density and localization precision in minutes without optical sectioning, it is worth pointing out that we have not yet experienced a fundamental limit with fluorogenic DNA-PAINT. We expect that further developments in probe chemistry and design should yield even better fluorogowency and faster off rates. Similarly, effective on rates can be further optimized as recently demonstrated for regular DNA-PAINT probes\(^ {12,28,29}\). The two probes that we report in this study are not only compatible with existing DNA-PAINT instruments and modalities but liberate DNA-PAINT imaging from the dependency on optical sectioning. Importantly, imaging with the fluorogenic probe retains robustness against photobleaching; images with high signal-to-noise ratios can be acquired at essentially arbitrarily high sampling densities by extending imaging time (Extended Data Figs. 3a and 4). Based on these strengths, we believe that fluorogenic DNA-PAINT will become the preferred form of single-molecule localization microscopy.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41592-022-01464-9.

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**Methods**

**Imager probes and docking strands.** The sequences for the real-time imager probe and the docking strand were taken from Auer et al.\(^{14}\) and share a complementary region of nine bases.

Two sets (A and B) of fluorogenic imager probes and docking strands that are orthogonal in color (orange and far red) and binding affinity were developed (Supplementary Note 3). The imager probes are 15 bases long and conjugated with a fluorophore at the 5’ end and a quencher at the 3’ end (Extended Data Fig. 1). The docking strands are partially mismatched to their corresponding imager probe sequences (Extended Data Table 2). Slightly different versions of the docking strand with different padding bases were developed within set A. Specifically, version 2 is optimized for multiplex imaging as it is predicted to have minimal cross-specific binding against set B. All oligonucleotides were ordered from Integrated DNA Technologies.

**DNA origami.** The ring-shaped DNA origami structure is based on a previously described structure\(^{22}\) with only minor modifications in the positions of the handles. The structure was designed using cdNAano (http://cdDNAano.org), with an expected outer diameter of ~62 nm. DNA scaffold strands (8,064-nucleotide circular ssDNA) were produced using *Escherichia coli* (gift from M. Davidson, Florida State University; Addgene plasmid 70008732) as previously described\(^{25}\). (Extended Data Fig. 1, D1-1a) using azide–DBCO click chemistry\(^{10}\). The cells were then labeled with mouse anti-IGG secondary antibody (Jackson ImmunoResearch, 115-005-144) and goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, 111-005-144) conjugated with oligonucleotide docking strands (Extended Data Fig. 1, D1-1c and D2-1a, respectively) both at a dilution of 1:200 for 1 h at room temperature.

**Fluorescence measurements in solution.** Fluorescence measurements of the probes in solution were performed with a microscope (the same microscope as described for imaging DNA origami structures) under widefield illumination provided by a xenon arc lamp (Sutter Instrument, Lambda LS). Fluorescence intensity was measured ~10 μm deep past the coverslip. Buffer-only blanks were measured for background correction. Samples were prepared in a high isotropic-strength PBS-based buffer (PBS, 500 mM NaCl) in channel slides (ibidi, µ-Slide VI, 80607).

To measure quenching efficiency, fluorophore-conjugated samples were prepared at 0.2 μM and saturated with adaptor strands at 0.3 μM and quencher-conjugated strands at 0.45 μM (Supplementary Table 2 and Supplementary Fig. 30).

**Unbound probe** samples contained only the imager probe (0.2 μM), and ‘bound probe’ samples were prepared with the probe and its complementary sequence in excess (20 μM) (Fig. 1b and Supplementary Fig. 3c).

All oligonucleotides were ordered from Integrated DNA Technologies.

**Microscope setup.** DNA origami samples and microtubule samples (for 2D imaging) were imaged on a modified Nikon Ti-E inverted microscope with a x100 1.45-numerical aperture (NA) oil-immersion objective with an sCMOS camera (Andor, Zyla 4.2). For illumination, a 561-nm laser with a built-in acousto-optic modulator for intensity modulation (Omicron Lux) was used with a dichroic (Semrock, D02-R488-25×36) and a bandpass (Semrock, FF01-524/45) filter. For imaging with the regular probe, data was recorded at 4 fps and ~0.2 kW cm\(^{-2}\). With the fluorogenic probe, data were recorded at 100 fps and ~2 kW cm\(^{-2}\). A custom image-based focus-lock system based on a previously described design\(^{11}\) was used.

