Repeat DNA-PAINT suppresses background and non-specific signals in optical nanoscopy

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DNA-PAINT is a versatile optical super-resolution technique relying on the transient binding of fluorescent DNA ‘imagers’ to target epitopes. Its performance in biological samples is often constrained by strong background signals and non-specific binding events, both exacerbated by high imager concentrations. Here we describe Repeat DNA-PAINT, a method that enables a substantial reduction in imager concentration, thus suppressing spurious signals. Additionally, Repeat DNA-PAINT reduces photoinduced target-site loss and can accelerate sampling, all without affecting spatial resolution.
Super-resolution optical microscopy methods have become essential tools in biology, and among these DNA-PAINT\textsuperscript{1–3} has proved especially versatile\textsuperscript{4,7}. In DNA-PAINT, epitopes of interest are labeled with ‘docking’ DNA motifs, while dye-modified ‘imager’ oligonucleotides are introduced in solution. Transient hybridization to docking motifs immobilizes imagers for long enough to generate ‘blinks’ (events) in a camera frame, which can then be fitted to localize target epitopes with sub-diffraction resolution\textsuperscript{2}. DNA-PAINT carries several advantages compared to competing approaches such as STORM\textsuperscript{8,9} and PALM\textsuperscript{10,11}, eliminating the need for photo-switchable or chemically-switchable dyes and effectively circumventing photobleaching, due to fresh imagers continuously diffusing in from the bulk.

The unparalleled flexibility of DNA-PAINT comes at a cost, in the form of a number of serious drawbacks currently limiting the applicability and performance of the technology when imaging biological cells and tissues. The presence of free imagers in solution produces a diffuse fluorescent background, which compromises event detection and localization precision. The impact of free-imager signals is particularly severe when imaging deep in biological tissues, where efficient background-rejection methods such as TIRF cannot be used.

In addition, imagers often exhibit substantial non-specific binding to biological preparations, which complicates data interpretation\textsuperscript{7} and can prevent detection of sparse targets\textsuperscript{12}.

Both imager-induced background and non-specific events can be reduced by decreasing imager concentration. However, such a reduction also decreases event rates and extends image-acquisition timescales, which is often prohibitive due to limitations in mechanical and chemical sample stability.

Finally, despite it being effectively immune to photobleaching, DNA-PAINT has been shown to suffer from photo-induced inactivation of docking strands\textsuperscript{13}.

Here, we introduce repeat DNA-PAINT, a straightforward strategy that mitigates all these critical limitations of DNA-PAINT.

Results

Repeat DNA-PAINT affords an increase in event rate. As demonstrated in Fig. 1a, c, we employ docking motifs featuring $N$ identical Repeated Domains (NxD, $N$ = 1, 3, 6, 10) complementary to imagers. Unless otherwise specified, we use a 9-nucleotide (nt) imager (P1) whose concentration is referred to as $[I]$.

In the super-resolution imaging regime, only a small fraction of docking sites is occupied by imagers at any given time. In these conditions, and if all repeated docking domains are equally accessible to imagers as in a 1x RD motif, the spatial event density $E$ is expected to be proportional to the product of imager concentration and repeat domain number $N$:

$$E = \rho_{DS} \cdot N \cdot \frac{[I]}{K_d},$$

where $\rho_{DS}$ is the docking strand density (set by the density of markers in the sample) and $K_d$ the binding affinity of imagers to a single docking domain (see also Supplementary Note 1).

In agreement with Eq. 1, tests performed on functionalized microspheres demonstrate a linear growth in event rate with increasing $N$, for fixed imager concentration $[I] = 50$ pM (Fig. 1b). The experimental findings are confirmed by molecular simulations, relying on the oxDNA\textsuperscript{14} model and the Forward–Flux Sampling method to estimate imager-docking binding rates\textsuperscript{15} (Fig. 1b).

Simulations further highlight that, as expected, imagers bind all individual domains on the repeat-docking motifs with similar probability, proving that the elongation of docking motifs does not hinder their accessibility (Supplementary Fig. 1).

Equation 1 also indicates that, when using docking motifs with $N$ repeats, the imager concentration can be reduced $N$-fold while preserving the event density $E$, or equivalently the event rate (when summed over a region of interest and quantified per frame).

