

Fluorescence-lifetime encoding for scalable single-protein-resolution imaging

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Fluorescence-lifetime encoding for scalable single-protein-resolution imaging

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ABSTRACT

Resolving individual subunits within protein complexes requires precision beyond DNA-PAINT's ~15 nm limit. RESI reaches this by averaging localisations across orthogonal DNA barcodes, but the imaging rounds needed scale quadratically with subunit number. RE-FLIM reduces this cost threefold by resolving three lifetime-distinct barcodes per round. Validated on DNA origami at 3 nm separations, RE-FLIM provides, to our knowledge, the first protomer-resolved view of HIV-1 Env trimers in intact cell membranes.

33 MAIN

34 Most protein complexes are organized at length scales of 1–20 nm, below the ~15 nm
35 resolution of DNA-PAINT single-molecule localization microscopy (SMLM)¹. Resolution
36 Enhancement by Sequential Imaging (RESI)² overcomes this by distributing identical
37 proteins across orthogonal DNA barcodes imaged sequentially, so that localizations from
38 each barcode can be averaged into a centre of sub-nanometre precision (Fig. 1a).
39 However, as protomer number m increases, the probability that each receives a distinct
40 barcode collapses unless the number of orthogonal sequences N grows quadratically, as
41 $N \propto m(m-1)$ (Fig. 1b). Because RESI images one sequence per round, acquisition time
42 grows with N accordingly, a combinatorial constraint that limits scalability.

43 Fluorescence lifetime offers a route to reading DNA barcodes in parallel. Lifetime-based
44 DNA-PAINT (FL-PAINT) has been used to separate distinct molecular targets within a
45 single acquisition, at conventional SMLM resolution³. Here, we introduce Resolution
46 Enhancement by Fluorescence Lifetime Imaging Microscopy (RE-FLIM), which combines
47 lifetime-based separation with RESI's localization-averaging step to resolve multiple
48 barcodes simultaneously at separations well below the DNA-PAINT resolution limit,
49 without additional laser lines or spectral channels (Fig. 1c).

50 We first validated RE-FLIM on DNA origami containing two pairs of orthogonal docking
51 strands separated by 3.2 nm (Fig. 1d), a distance inaccessible to DNA-PAINT. Cy3B- and
52 Atto 550-labelled imager strands were imaged using a wide-field single-photon-counting
53 camera, achieving localization precision (σ_{SMLM}) ~ 8 nm (Fig. 1e). Lifetime-based species
54 assignment confidently classified 92% of binding events, and centroid localization yielded
55 $\sigma_{\text{RE-FLIM}}$ ~ 1 nm, an eightfold gain in precision over σ_{SMLM} (Fig. 1e, Extended Data 1). First
56 nearest-neighbour distance (NND) analysis recovered the expected 3.2 nm spacing (Fig.
57 1f; 1st-NND = 3.5 nm), matching two-rounds RESI measurements (Extended Data 2 and
58 Supplementary Fig. 1; 1st-NND = 3.3).

59 We next extended RE-FLIM to simultaneous three-barcode imaging using DNA origami
60 bearing three orthogonal docking strands arranged in ~5.0 × 5.6 × 5.6 nm triangles (Fig.
61 1g). Three lifetime-distinct imagers were simultaneously resolved in a single acquisition
62 round, with 92% of binding events assigned to one of the three species.
63 Localizations assigned to each vertex reproduced the expected triangular geometry (Fig.
64 1h, Extended Data 3), with 1st- and 2nd-NND distributions peaking at 6.0 and 7.7 nm,
65 respectively (Fig. 1i), consistent with three-round RESI measurements (Extended Data
66 4 and Supplementary Fig. 2). Thus, RE-FLIM preserves structural accuracy while
67 reducing the required number of imaging rounds threefold.

We then applied RE-FLIM to HXB2 HIV-1 envelope glycoprotein (Env), a homotrimeric spike protein whose GFP_{OPT}-labelled⁴ protomers are separated by 15.3 nm in the closed, prefusion conformation⁵ (Fig. 2a, Supplementary Fig. 3). Expressed in U2OS cells, Env was stochastically labelled with anti-GFP nanobodies bearing one of six orthogonal docking strands. At ~50% labelling efficiency⁶, this yielded trimer, dimer-, and monomer-like configurations (Fig. 2b). Two RE-FLIM rounds, each resolving three lifetime-distinct species simultaneously (Fig. 2c, d), generated six barcode populations (R1–R6).