**Astigmatic 3D imaging of microtubule samples was performed with a custom-built microscope as previously described\(^{24}\). Briefly, fluorescent signal was collected by an oil-immersion objective lens (Olympus, X100, 1.49 NA) and imaged with an sCMOS camera (Hamamatsu, ORCA-Flash4.0) at an intensity of ~13 kW cm\(^{-2}\). Fluorescence microspheres 100 nm in diameter (Thermo Fisher, 580/665, F8801) were imaged to generate reference PSFs. A custom-built focus-lock system based on tracking a reflected infrared laser was used to correct for axial drift.

Mitochondrial samples were imaged with a custom-built 4Pi-SIM system as previously described\(^{11}\). Briefly, the fluorescent signal was collected coherently between two opposing objectives (Olympus, ×100, 1.35 NA, silicone oil immersion) and imaged with an sCMOS camera (Hamamatsu, ORCA-Flash4.0 version 2). Data were acquired at 100 fps with a 560-nm laser (MPB Communications, 500 mW) at an intensity of ~13 kW cm\(^{-2}\). Fluorescence microspheres 100 nm in diameter (Thermo Fisher, TetraSpeck, 100 nm in diameter, T7284 or T7279) were imaged to generate reference PSFs. A custom-built focus-lock system based on tracking a reflected infrared laser was used to correct for axial drift.

Mitochondrial samples were imaged with a custom-built 4Pi-SIM system as previously described\(^{11}\). Briefly, the fluorescent signal was collected coherently between two opposing objectives (Olympus, ×100, 1.35 NA, silicone oil immersion) and imaged with an sCMOS camera (Hamamatsu, ORCA-Flash4.0 version 2). Data were acquired at 100 fps with a 560-nm laser (MPB Communications, 500 mW) at an intensity of ~13 kW cm\(^{-2}\). Fluorescence microspheres 100 nm in diameter (Thermo Fisher, TetraSpeck, 100 nm in diameter, T7284 or T7279) were imaged to generate subsidraction alignment maps between the two color channels.

**Imaging buffer.** For imaging origami structures, a Tris-based buffer (5 mM Tris, 10 mM MgCl\(_2\), 1 mM EDTA, pH 8.0, with 16 mM MgCl\(_2\) using an 18-h thermal annealing program (85–25 °C). Folded structures were concentrated by resuspending in half the original strands on the bottom of the ring for biotin functionalization.

′handles (Supplementary Table 1, biotin handle) extend from the 3′ end (Extended Data Fig. 1, D1-1b) and then seeded onto ozone-cleaned 25-mm round coverslips. Mitochondria25 with the following changes: the cells were labeled with mouse anti-GFP primary antibody (Invitrogen, A-11120) at 1:100 and rabbit anti-TOM20 antibody (Abcam, ab78474) at 1:2000 overnight at 4°C. Fluorescence samples were prepared at 0.2 μM and labeled with goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch, 115-005-146) and goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, 111-005-144) conjugated with oligonucleotide docking strands (Extended Data Fig. 1, D1-1c and D2-1a, respectively) both at a dilution of 1:200 for 1 h at room temperature.

**Data analysis.** Data acquired by the single-objective microscope systems were analyzed and visualized with PYME\(^{18}\) (https://python-microscopy.org) and custom code written in Python. Localizations in 2D were performed by a weighted least-square fit with a 2D Gaussian PSF model\(^{11}\). Astigmatic 3D localizations were performed by superimposing against a PSF experimentally derived from bead images\(^{24}\). For two-color DNA PAINT, molecules were localized independently in each channel and subsequently recombinated using an experimentally acquired alignment map.
The localization routine for 4Pi-SMS microscopy data has been previously described in detail. Images were rendered using Vutara SRX software (Bruker). Localizations that appeared in consecutive frames (allowing for misdetection of one frame) at the same position (distance <2σxy-localization precision) were combined into ‘blinks’. This correction takes into account that these localizations most likely do not represent independent samples of the docking strands (Supplementary Note 2). Although this results in fewer localization counts, blinks provide better estimates of the position at higher precision, and this approach avoids artificially inflated blinking rates and overcounting artifacts due to fast camera frame rate and/or slow blinking.