To confirm this hypothesis we constructed DNA origami test tiles that display a number of “anchor” overhangs, initially connected to 1x RD docking motifs. The former could then be displaced through a toe-holding reaction, and were replaced with a 10x RD strand (Fig. 1c). The event rate per origami tile was preserved when changing from 1x RD docking sites with 0.4 nM imager concentration to 10x RD docking sites but 10-times lower imager concentration of 40 pM (Fig. 1d). The same strategy was applied to biological samples, specifically cardiac tissues\textsuperscript{6} where we labeled ryanodine receptors (RyRs) with the common anchor strand that initially held a 1x RD motif. As expected, we find near identical event rates when imaging 1x RD with $[I] = 0.4$ nM versus replacing these with 10x RD with $[I] = 40$ pM (Supplementary Fig. 2).

Repeat DNA-PAINT suppresses backgrounds and enhances resolution. The ability of Repeat DNA-PAINT to function optimally with a substantial (up to 10-fold) reduction in imager concentration would be especially beneficial if imaging deep in biological tissues.
concentration makes it ideal for mitigating issues resulting from imagers in solution, the most direct being the fluorescent background produced by unbound imagers.

In Fig. 2 we therefore investigate the fluorescent background in cardiac tissue samples with conventional docking strands (1x RD) and repeat domains (10x RD). Visual assessment demonstrates a clear improvement in contrast between the two imaging modes, as shown by example frames in Fig. 2ai (1 RD) and Fig. 2a(ii) (10x RD), to an extent that substantially improves the detectability of individual binding events and their localization precision16.

For a quantitative assessment, we measured background signals produced with $[I] = 40$ pM and $0.4$ nM in optically thick tissues labeled with common anchor overhangs, but lacking docking motifs. Figure 2b (left pair of bars), demonstrates a near linear increase of the fluorescent background with $[I]$. Once the markers were functionalized with docking strands, either 1x RD or 10x RD, the ratio of background levels was slightly lower, apparently due to an additional offset background (Fig. 2b, right pair of bars). We hypothesize that the additional background is generated by specific binding events occurring out of the plane of focus. These events are indeed expected to produce an out-of-focus signal proportional to the event rate, and thus similar when using 1x RD with $0.4$ nM versus 10x RD with $40$ pM of imager (by design).

It is expected that the substantial reduction in background afforded by Repeat DNA-PAINT translates into a significant improvement in resolution. To quantify this improvement we imaged deep (several microns) into optically thick (~20 µm) cardiac tissue using this technique. We performed a two-stage experiment as exemplified in Fig. 1c, first imaging with 1x RD at high $[I]$ and then with 10x RD at low $[I]$. In both cases, we carried out Fourier Ring Correlation (FRC) measurements of the optical resolution in $2 \times 2 \mu m^2$ regions across the ~24 × 20 µm$^2$ imaging region (Fig. 2c). This yielded a mean FRC resolution measurement (Fig. 2d) of $123.7 \pm 3.0$ nm (SEM) for 1x RD, $[I] = 0.4$ nM, and $78.0 \pm 1.8$ nm (SEM) for 10x RD, $[I] = 40$ pM, confirming the substantial improvement in resolution with Repeat DNA-PAINT when background from imagers in solution cannot be effectively rejected, e.g., when imaging deep in thick tissue with widefield illumination (Fig. 2e).
Repeat DNA-PAINT suppresses non-specific binding. Having proven the benefits of Repeat DNA-PAINT in reducing backgrounds and improving resolution, we assessed its impact on non-specific imager-binding events at unlabeled locations of biological samples. These non-specific events produce spurious blinks that are often difficult to distinguish from proximal specific signals. Expectedly, Fig. 3a shows that the rate of non-specific events, as detected in unlabeled cardiac tissue, scales linearly with \([I]\). Similar trends are observed for different imager sequences (Supplementary Fig. 4).

In Fig. 3b we study the time-sequence of imager-attachment events recorded in cardiac tissue, as a potential way of separating specific from suspected non-specific events. We compare a trace recorded within a likely unlabeled area, where only suspected non-specific events are observed, based on only one brief attachment phase (Fig. 3b, red region), with one measured at a location where docking strands are present and specific binding is detected (Fig. 3b, yellow region). We observe a qualitative difference between the two situations, with specific binding occurring steadily and suspected non-specific events being often localized in time, similar to the time courses of imager attachment observed in data from unlabeled cardiac tissue, which underlies the summary data in Fig. 3a.

Although occasionally applicable, this identification strategy is only robust if specific and suspected non-specific binding sites are spatially isolated. In samples where docking strands are more densely packed and/or evenly distributed, non-specific events cannot be easily separated (Supplementary Fig. 5), introducing potential artifacts in the reconstructed images and distorting site-counting as performed, e.g., via qPAINT.