RE-FLIM resolved individual Env protomers with $\sigma_{\text{RE-FLIM}} \sim 3$ nm, whereas DNA-PAINT could not distinguish protomers within the 15.3 nm trimer (Fig. 2e, f; $\sigma_{\text{SMLM}} \sim 11$ nm). The six recovered barcode populations (Fig. 2g) were assigned to individual centroids (Fig. 2h, Supplementary Fig. 4). 1st- and 2nd-NND distributions skewed shorter than density-matched complete spatial randomness (CSR) simulations, with a dominant 1st-NND peak < 20 nm, revealing short-range nanoscale organization of Env protomers in the plasma membrane (Fig. 2i, Supplementary Fig. 5). Graph-based clustering (25 nm linking radius; Methods) classified centres as trimer, dimer-, and monomer-like (Fig. 2j, k), recovering all three as expected for partially labelled trimers. Pairwise edge lengths pooled across trimer and dimer-like configurations showed a median of 16.3 nm and a mode of 17.2 (Fig. 2l), consistent with the 15.3 nm protomer spacing of closed Env. Across linking radii of 20–40 nm, the Env mode remained confined to 16.0–19.5 nm, whereas the CSR mode tracked the linking radius itself, confirming an intrinsic, radius-independent length scale (Supplementary Fig. 6).

Together, these results establish RE-FLIM as a scalable route to protomer-resolved imaging of native protein complexes at one third of the acquisition rounds required by RESI, providing, to our knowledge, the first such view of individual HIV-1 Env trimers in plasma membranes.