Drift corrections were performed using the redundant cross-correlation method. Blinks were rendered as 2D or 3D Gaussian functions in images and movies. Fourier ring correlation and FSC were computed based on the method described by Nieuwenhuizen et al. A threshold of 0.143 was used. A subregion of 5×5×0.6 μm was used for the FSC calculations presented in Fig. 4c and Extended Data Fig. 3c.

Coordinate-based colocalization was computed based on the method described by Malkusch et al., and code was published as part of LocAlization Microscopy Analyzer (LAMA) reported by Malkusch and Heilmann.

Analytic study of DNA-PAINT imaging speed and simulations of multiemitter artifact were performed with code written in Python (shared on GitHub; https://github.com/bewersdorflab/fluorogenic-dna-paint-manuscript-supplement/blob/main/imagerb_docking_strand_screen.ipynb) that used tools provided in BioPython.

Statistics. Statistical tests were performed using functions from the SciPy library (scipy.stats). Assumption of normality was tested for -tests for which sample size was >5 using the Shapiro–Wilks test. No assumptions were tested for the Mann–Whitney U-test or the Wilcoxon signed-rank test. No multiple-comparison corrections were performed.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The majority of datasets generated during and/or analyzed during the current study are available at the Zenodo repository (https://doi.org/10.5281/zenodo.6515537). Remaining raw datasets are available from the corresponding author on reasonable request.

Code availability
PYME is available at https://python-microscopy.org/. The PYME modules that we have developed are shared at https://github.com/bewersdorflab. Codes for simulating multi-emitters (Supplementary Fig. 2) and for screening docking strands (Supplementary Note 3) are available at the GitHub repository (https://github.com/bewersdorflab/fluorogenic-dna-paint-manuscript-supplement).

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Author contributions
K.K.H.C. and J.B. conceived the idea. Z.Z. designed the DNA origami structure. Z.Z., N.D.W. and Y.Y. prepared DNA origami samples. P.K. prepared cell samples. K.K.H.C. and J.B. wrote the manuscript with input from all authors.

Competing interests
J.B. discloses financial interest in Bruker, Hamamatsu Photonics and panluminate. J.B. is a co-inventor on a US patent (9,769,399) related to the 4Pi-SMS system and image analysis used in this work. Y.Z. and J.B. are co-inventors on a US patent (11,209,367) related to 4Pi-SMS microscopy. The remaining authors declare no competing interests.

Additional information
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Extended Data Fig. 1 | List of imager probes and docking strands used in this study.

| Regular DNA-PAINT (from Auer et al. 2017) | ID. | 5' to 3' direction | Used in: |
|-------------------------------------------|-----|--------------------|----------|
| Imager probe                              | P0-0| Cy3B - AGAAGTAATG  | Fig. 1, 3|
| Docking strand (antibody)                 | D0-0a| Azide - TTTATTACTTCT | Fig. 3   |
|-------------------------------------------|-----|--------------------|----------|

| Fast fluorogenic DNA-PAINT | ID. | 5' to 3' direction | Used in: |
|---------------------------|-----|--------------------|----------|
| Imager probe A            | P1-0| Cy3B - AGAAGTAATGTTGGAA - BHQ2 | Fig. 1-5, Ext. Data 3-6, Suppl. Fig. 2-3, Suppl. Video 1-3 |
| Docking strand A (antibody)| D1-1a| CCTTCAACATACCTCTAC - Azide | Fig. 3-4, Ext. Data 3, Suppl. Video 1-2 |
| Docking strand A (DNA origami) | D1-1b| CCTTCAACATACCTCTA - DNA origami | Fig. 2 |
| Docking strand A v.2 (antibody) | D1-1c| TTTCAACATACCTCTA - Azide | Fig. 5, Ext. Data 4-6, Suppl. Video 3 |
| Imager probe B             | P2-0| ATTO 643 - AAGAAGTAAAGGGAG - IBFQ | Fig. 5, Ext. Data 4-6, Suppl. Video 3 |
| Docking strand B (antibody) | D2-1a| CCTCGCTGAACCCCTTA - Azide | Fig. 5, Ext. Data 4-6, Suppl. Video 3 |
### Extended Data Fig. 2 | Alignment between imager probes and their corresponding docking strands