Repeat DNA-PAINT offers a solution that avoids the complexity of identifying non-specific events, by directly reducing their occurrence to negligible levels, as demonstrated in Fig. 3c. Specifically, owing to the 10-fold reduction in imager concentration, image data collected with 10x RD on our cardiac samples only feature ~0.9% non-specific events, whereas conventional DNA-PAINT, here implemented with 1x RD docking strands, yields a ~8% non-specific contamination. We thus conclude that Repeat DNA-PAINT offers a robust route for suppressing spurious events independent of sample characteristics.

Repeat DNA-PAINT mitigates photoinduced site damage. Despite its insensitivity to photobleaching, DNA-PAINT is subject to a progressive inactivation of docking sites, ascribed to their interaction with the free-radical states of photo-excited fluorochromes. The domain redundancy in Repeat DNA-PAINT can greatly slow down site loss, as we demonstrate with origami test tiles nominally featuring six anchor sites (Fig. 4a). For tiles with 1x RD and 10x RD motifs, we compare the average number of sites actually detected on the tiles in the first 20 K frames of long imaging runs, to those counted in the following 20 K frames. While for 1x RD tiles we observed a ~12.1% loss of docking sites between the two experimental intervals, 10x RD tiles just lose...
Fig. 4 Repeat DNA-PAINT reduces docking site-loss. a Photoinduced site loss as quantified with DNA origami tiles labeled with 10x RD or 1x RD by comparing the number of sites detected in the first half (0–20 K frames) versus the second half (20–40 K frames) of an experimental run. (left) Rendered images of typical tiles, origami designs as shown at top. (right) Histograms summarizing the percentage of lost sites, using (b) 10x RD or (c) 1x RD. Site loss is much more extensive when using 1x RD docking strands. Scale bars: 100 nm.

Fig. 5 Repeat DNA-PAINT preserves spatial resolution. a Simulated radial distributions of the fluorophore site on imagers hybridized to all possible sites on 1x RD, 3x RD, and 6x RD, with respect to the anchoring point of the docking motif. b Radial profiles of blinks as obtained by convolving the fluorophore-distributions with the microscope point-spread function (Supplementary Fig. 6). Insets: zoom in of the region around the first Airy minimum, showing very small broadening that is unlikely to be experimentally detectable. c Scheme of DNA-origami test tiles with red sites indicating the locations of 10x RD motifs and a rendered DNA-PAINT image, similar origami quality observed in n = 7 origami experiments. d Typical spatial profiles measured across the ’spots’ of origami tiles with 10x RD strands as in c, with full-width at half maximum (FWHM) spot diameters as indicated. The average FWHM is 12.28 ± 1.77 nm (mean ± SD), nearly identical to 12.56 ± 2.09 nm determined for 1x RD (Supplementary Fig. 7). e Fourier Ring Correlation (FRC) resolution measurements of DNA-PAINT images of origami tiles with 1x RD strands (12.12 ± 2.69 nm, mean ± SD) are indistinguishable from 10x RD (12.36 ± 2.67 nm). Boxplots show minima, maxima and median of the data. Scale bars: 30 nm.

Extended docking motifs do not affect spatial resolution. A potential issue deriving from the extension of the docking strands is the loss of spatial resolution\textsuperscript{17,18}, as the flexible docking-imager complexes undergo rapid thermal fluctuations during binding events (see Supplementary Note 2). We used oxDNA simulations to quantify the resulting ‘blurring’, by sampling the distance between the tethering point of the docking strand and the fluorophore location of imagers hybridized to each binding site in 1x RD, 3x RD, and 6x RD motifs. The results, summarized in Fig. 5a, demonstrate narrow fluorophore distributions for the binding sites closest to the tethering point, and broader ones for the more distal sites, peaking at ~8 nm for the furthest domain.

Although this level of broadening may appear significant compared to the resolution of DNA-PAINT in optimal conditions (~5 nm\textsuperscript{19}), it has little impact on the precision with which one can localize the labeled epitope by fitting the diffraction-limited image of a blink. The effect can be quantified by convolving the fluorophore distributions (Supplementary Fig. 6 and Supplementary Note 2) with the theoretical point-spread

\[ \text{FRC resolution} = \frac{1}{2} \left( \frac{1}{\text{FWHM}} - 1 \right) \]

\[ \text{FWHM} = \frac{\lambda}{2 \pi \text{NA}^2} \]

\[ \text{Diffraction limit} = \frac{\lambda}{2 \pi \text{NA}} \]
function (PSF) of the microscope, as shown in Fig. 5b. The PSF broadening is minute and produces, at most, a 0.12% shift in the location of the first Airy minimum.