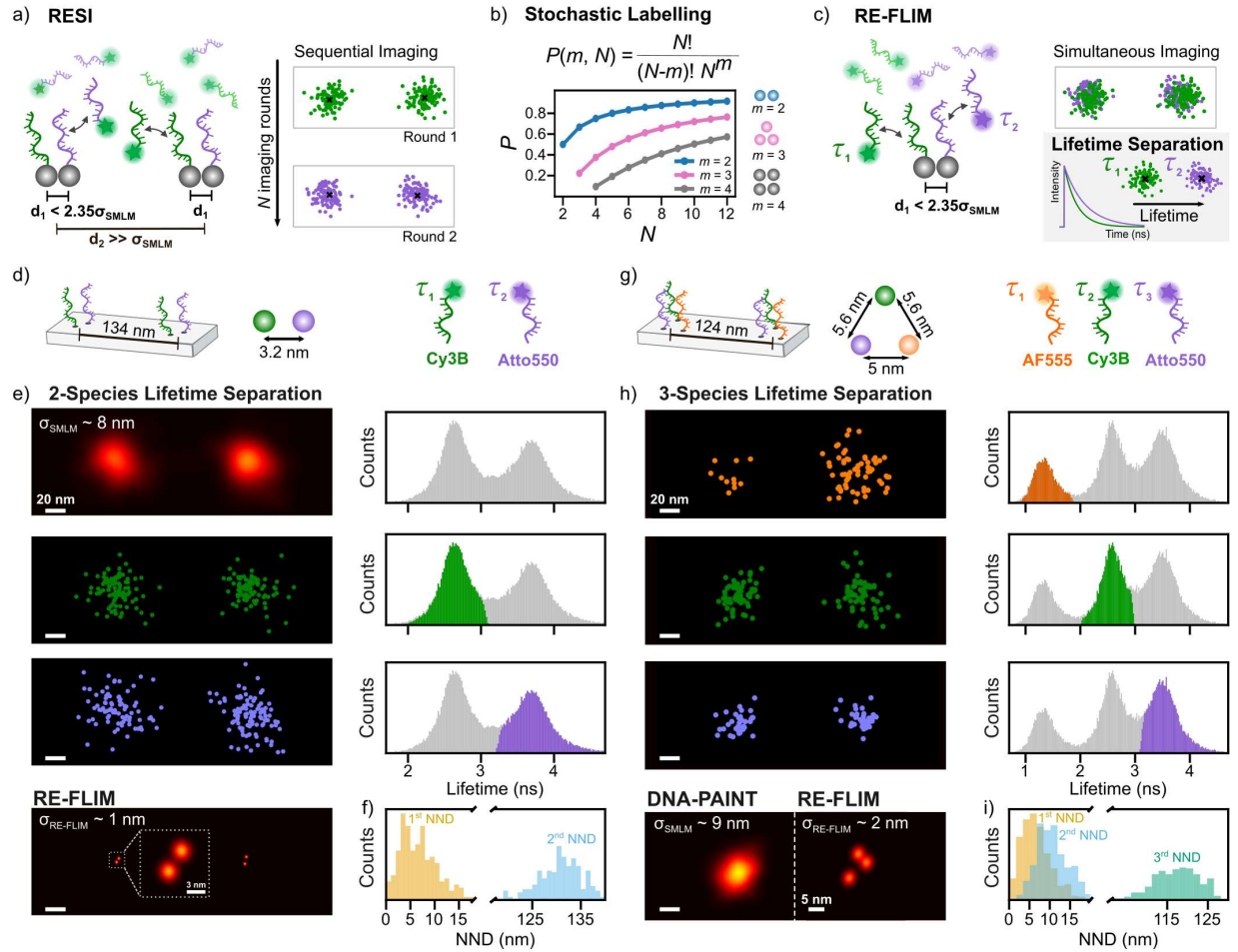


Figure 1. RE-FLIM resolves sub-5 nm separations via simultaneous fluorescence lifetime multiplexing. (a) In RESI, identical proteins are labelled with orthogonal DNA barcodes and imaged sequentially; averaging localizations within each barcode subset yields centres with precision far surpassing DNA-PAINT resolution ($\sim 2.35\sigma_{\text{SMLM}}$). (b) Probability $P(m, N)$ of uniquely assigning m protomers across N imaging rounds decreases rapidly with subunit number, limiting scalability. (c) RE-FLIM adds fluorescence lifetime as an orthogonal encoding axis, enabling simultaneous multi-species discrimination in one acquisition round. (d) Two-species (Cy3B/Atto 550) RE-FLIM on DNA origami with docking-strand pairs separated by 3.2 nm (inter-pair spacing 134 nm). (e) DNA-PAINT image ($\sigma_{\text{SMLM}} \sim 8$ nm) cannot resolve the 3.2 nm intra-pair distance (top); lifetime histograms confirm imager-species separation; RE-FLIM reconstruction ($\sigma_{\text{RE-FLIM}} \sim 1$ nm) resolves both sites (bottom). (f) 1st- and 2nd-NND analysis ($n = 216$ pairs) matches the design: 1st-NND = 3.2 nm; 2nd-NND = 130.5 nm. (g) Three-species (AF555/Cy3B/Atto 550) RE-FLIM on origami with docking-strand triangles ($5.0 \times 5.6 \times 5.6$ nm; inter-trimer spacing 124 nm). (h) Lifetime histograms confirm three-species separation; RE-FLIM ($\sigma_{\text{RE-FLIM}} \sim 2$ nm) resolves all three vertices whereas DNA-PAINT ($\sigma_{\text{SMLM}} \sim 9$ nm) resolves only inter-unit spacing. (i) 1st, 2nd and 3rd NND distributions ($n =$

179 docking triangles) match the design: 1st-NND = 6.0 nm; 2nd-NND = 7.7 nm; 3rd-NND = 118.6 nm.