Fluorogenic DNA-PAINT uses imager probes and docking strands with internal mismatches. Complementary base pairings are colored in blue whereas mismatches in red.
Extended Data Fig. 3 | Fast astigmatic 3D fluorogenic DNA-PAINT imaging without optical sectioning. The full dataset from which Fig. 4a–c were generated. (a) Fast 3D fluorogenic DNA-PAINT imaging of immunolabeled microtubules in COS-7 cells under widefield illumination at multiple time points. A reasonable image can be acquired in 30 s. (b) Bleaching is negligible, causing only a small reduction (30%) in blinking rate over an hour. (c) 3D resolution as quantified by Fourier shell correlation (FSC) improves with longer imaging duration as more blinking events are detected. The resolution reaches 34.3 nm after 1 hr. (d) The localization precision peaks at <5 nm for all three dimensions (X: 1.7 nm, Y: 1.7 nm, Z: 4.5 nm). (e) Fitting an exponential decay function to blink durations (blinks that are only 1 frame in duration were ignored for fitting) estimates the mean off-rate at 46.7 s⁻¹.
Extended Data Fig. 4 | Time series of fast 2-color fluorogenic DNA-PAINT imaging without optical sectioning. The full dataset from which Fig. 5b-f were generated, rendered at various timepoints. (a–d) Fast 2-color fluorogenic DNA-PAINT imaging of immunolabeled endoplasmic reticulum (ii; [imager probe A] = 10 nM) and mitochondria (iii; [imager probe B] = 1 nM) in U-2 OS cells under widefield illumination (100 Hz frame rate). There is no well-defined minimum imaging time as it depends on a multitude of factors including the biological question being addressed. The timepoint we reported in the main text (600 s) is more densely sampled than typical single-molecule localization microscopy images. Negligible changes are observed with prolonged imaging (d; 1,200 s) which would suggest oversampling.
Extended Data Fig. 5 | Analysis of fast 2-color fluorogenic DNA-PAINT imaging without optical sectioning. Detailed analysis of the full 20-minute 2-color fluorogenic DNA-PAINT dataset from which Fig. 5 and Extended Data Fig. 4 were generated. Image colored by the correlation parameter (C) based on Coordinate-Based Colocalization (CBC) analysis at low (a) and high (b) magnification. (c) Histogram of the correlation parameter, C. A value of zero indicates a lack of correlation between the two color channels ($C = -0.09 \pm 0.30$; $n_{baks} = 2,322,207$; two-sided one-sample Wilcoxon signed-rank test against zero, $T$-statistic=$9\times10^{11}$, $p < 0.001$). (d) Minimal bleaching is observed over a 20-minute timeframe (~20%). (e) The lateral localization precision peaks at <5 nm for both channels. (f) Blink durations fitted with an exponential decay function to estimate the binding off-rate (blinks that are only 1 frame in duration were ignored for fitting).
Extended Data Fig. 6 | Additional examples of fast 2-color fluorogenic DNA-PAINT imaging without optical sectioning. Fast 2-color fluorogenic DNA-PAINT imaging of immunolabeled endoplasmic reticulum (green; [imager probe A] = 10 nM) and mitochondria (magenta; [imager probe B] = 1 nM) in U-2 OS cells under widefield illumination (100 Hz frame rate for 10 minutes) (n = 5 including the dataset presented in detail in Fig. 5 and Extended Data Figs. 4,5).
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☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

☐ Give P values as exact values whenever suitable.

☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

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☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

| Data collection | Data collection was performed using Python Microscopy Environment 17.02.27 [PYME; https://python-microscopy.org/] and custom code written in LabVIEW 2010 [National Instruments]. |
|-----------------|----------------------------------------------------------------------------------------------------------------------------|
| Data analysis   | Image data analysis was performed using Python Microscopy Environment 19.02.27 [PYME; https://python-microscopy.org/], and custom code written in Python 2.7 and 3 (with Numpy 1.16 and 1.19), and MATLAB 2017b (MathWorks). NUPACK web and Biopython 1.79 was used for oligonucleotide analysis. PYME, Vutara SHX 6.04.07 [Bruker], Matplotlib 3.3, and custom code written in Python was used for visualization. Custom PYME modules are shared at https://github.com/bewersdorflab. Custom code specific to this study are shared at https://github.com/bewersdorflab/flurogenic-dna-paint-manuscript-supplement. |

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- A description of any restrictions on data availability
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The majority of datasets generated during and/or analyzed during the current study are available in the Zenodo repository, https://doi.org/10.5281/zenodo.6315337. Remaining raw datasets are available from the corresponding author on reasonable request.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
This is a methods paper describing a technological development. No sample size calculation was performed a priori. Our sample size was selected to adequately describe the technical performance of our methodology.

Data exclusions
Qualitative exclusion criteria for accepting or rejecting imaged samples were pre-established based on comparisons to previously published images and preliminary experiments performed during the optimization process of the method development. DNA origami structures were selected from images based on consistency with the expected structure shape and localization counts. Unclassified objects were rejected.

Replication
The data presented here are the optimal images generated among iterations of experiments as we optimized our method. Typically, sample size was n=2-3 in the final experiment from which we picked our final representative dataset (n=5 for the 2-color experiments). Each n is an independently acquired dataset.

Randomization
No randomization was performed. Randomization was considered not to be necessary as the purpose of the study was to demonstrate a new technique and not report biological results.

Blinding
No blinding was performed. Blinding was considered not to be necessary as the purpose of the study was to demonstrate a new technique and not report biological results.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a | n/a |
| ☐ X Antibodies | X X ChiP-seq |
| ☐ X Eukaryotic cell lines | ☐ X Flow cytometry |
| ☒ X Palaeontology and archaeology | ☒ X MRI-based neuroimaging |
| ☐ X Animals and other organisms | |
| ☒ X Human research participants | |
| ☒ X Clinical data | |
| ☒ X Dual use research of concern | |

Antibodies

| Antibodies used | Validation |
|-----------------|------------|
| Primary antibodies: mouse anti-alpha tubulin primary antibody (Sigma Aldrich, T5168) rabbit anti-GFP primary antibody (Invitrogen, A-11122) rabbit anti-TOMM20 primary antibody (Abcam, ab78547) mouse anti-GFP primary antibody (Invitrogen, A-11120) | The results of all primary antibodies are consistent with the information shown on respective manufacturer’s websites, where many references are listed. mouse anti-alpha tubulin primary antibody (https://www.sigmaaldrich.com/US/en/product/sigma/T5168) rabbit anti-GFP primary antibody (https://www.thermofisher.com/antibody/product/GFP-Antibody-Polyclonal/A-11122) rabbit anti-TOMM20 primary antibody (https://www.abcam.com/tomm20-antibody-mitochondrial-marker-ab78547.html) mouse anti-GFP primary antibody (https://www.thermofisher.com/antibody/product/GFP-Antibody-clone-3E6-Monoclonal/A-11120) |

Unconjugated secondary antibodies: goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch, 115-005-146) goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, 111-005-144)
# Eukaryotic cell lines

**Policy information about cell lines**

| Cell line source(s)          | COS-7 ATCC lot #63624240, U-2 OS ATCC lot #70008732 |
|-----------------------------|---------------------------------------------------|
| Authentication              | Cell lines were purchased from ATCC directly and not independently authenticated. |
| Mycoplasma contamination     | Cell lines were not tested for mycoplasma contamination. |
| Commonly misidentified lines| None |

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