We thus do not expect that the larger physical size of multi-repeat docking motifs cause any loss of experimental resolution. We confirmed this prediction with DNA-origami test samples (Fig. 5c), showing no detectable resolution difference between 1x RD and 10x RD, both rendering spots with apparent diameter of ~13 nm (Fig. 5d and Supplementary Fig. 7). Similarly, the Fourier Ring Correlation (FRC) measure of resolution was essentially unaltered between 1x RD (12.2 ± 2.7 nm) and 10x RD (12.4 ± 2.7 nm) images, as shown in Fig. 5e. Note that when imaging origami test samples, the resolution is virtually unaffected by the higher imager concentration used with 1x RD and the consequent strong free-imager background, as instead demonstrated for the case of thick biological tissues (Fig. 2). Indeed, origami represent a highly ideal scenario in which imaging can be carried out in TIRF mode, which is highly effective in rejecting out-of-focus backgrounds. Other imaging modes, necessary to investigate thicker biological samples, do not perform nearly as well, leading to the substantial benefits in terms of background and resolution associated with reducing imager concentration.

Additional advantages of Repeat DNA-PAINT: qPAINT, enhanced imaging rate and photobleaching-free wide-field imaging. Repeat DNA-PAINT is also fully compatible with extensions of DNA-PAINT, such as qPAINT, a technique that estimates the number of available docking sites within a region of interest. We confirm the accuracy of qPAINT with origami tiles displaying five 10x RD motifs, where the technique estimates 4.93 ± 0.16 sites/tile (see Fig. 6a, and “Methods” section).

In addition, we point out that the boost in event-rate afforded by Repeat DNA-PAINT can also be exploited to increase image acquisition rate. The key for increasing imaging frame rate is using weakly binding imagers, which thanks to a larger $K_d$, and associated larger off-rate, produce shorter events. In parallel, however, one would have to increase imager concentration in direct proportion to $K_d$, in order to retain a sufficiently high binding frequency, see also Eq. 1 and Supplementary Note 1. The concomitant increase in background (see also Fig. 2) would normally be prohibitive but the event-rate acceleration afforded by Repeat DNA-PAINT allows imaging to be carried out at “normal” imager concentrations, in the sub nanomolar range. Figure 6b indeed demonstrates that by simply replacing 1x RD with 10x RD at ‘conventional’ imager concentration ($[I] = 0.3$ nM), and using a shorter (low-affinity) 8 nt imager P1s, one can increase frame rate 10-fold (from 100 ms to 10 ms), and reduce the overall imaging time ~6-fold. When performing accelerated imaging, we observe a slightly lowered limiting spatial resolution, from ~80 nm at 100 ms acquisition time to ~100 nm at 10 ms, see Supplementary Fig. 7. Note however that high frame rate acquisition can be further improved by optimizing illumination conditions, so that the number of photons collected from a dye molecule in a short-exposure frame equals that achieved at longer integration time. The ability of repeated-docking motifs to accelerate imaging has been recently confirmed by Straus et al.21, which however do not discuss the associated improvements in terms of background, resolution and non-specific signals.

Finally, Repeat DNA-PAINT enables effectively photobleaching resistant, high-contrast, diffraction-limited imaging. In all the super-resolution applications described above, low imager concentrations are used so that only a small fraction of docking sites is occupied at any given instant. At higher imaging concentrations, a significant fraction of the sites are occupied by imagers. Since imagers are still constantly exchanged with the surrounding solution, operating under these conditions would in principle allow for photobleaching-free diffraction-limited fluorescence imaging, including wide-field and point-scanning confocal. However, to achieve a sufficient docking-site occupancy with conventional 1x RD docking strands, one would have to increase imager concentration to a point where the free-imager background massively reduces contrast. Repeat DNA-PAINT performed with 10x RD motifs solves this issue thanks to the intrinsically higher imager binding rates, which enables wide-field imaging at the imager concentrations normally used for conventional DNA-PAINT. This translates in a straightforward strategy for collecting high-contrast, photobleaching-free images of staining patterns (Supplementary Fig. 9).

Discussion

In summary, we demonstrate that Repeat DNA-PAINT mitigates all key limitations of DNA-PAINT, namely non-specific events (10x reduction), free-imager background (~5x reduction) and photoinduced site loss (5x reduction) while also being able to accelerate data acquisition (6–10x). We also show that there is no
observed impact on spatial resolution from “long” docking strands containing many repeat domains which greatly extends the design space of Repeat DNA-PAINT. Notably, the implementation of Repeat DNA-PAINT is straightforward and does not carry any known drawbacks, it is routinely applicable, consolidating the role of DNA-PAINT as one of the most robust and versatile SMLM methods.