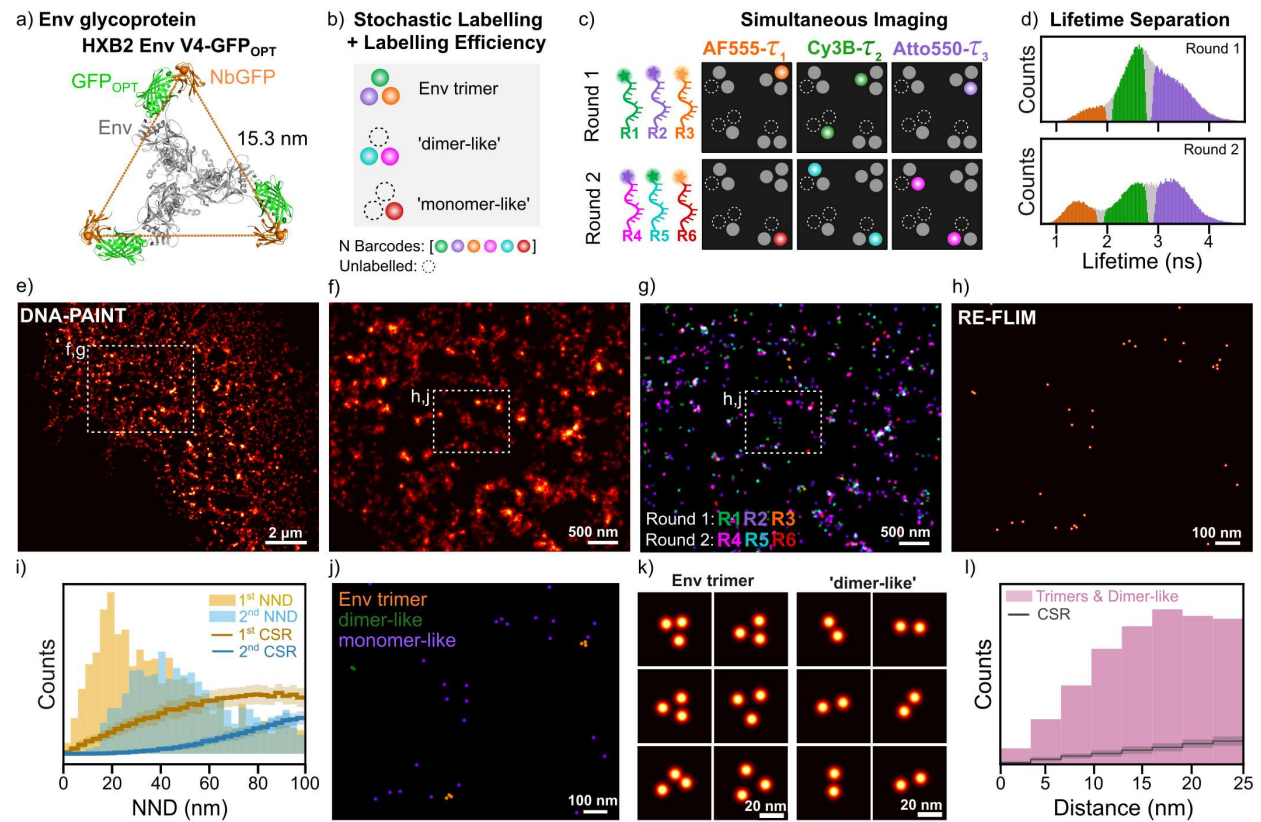


Figure 2. RE-FLIM resolves individual protomers within native HIV-1 Env trimers. (a) AlphaFold model of HXB2 Env-V4-GFP_{OPT}-nanobody superposed on closed Env (PDB: 5T3X), showing 15.3 nm separation between nanobody Cα atoms (Val114) across protomers. (b) Stochastic labelling with six orthogonal nanobody-DNA barcodes at ~50% labelling efficiency yields trimer, dimer- and monomer-like labelled configurations. (c) Two RE-FLIM rounds, each resolving three lifetime-distinct species simultaneously. (d) Lifetime histograms confirm species separation within each round. (e) DNA-PAINT reconstruction of a representative cell ($\sigma_{\text{SMLM}} \sim 11$ nm). (f) Magnified region from (e); unresolved Env clusters. (g) Composite image of six barcode populations across both rounds. (h) Zoomed RE-FLIM reconstruction resolving individual protomers ($\sigma_{\text{RE-FLIM}} \sim 3$ nm). (i) 1st- and 2nd-NND distributions ($n = 3067$ centres) vs. CSR, revealing short-range clustering for a representative FOV. (j) Graph-based classification into trimer, dimer- and monomer-like configurations (25 nm linking radius; Methods). (k) Representative galleries of classified trimer and dimer-like configurations. (l) Pairwise separations within classified trimers ($n = 62$) and dimer-like ($n = 711$) configurations, pooled across 4 FOVs from 3 independent experiments ($n = 897$ distances), showing an Env-specific length scale consistent with the 15.3 nm prediction (median = 16.3 nm, mode = 17.2 nm).

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Contributions

S.S. and S.P.-P. conceived the study and designed the experiments. A.G. performed the RE-FLIM experiments, analysed the data, and developed the RE-FLIM analysis workflow. A.G. and C.Z. developed and implemented the optical setup for RE-FLIM and RESI experiments. R.H. performed preliminary RE-FLIM DNA-origami experiments and developed the event-analysis code. C.Z. and H.H. performed RESI experiments on DNA-origami and HIV-1 Env samples. H.H. prepared the Env samples, using plasmids and reagents generated by D.J.W. and I.C.-A. T.M. performed AlphaFold modelling of Env-GFP-Nb complexes. K.K. and G.P.A. prepared DNA-origami structures. A.G., R.H., C.Z., H.H., and S.S. interpreted the data and wrote the manuscript with input from all authors. All authors reviewed and approved the final manuscript.

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METHODS

DNA origami synthesis

The rectangular DNA origami structures (Fig. 1d and 1g) were designed using CaDNAo2 and it is accessible at <https://nanobase.org/structure/146>. It is modified with six biotin staples going out of the structure for binding to the surface. For the DNA origami with edge pairs separated by 3 nm (Fig. 1d) 2 pairs of DNA PAINT docking strands (TTTCCTCCTCCTCCTCCTCCT and CAACACACACACACACACA) were used. For the DNA origami featuring edge triangles (Fig. 1g) 3 DNA docking strands (TTTCCTCCTCCTCCTCCTCCT, AAACCACCACCACCACCA and TCCTCTCTCTCTCTCTCTC) were used. The origami design is based on a 7249-nucleotide long scaffold extracted from the M13mp18 bacteriophage (Tilbit Nanosystems GmbH) and folded into the desired shape using 243 staples folded in 1× TAE (Alfa Aesar, #J63931) and 12 mM MgCl₂ (Alfa Aesar, #J61014) buffer. It was mixed in a 10-fold excess of staples over scaffold, and 100-fold for modified staples. is based on a 7249-nucleotide long scaffold extracted from the M13mp18 bacteriophage (Tilbit Nanosystems GmbH) and folded into the desired shape using 243 staples folded in 1× TAE (Alfa Aesar, #J63931) and 12 mM MgCl₂ (Alfa Aesar, #J61014) buffer. It was mixed in a 10-fold excess of staples over scaffold, and 100-fold for modified staples.