Methods

Experimental methods and materials

DNA-PAINT oligonucleotides. Oligonucleotide sequences were designed and checked with the NUPACK web application22 (www.nupack.org). Oligonucleotides were then purchased from either Integrated DNA Technologies (IDT, Belgium) or Eurofins Genomics (Eurofins, Germany) with HPLC purification. See Supplementary Table 1 for a full list of oligonucleotide sequences used in.

DNA origami production and sample preparation. All oligonucleotides (staples) used were purchased from IDT and after desalting, pre-reconstituted in Tris EDTA (10 mM Tris + 1 mM EDTA, TE) buffer (pH 8.0) at 100 µM concentration. Rothemund Rectangular Origami (RRO) with various staple overhangs were manufactured following standard methods23. Picasso24 was used to generate staple sequences which yield an RRO with 3 staple overhangs in specific positions on the reverse face of the origami. We designed overhangs which would then hybridize to 1x RD or 10x RD docking motifs (see anchor in Supplementary Table 1). Eight DNA strands had 5’ biotin modifications on the reverse face for anchoring. RROs were prepared by mixing in TE + 12.5 mM MgCl2, the scaffold (M13mp18, New England Biolabs, USA) at a concentration of 10 nM, biotinylated staples at 10 nM, staples featuring the “anchor” 3’ overhangs at 1 µM, and all other staples at 100 nM. Assembly was enabled through thermal annealing (Techne, TC-512 thermocycler) bringing the mixture to 80 °C and cooling gradually from 60 °C to 4 °C over the course of 3 h. A full list of staple cations on the anchor strand initially harboring 1x RD prior to being displaced and re-dispersion steps. These microspheres were attached via non-specific antibody was incubated overnight (4 °C) with the sample at 5 µg ml−1 in a PBS incubation solution buffer containing 1% BSA, 0.05% Triton X-100 and 0.05% sodium azide, alpha-actinin (A7732, Sigma) was diluted 1:200 in incubation buffer and treated in the same manner. Samples were washed in PBS 3–4 times for 10–15 min each. Secondary antibodies, previously conjugated to oligonucleotides and stored at 1 mg ml−1 were diluted 1:200 in incubation solution added to the samples, and left for 2 h at RT. The tissue was then finally washed for a further 3 times in PBS.

Microsphere functionalization and sample preparation. Streptavidin-functionalized polystyrene particles with a diameter of 500 nm (MicroParticles GmbH, Germany) were labeled with biotinylated oligonucleotides (Fig 1a: docking motifs 1x RD, 3x RD, and 6x RD, see Supplementary Table 1) as described elsewhere24. Briefly the microspheres were dispersed in TE buffer containing 300 mM NaCl and the docking strands in 4x excess concentration as compared to the binding capacity of the beads. Unbound oligonucleotides were removed by a series of centrifugation and re-dispersion steps. These microspheres were attached via non-specific adhesion to cover slips cleaned as described above and coated by incubating them for 30 min with a 0.1 mg ml−1 solution of PLL-g-PEG (SuSoS, Duebendorf) in PBS.

Oligonucleotide to antibody conjugation. Anchor oligonucleotides (Supplementary Table 1) were conjugated to secondary antibodies for immunolabeling of cardiac samples. Lyophilized oligonucleotides were resuspended in PBS (pH 7.4) to 100 µM and kept at −20 °C for long term storage required for conjugation. AffiniPure Goat Anti-Mouse secondary antibodies (affinity purified, #115-005-003, Jackson ImmunoResearch, PA) were conjugated using click-chemistry as described by Schnitzbauer et al.25. Briefly, the antibody was incubated with 10-fold molar excess DBCO-sulfo-NHS-ester (Jenabioscience, Germany) for 45 min. The reaction was quenched with 80 mM Tris-HCl (pH 8.0) for 10 min and then desalted using 7 K MWCO Zeba desalting columns (Thermo Fisher). A 10-fold molar excess of the azide modified oligonucleotide was then incubated with the DBCO-antibody mixture overnight at 4 °C. Subsequently the antibody was purified using 100 K Amicon spin columns (Sigma). The absorbance of the oligonucleotide-conjugated fluorophores (Cy3 or Cy5) was recorded with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and used to quantify the degree of labeling for each conjugation, typically achieving >1–3 oligonucleotides per antibody.

Biological sample preparation and labeling. Cardiac tissue (porcine) was fixed with 2% paraformaldehyde (PFA, pH 7.4, Sigma) for 1 h at 4 °C. Samples were then washed in PBS and kept in PBS containing 10% sucrose for 1 h before being moved to 20% (1 h) and finally 30% sucrose overnight. The tissue was then frozen in cryotubes floating in 2-Methylbutane cooled by liquid nitrogen for 10–15 min. Preparations were incubated in 1.5 glass cover slips for 15 min using 50% poly-L-lysine (Sigma). Tissue cryosections with thicknesses of 5–20 µm were adhered to the cover slips and kept at −20 °C until used. For DNA-PAINT experiments, the tissues were labeled with mouse primary anti ryadnoine or anti actinin antibodies, and targeted by the oligonucleotide conjugated secondary antibodies. Immunohistochemistry was performed as described above in imaging chambers as described previously26 permeabilizing the tissue with 0.1% Triton X-100 in PBS for 10 min at room temperature (RT). The samples were blocked with 1% bovine serum albumin (BSA) for 1 h in a hydration chamber. The monoclonal mouse anti-ryadnoine receptor (Fisher, MA3-916, Thermo Fisher) primary antibody was incubated overnight (4 °C) with the sample at 5 µg ml−1 in a PBS incubation solution buffer containing 1% BSA, 0.05% Triton X-100 and 0.05% sodium azide, alpha-actinin (A7732, Sigma) was dilutet for 2 h in incubation buffer and treated in the same manner. Samples were washed in PBS 3–4 times for 10–15 min each. Secondary antibodies, previously conjugated to oligonucleotides and stored at 1 mg ml−1 were diluted 1:200 in incubation solution added to the samples, and left for 2 h at RT. The tissue was then finally washed for a further 3 times in PBS.

Microscopy experiments. A modified Nikon Eclipse Ti-E inverted microscope (Nikon, Japan) with ×60 1.49NA APO oil immersion TIRF objective (Nikon, Japan) was used to acquire super-resolution data. Images were taken using an Andor Neo.4.2.2 (80 nm) camera (Andor, UK) using a camera integration time of 100 ms, or 10 ms for accelerated acquisition (Fig 6b and Supplementary Fig. 8). A tunable LED-light source (CoolLED, UK) was used where possible to illuminate the widefield fluorescence and check labeling quality prior to super-resolution imaging. A 642 nm continuous wave diode laser (Omnikron LuxX, Germany) was used to excite the ATTO 655 imager strands for DNA-PAINT imaging. Microspheres and DNA-origami tiles were imaged in total internal reflection fluorescence (TIRF) mode, whilst tissue samples required highly inclined and laminated optical sheet (HIL) mode. An auxiliary camera (DCC3240N, Thorlabs) was used in a feedback loop to monitor and correct for focal drift, similar to McGorty et al.27, and previously implemented in ref. 6. Red fluorescent beads with a diameter of 200 nm (F8887, Thermo Fisher Scientific) were introduced to the samples prior to DNA-PAINT imaging and later used in post-analysis to correct for lateral drift.

Operation of the microscope components, image acquisition and image analysis were conducted using the Python package PyMIB (Python Microscopy Environment), which is available at https://github.com/python-microscopy/pymicroscopy. Single molecule events were detected and fitted to a 2D Gaussian model. Localization events were rendered into raster images that were saved as tagged image file format (TIFF) either by generating a jittered triangulation of events or by Gaussian rendering22.
removal of excess 10x RD and its imaging. The number of localized events were then used to generate two rendered Gaussian images which were compared. Localization precision, as shown in Fig.2d, was used to conduct qPAINT analysis. To calculate the number of binding sites per tile, the highly correlated degrees of freedom. The maximum VMMC cluster-size was set to 12 nucleotides, with translational moves of 0.05 oxDNA units, and rotational moves of 0.22 oxDNA units. Temperature was set to 300 K. We run simulations at effective monovalent salt concentrations of 640 mM.

Separate simulations were initialized with the imager bound to each of the possible locations on docking strands 1x RD, 3x RD, and 6x RD. Large artificial biases were used to ensure that at least 7 of the 9 imaging-docking bonds were formed, so that the two strands remained bonded for the duration of the simulation. The end-nucleotide of the docking motif corresponding to its anchoring point, was confined to point with a 3D harmonic potential. Each system was simulated in 16 replicas, for between 9 × 10⁵ and 2.7 × 10⁶ MC steps. The position of the fluorophore-bearing nucleotide on the imager was taken as a proxy for that of the fluorophore (which cannot be simulated in oxDNA), and each nucleotide as a site with 6 anisotropic interactions: excluded volume, stacking, cross-stacking, hydrogen bonding, backbone connectivity and electrostatic repulsion. Here we used the updated oxDNA2 force field with explicit electrostatics.