Unmodified staple strands were purchased from IDT; biotin-functionalized staples and DNA-PAINT docking strands were purchased from Biomers GmbH. The scaffold and the staple mix were self-assembled into the designed structure using a temperature ramp. The combination was initially heated to 95 °C, where it remained for 5 min, before cooling

to 20 °C during a 19 h linear ramp. A 1% (w/v) agarose gel electrophoresis was used as a purification procedure to remove excess staple strands. In a 1× TAE 12 mM MgCl₂ buffer, the gel was run at 70 V for 3 h in an ice-water bath. After electrophoresis, the pure DNA origami structures were extracted by cutting out the bands in the gel containing the DNA origami structure and squeezing them out using a parafilm-wrapped glass slide. The final concentration of the DNA origami structures was determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). The list of modified staples used can be found in Supplementary Table 2 and Supplementary Table 3.

Buffers

Buffer A+: 10 mM Tris (VWR), 100 mM NaCl (Sigma-Aldrich) and 0.05% (v/v) Tween-20 (Sigma-Aldrich), adjusted to pH 8.0.

Buffer B+: 10 mM MgCl₂ (LifeTech), 5 mM Tris-HCl pH 8.0, 1 mM EDTA (LifeTech) and 0.05% (v/v) Tween-20 (Sigma-Aldrich) pH 8.0, supplemented with 1× Trolox.

Buffer C+: 1× PBS, 1 mM EDTA (Invitrogen), 500 mM NaCl (Sigma-Aldrich) and 0.02% (v/v) Tween-20 (Sigma-Aldrich), adjusted to pH 7.4. Where indicated, C+ buffer was supplemented either with 1× Trolox alone or with an oxygen-scavenging system comprising 1× Trolox, 1× protocatechuic acid (PCA) and 1× protocatechuate 3,4-dioxygenase (PCD).

Oxygen-scavenging system

The oxygen-scavenging system consisted of 1× PCA (prepared from a 40× stock), 1× PCD (prepared from a 100× stock) and 1× Trolox (prepared from a 100× stock). The imaging buffer was prepared in 1× PBS + 500 mM NaCl buffer immediately before use and incubated in the dark for 1 h before imaging.

The 40× PCA stock solution was prepared by dissolving 154 mg PCA (Sigma-Aldrich) in 10 ml distilled water and adjusting the pH to 9.0 with NaOH (Avantor). The 100× PCD stock solution was prepared by dissolving 2.2 mg PCD (Sigma-Aldrich) in 3.4 ml of 50% glycerol containing 50 mM KCl, 1 mM EDTA and 100 mM Tris buffer. The 100× Trolox stock solution was prepared by dissolving 100 mg Trolox (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; Sigma-Aldrich) in 0.43 ml methanol, 0.345 ml 1 M NaOH and 3.2 ml distilled water.

DNA-origami sample preparation

For sample immobilization, commercial chambers (IB-80607, µ-Slide VI 0.5 Glass Bottom, Sterile) were used. Chambers were washed 3 times with buffer A+, then the surface was passivated with a solution of BSA-biotin (1 mg mL⁻¹, A4503-10G, Sigma-Aldrich) in buffer A+ for 5 minutes at room temperature. After 3 washes with buffer A+, slides were incubated with a solution of neutravidin (1 mg mL⁻¹, 31000, Thermo Fisher Scientific) in

buffer A+ and incubated for 5 min at room temperature. After 1 wash with buffer A+ and 2 washes with buffer B+, 100 pM of DNA-origami structure was added to the chamber and incubated for 10 min for immobilization via biotin binding to the functionalized surface. The sample was washed again 3 times with buffer B+ and 100 μ l of gold nanoparticles (90 nm, G-90-100, Cytodiagnostics) was flushed through and incubated for 5 min before washing with buffer B+. Imaging was performed after the addition of the corresponding DNA imager solution.

For RESI imaging, DNA imagers carrying a 3'-Cy3B fluorophore were sequentially exchanged in buffer B+ supplemented with 1 $\times$ Trolox, 1 $\times$ PCA and 1 $\times$ PCD. For the 3 nm dimer origami, R1 and R4 imagers were used at concentrations of 0.5 nM and 1 nM, respectively. For the three-site triangle origami, R1, R2 and R3 imagers were used at concentrations of 1 nM each.

For RE-FLIM imaging, multiple DNA imager species were simultaneously introduced in each acquisition round to enable lifetime-based species separation. For the two-site dimer origami, R1 and R4 docking strands were imaged using Cy3B- and ATTO550-labelled imagers, respectively, at concentrations of 1.5 nM each. For the three-site triangle origami, R1, R2 and R3 were simultaneously imaged using Cy3B-, ATTO550- and Alexa Fluor 555-labelled imagers at concentrations of 1 nM, 1 nM and 7 nM, respectively. All RE-FLIM imager solutions were prepared in buffer B+ supplemented with 1 $\times$ Trolox.

Cell Culture

U2OS cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; ThermoFisher, 21041025) supplemented with 10% fetal bovine serum (FBS). Cells were maintained at 1 $\times$ 10⁵ cells/ml and passaged every 3 days using Accutase (Promo Cell, C-41310).

HIV-1 Env-GFP expression in U2OS cells

2 $\times$ 10⁴ cells were seeded per well into 8-well Ibidi μ -slides (IB-80807) and incubated for 16 h prior to transfection. Cells were transfected with 80 ng of pCMV-HXB2-Env-v4.1-GFP_{OPT} (a derivative of super folded GFP introduced in the v4 loop of HXB2 Env, as described previously)⁴, using GeneJuice (Merck, 70967) according to the manufacturer's instructions in Opti-MEM reduced serum medium (Thermo Fisher Scientific, 11058021). Following transfection cells were incubated for 24 h in DMEM/F-12 supplemented with 10% FBS.

DNA-PAINT labelling of HIV-1 Env-GFP

Cells expressing HIV-1 Env-GFP were fixed with 2% paraformaldehyde (PFA) in PBS for 15 mins, washed three times with PBS, and blocked with 5% bovine serum albumin (BSA) containing 100 μ g/ml sheared salmon sperm DNA (15632011, Thermo Fisher Scientific).

Samples were then stained with a mixture of six DNA-conjugated anti-GFP nanobody probes (37.5 nM total; Massive-sdAB-FAST 2-Plex, Massive Photonics GmbH), in which the same anti-GFP nanobody was functionalized with one of six orthogonal DNA docking strands (R1–R6; 6.25 nM each) in 5% BSA. Following labelling, samples were washed three times with PBS and post-fixed with 4% PFA for 5 min. Residual autofluorescence was quenched by washing with 60 mM glycine in PBS. Finally, 90 nm gold nanoparticle fiducials (Cytodiagnostics, G-90-100) were added, followed by two washes with PBS and one wash with C+ buffer prior to imaging.

For RESI imaging, DNA imager strands carrying a 3'-Cy3B fluorophore were diluted in C+ imaging buffer supplemented with 1× Trolox, 1× PCA and 1× PCD. Imager concentrations were 1.5 nM for R1 and R2, 3 nM for R3-R5 and 6 nM for R6. Six sequential exchange rounds were performed, with a single orthogonal imager strand present during each round. Between rounds, the imaging chamber was washed thoroughly with C+ buffer before addition of the subsequent imager solution.

For RE-FLIM imaging, six orthogonal docking strands were imaged in two exchange rounds, each simultaneously containing three lifetime-distinct DNA imager species. In the first round, R1, R2 and R3 were imaged using AF555-, Cy3B- and ATTO550-labelled imager strands, respectively. In the second round, R4, R5 and R6 were imaged using ATTO550-, Cy3B- and AF555-labelled imager strands, respectively. All imager strands carried a 3' fluorophore and were diluted in C+ imaging buffer supplemented with 1× Trolox. In the first exchange round, R1 (Cy3B), R2 (ATTO550) and R3 (Alexa Fluor 555) imager strands were added at concentrations of 1.5 nM, 0.5 nM and 3 nM, respectively. In the second exchange round, R4 (ATTO550), R5 (Cy3B) and R6 (Alexa Fluor 555) imager strands were added at concentrations of 1.5 nM, 0.5 nM and 3 nM, respectively. Between exchange rounds, the imaging chamber was washed thoroughly with C+ buffer before addition of the subsequent imager mixture.