The systems were simulated using Monte-Carlo (MC) sampling, and moves were proposed with the Virtual Move Monte Carlo (VMMC) scheme to better sample the highly correlated degrees of freedom. The maximum VMMC cluster-size was set to 12 nucleotides, with translational moves of 0.05 oxDNA units, and rotational moves of 0.22 oxDNA units. Temperature was set to 300 K. We run simulations at effective monovalent salt concentrations of 640 mM.

Separate simulations were initialized with the imager bound to each of the possible locations on docking strands 1x RD, 3x RD, and 6x RD. Large artificial biases were used to ensure that at least 7 of the 9 imaging-docking bonds were formed, so that the two strands remained bonded for the duration of the simulation. The end-nucleotide of the docking motif corresponding to its anchoring point, was confined to point with a 3D harmonic potential. Each system was simulated in 16 replicas, for between 9 × 10⁵ and 2.7 × 10⁶ MC steps. The position of the fluorophore-bearing nucleotide on the imager was taken as a proxy for that of the fluorophore (which cannot be simulated in oxDNA), and its location relative to the harmonic trap anchoring the docking motif was sampled every 5,000 steps. The fluorophore location was then projected onto the x-y plane to produce the 2D probability distributions in Supplementary Fig. 6, with uncertainties calculated between replicas (which however are negligible and
unnoticeable in Fig. 5a). The probability distributions in Fig. 5a are obtained by radial averaging.

In Supplementary Note 2 we show that the timescales of relaxation of the imager-docking configuration into equilibrium are orders of magnitude faster than those of photon emission. One can thus assume that the physical locations from where photons are emitted are randomly drawn from the distributions of dye locations. The photon spatial distribution sampled by the microscope during each blink can therefore be estimated by converting the distribution of fluorophore locations with the PSF, here approximated with an Airy disk whose full width half maximum (FWHM) is 250 nm. Convolution between the PSF and fluorophore distributions is performed in 2D, and the radial cross sections are shown in Fig. 5b. This approximate PSF, as defined by the FWHM of an Airy disk occurs at 0.51λ/NA = 250 nm, using values of λ = 700 nm and NA = 1.45 that correspond to the experimental conditions in this study.

Evaluation of hybridization rate using forward flux sampling. We use molecular dynamics (MD) simulations performed with the oxDNA model to estimate the relative rates of hybridization of imagers to docking motifs with variable number of repeats (1x RD, 3x RD, and 6x RD) as shown in Fig. 1b. The absolute rates are not accessible, since diffusion rates in the coarse-grained representation oxDNA are not necessarily realistic.

For these simulations, the oxDNA force field is manually modified to eliminate intra-strand hydrogen bonding. Such a modification is necessary to prevent the appearance of a hairpin loop in 6x RD. Said loop is predicted not to occur by standard Nearest-Neighbor nucleic acid thermodynamics, as implemented in NUPACK.34 We suspect the loop formation in oxDNA is an artifact related to identical excluded volume for purines and pyrimidines, so that duplex destabilisation due to base pair mismatch is underestimated.

Our objective is to estimate the first order rate constant of imager hybridization to any binding domain of a tethered docking strand. Even with the highly coarse-grained oxDNA model, hybridizations are still rare over simulated timescales. To enhance sampling of hybridization events, we use Direct Forward Flux Sampling (FFS)35, 36. FFS relies on defining a reaction coordinate onto which the state of the system can be projected. Along this coordinate one then identifies a number of intermediate system configurations between the initial and final states of interest. The rate for the system to evolve between the initial and final states can then be decomposed over the intermediate steps, which can be sampled more effectively.

The calculation of FFS is based on that of Ouldridge et al.27 We define a reaction coordinate Q which can take all integer values between Q = −2 and Q = 4. For Q = −2, −1, 0 the reaction coordinate is defined based on the minimum distance d_min between the imager and the docking motifs, calculated considering any of the nucleotides on either strand. This includes nucleotide pairs that are non-complementary. For Q = −1, 0, the coordinate is also dependent on Nbbox, the number of nucleotide bonds between docking strand and imager. Following ref. 27 we assume that two nucleotides are bound if their energy of hydrogen bonding is more negative than 0.1 simulation units, equivalent to 2.5 kJ mol⁻¹. Q = 4 corresponds to our target state in which all 9 imager nucleotides are hybridized to the docking strand. Conditions associated to all values of Q are summarized in Supplementary Table 2. We indicate as λ_i the non-intersecting interfaces between states with consecutive values of the reaction coordinate, where i = −2, −1, 0, 1, 2. E.g. λ_i is the interface between states with Q = 0 and those with Q = 1. Note that for the system to transition from Q = −2 to Q = 4 it is necessary that all intermediated values of the reaction coordinate are visited.