Microscopy setups

DNA-PAINT/RESI microscopy setup

DNA-PAINT and RESI imaging were performed on a custom built wide-field total internal reflection fluorescence (TIRF) microscope based on a Nikon Eclipse Ti-2 inverted microscope equipped with a 100× oil immersion TIRF objective (Apo TIRF, NA 1.49) and a Perfect Focus System. TIRF illumination was provided by a continuous wave 561 nm laser (MPB Communications, 1 W). The laser beam was spatially filtered using a 50 μm pinhole, circularly polarized using a polarizer and quarter-wave plate, and expanded using a variable beam expander (Thorlabs) to a 1/e² beam diameter of 6 mm. The beam was then passed through a beam-shaping element (piShaper 6_6_VIS, AdlOptica, Berlin,

Germany) to generate a collimated flat-top illumination profile before further expansion with a custom-built telescope to define the field of view at the sample. The excitation beam was focused on the back focal plane of the objective, passed through an excitation filter (FF01-390/482/563/640-25, Semrock), and coupled into the objective using a beam splitter (Di03-R405/488/561/635, Semrock). Fluorescence emission was filtered using an emission filter (FF01-446/523/600/677-25, Semrock) and detected using an sCMOS camera (ORCA-Fusion BT, Hamamatsu). Images were acquired with 2×2 camera binning, resulting in an effective pixel size of 130 nm in the sample plane.

The 561 nm excitation laser was operated at a measured power of 4.5 mW after the objective, corresponding to a power density of 470 W cm^{-2} at the sample. All RESI datasets were acquired with 100 ms exposure times. DNA origami datasets comprised 20,000 frames per exchange round for the 3 nm dimer design and 15,000 frames for the 5.5 nm triangle design, whereas HIV-1 Env datasets comprised 20,000 frames per exchange round. Samples were washed with at least 2 ml of C+ buffer between exchange rounds before addition of the subsequent DNA imager solution.

RE-FLIM microscopy setup

RE-FLIM imaging was performed on a custom-built wide-field total internal reflection fluorescence (TIRF) microscope based on a Nikon Eclipse Ti-2 inverted microscope equipped with a 100 $\times$ oil immersion TIRF objective (Apo TIRF, NA 1.49) and the Perfect Focus System. Excitation was provided by a 532 nm pulsed diode laser (LDH-P-FA-530XL, PicoQuant) operated using a Sepia II laser driver and controller (PicoQuant). The laser beam was passed through a cleanup filter (FF01-532/3, Semrock), directed through a periscope, and focused on the back focal plane of the objective using a multiband dichroic mirror (ZT405/488/532/640rpc-XT, Chroma). Fluorescence emission was filtered using a quad-band emission filter (FF01-446/510/581/703, Semrock) and detected using a wide-field time-correlated single-photon counting (TCSPC) camera (LINCam, Photonscore), which records the position and arrival time of each detected photon. Images were acquired through the microscope's 1.5 $\times$ magnification and an additional 4 $\times$ magnification adapter (VM4X, Nikon), resulting in a total magnification of 600 $\times$.

The 532 nm laser was operated at a repetition rate of 40 MHz with a measured power of 0.5 mW after the objective, corresponding to a power density of 141 W cm^{-2} at the sample. Photon events were recorded continuously using the LINCam acquisition software (Photonscore). Each detected photon was stored together with its detector coordinates and arrival time relative to the excitation pulse. Photon arrival times were digitized into 10 ps time bins and detector positions assigned to a 4096×4096 spatial grid spanning the 17 mm active detector area, corresponding to an effective sampling of 7.2 nm in the sample plane at 600 $\times$ magnification.

For RE-FLIM imaging of DNA origamis, photon events were acquired continuously for ~ 40 min. For RE-FLIM imaging of HIV-1 Env, two sequential exchange rounds were acquired, each consisting of ~ 30 min continuous photon acquisition.

RE-FLIM data analysis

RE-FLIM data analysis consisted of sequential photon preprocessing, preliminary single-molecule localization, DNA-PAINT binding event identification, fluorescence lifetime-based species assignment, and RESI-style cluster reconstruction. Raw photon data were processed using custom MATLAB and Python scripts, with localization and clustering performed using the Picasso software package.

Photon preprocessing and preliminary localization

The LINCam acquisition software (Photonscore) acquires photons continuously, recording their detector position and photon arrival time information. Raw photon data were processed using custom MATLAB scripts to generate two-dimensional intensity frames by applying 16× spatial binning, resulting in an effective pixel size of 115 nm. Frames were generated using a sliding integration window, with 200 ms integration frames produced every 20 ms, yielding a 10× temporal oversampling that increases sampling while retaining sufficient photons per frame for single-molecule localization. In parallel, photon arrival times were indexed according to acquisition time, preserving the temporal information of the original photon stream for subsequent event-based analysis. The reconstructed intensity frames were localized using the Picasso software package¹ to obtain preliminary single-molecule positions. Drift correction was performed using adaptive intersection maximization, and outlier localizations were removed prior to downstream analysis.

DNA-PAINT binding event identification and lifetime estimation

Preliminary localizations were used to define spatial search regions for photon assignments. DNA-PAINT-resolved structures were grouped prior to event analysis. DNA origami structures were manually selected from reconstructed images, whereas HIV-1 Env regions were identified using the Exchange-PAINT-specific workflow described below. Each selected region was processed using a custom Python-based event analysis pipeline (Supplementary Note 1).