The rate of imager-docking hybridization can then be calculated as

$$r = \Phi_{-2\to0} \prod_{i=1}^{4} p(i-1)$$

Here, $\Phi_{-2\to0}$ is the flux from interface $\lambda_{-2}$ to $\lambda_{0}$, and $p(i-1)$ are the probabilities that when at interface $\lambda_{i}$, the system crosses interface $\lambda_{i+1}$ before reverting back to interface $\lambda_{i}$.

The flux $\Phi_{-2\to0}$ is estimated from a simulation run as $\Phi_{-2\to0} = \frac{N_{-2\to0}}{\Delta t_{\text{simulation}}}$, where $N_{-2\to0}$ is the number of successful transitions from states with $Q = -2$ to states with $Q = 0$ observed after simulating the system for $\Delta t_{\text{simulation}}$ time steps. A successful transition is recorded every time the system first visits a state with $Q = 0$ after having occupied one with $Q = -2$. Prior to beginning to sample transitions, the system is equilibrated for 10⁶ time steps. Note that generating $\Phi_{-2\to0}$ at experimentally relevant (low nM) imager concentrations would be inefficient. Instead, we place one imager and one docking strand in a cubic (periodic) box of side length 42.5 nm corresponding to an effective concentration of 21.6 μM. Time spent in hydrogen bonded states is not included in $\Delta t_{\text{simulation}}$.

Subsequently, we evaluate the crossing probabilities of individual interfaces $p(i|j)$. We start by randomly choosing saved trajectories at $\lambda_{i}$ and simulating until we either reach $\lambda_{j}$ (success) or $\lambda_{0}$ (failure), then record the probability of success, $p(i|j)$, as well as the instantaneous configuration on passing through $\lambda_{i}$. Then, we randomly choose from those saved trajectories at $\lambda_{i}$ and simulate until either at $\lambda_{j}$ (success) or $\lambda_{0}$ (failure), saving trajectories at $\lambda_{i}$ as well as the success probability $p(j|i)$. We continue this procedure for the subsequent interfaces $\lambda_{j}$ and $\lambda_{0}$, and finally obtain the imager-docking hybridization rate in Eq. 3.

Details for the number of trials and successful transitions across each interface are summarized in Supplementary Tables 3 and 4.

The on-rates in Fig. 5a are averaged between two simulation repeats of 20,000 transitions through each interface.

The relative hybridization rates of imager strands to each individual binding site on the multi-repeat docking motifs, shown in Supplementary Fig. 1, are extracted from the distribution of terminal states in FFS. Note that the terminal state $Q = 4$ in our reaction coordinate is defined as one in which 9 nucleotide bonds are formed between the imager and docking strand, regardless of which nucleotides are hybridized (in Supplementary Table 2). To determine which one of the binding sites is occupied in a given FFS terminal configuration we therefore analyzed the secondary structure of the FHWM of all terminal configurations. We defined the imager as being bound to a given domain if the majority of the docking nucleotides participating in bonding belonged to that domain. Approximately 20,000 terminal secondary structures were analyzed for the two separate simulation runs.

Concerning precise parameters needed to replicate these simulations: MD timesteps were set to 0.003 oxDNA time units (0.1 femtoseconds) with an oxDNA diffusion coefficient set to 1.25 oxDNA. Major-minor grooving was turned off. Temperature was set to 300 K and the standard oxDNA thermostat used and set to thermalize a fraction of velocities every 51 timesteps.

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

Data availability
All data supporting this study are available upon reasonable request. Source data are provided with this paper.

Code availability
Experimental data was collected using the Python software package PyMe (Python Microscopy Environment), which is available at https://github.com/pyme-microscopy/python-microscopy.37 Simulations were carried out using a modified version of the oxDNA package available at https://github.com/WillTKaufhold1/oxDNA-no-self-bonds.38 Data was analyzed using the Python software package PyMe and the set of plugins available at https://github.com/csoeller/PyMe-extra.29

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**Author contributions**

C.S. and L.D.M. supervised the research progress. Data acquisition was performed by A.H.C., T.L. W.T.K., A.M. and data analysis was performed with contributions from all authors. T.L. prepared and imaged microspheres, W.T.K. made the origami tiles, A.H.C. and T.L. prepared and imaged the origami tiles, A.H.C. and A.M. prepared and imaged biological samples. Simulations and modeling were developed by W.T.K. and L.D.M. The manuscript was written by A.H.C., W.T.K., L.D.M., C.S. and all authors reviewed the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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