Briefly, localizations appearing in consecutive overlapping frames were linked based on spatial proximity to generate candidate DNA-PAINT binding events. Event boundaries were subsequently refined by change-point detection applied to the corresponding photon intensity traces, allowing accurate determination of binding onset and termination times. Photons falling within the spatial and temporal boundaries of each event were retrieved

from the indexed photon stream. Event positions were estimated by calculating the centre of mass within a 4.5-pixel-diameter region (1 pixel = 115 nm). The localization precision of each binding event (σ_{SMLM}) was calculated as the standard error of the mean of the detected photon coordinates. Fluorescence lifetime was estimated from the Gaussian-weighted mean photon arrival time within a 5.5-pixel-diameter region. Lifetime values were referenced to the peak of the photon arrival-time distribution, which was determined once for each dataset.

For HIV-1 Env Exchange-PAINT datasets, exchange rounds were first aligned in Picasso prior to event analysis. To identify membrane regions consistently detected across exchange rounds, localizations from the first round were matched to those from the second round using a 40 nm nearest-neighbour distance criterion, approximately twice the localization precision of the preliminary frame-based localizations. Only Env regions detected in both exchange rounds were retained for subsequent analysis. These matched localizations were subsequently grouped into individual Env regions using DBSCAN clustering with a radius of 40 nm and a minimum cluster size of 100 localizations in the oversampled preliminary localization file (10× temporal oversampling from the 200 ms/20 ms sliding-window frame generation, see Supplementary Note 1). The resulting clusters defined the ROIs for independent event analysis of each exchange round using the RE-FLIM event-analysis pipeline.

Lifetime-based species separation

DNA-PAINT binding events were assigned to individual imager species based on their measured fluorescence lifetime and associated uncertainties. Reference lifetimes values for each species were obtained from independent single-species measurements and refined for each dataset using an expectation-maximization procedure weighted by the inverse square of the lifetime uncertainty to account for experiment-to-experiment variation. For each event, the distance to each reference lifetime was calculated in units of the event-specific lifetime uncertainty. For DNA origami datasets, events with fewer than 50 detected photons were excluded, and the remaining events were assigned to the closest lifetime species when the measured lifetime fell within 2.5 uncertainty units of that species and the corresponding standardized distance was at least 0.5 lower than that to the next closest species. For HIV-1 Env datasets, where the lifetime distributions are broader and exhibit greater overlap, reference lifetimes were refined from the 20% of events with the lowest lifetime uncertainty. In both cases, events not meeting these criteria were classified as unassigned and excluded from the classified set. For Exchange-PAINT experiments, lifetime classification was performed independently for each exchange round, with three imager species analysed per round.

RESI cluster analysis

Lifetime-assigned localizations (RE-FLIM) and sequential-round localizations (RESI) were processed using the Picasso SMLM clusterer following the RESI workflow². For RE-FLIM, clustering was performed independently for each lifetime-assigned species; for RESI, independently for each sequential imaging round.

The clustering radius was selected based on the localization precision of each dataset (~2.5 times the mode of the localization precision distribution), with a minimum of 10 localizations required per cluster. Cluster centres were calculated as the mean position of all localizations assigned to each cluster. The RE-FLIM or RESI localization precision ($\sigma_{\text{RE-FLIM}}$ or σ_{RESI}) was defined as the standard error of the mean of clustered localizations. For each species, $\sigma_{\text{RE-FLIM}}$ and σ_{RESI} values were reported as the modes of their corresponding localization precision distributions.

Nearest-neighbour distance analysis

Inter-site distances were quantified by nearest-neighbour distance (NND) analysis of RE-FLIM or RESI cluster centres. For DNA-origami datasets, individual structures were manually grouped in Picasso, and only structures containing the expected number of resolved sites were included in the analysis. Nearest-neighbour distances between cluster centres were calculated within each structure. Distance distributions were generated using bin widths comparable to the localization precision of the corresponding dataset (i.e., RE-FLIM or RESI).

Structural modelling of Env-GFP–nanobody complexes

The binary complex of the HXB2 Env-V4-GFPopt fusion construct and an anti-GFP nanobody was modelled using the AlphaFold 3 (AF3) web server (PMID: 38718835). Sixteen predicted N-linked glycosylation sites on Env were predicted using the the NetNGlyc 1.0 web server and modelled as branched glycans using AF3 (code NAG(NAG(MAN(MAN(MAN)(MAN(MAN)(MAN))))) (PMID:11928486). The amino acid sequence of the anti-GFP nanobody from PDB ID 3OGO was used for modelling (PMID: 20945358). Three binary Env-GFPopt–nanobody models were superposed onto the HIV-1 Env trimers in the 5T3X (PMID: 27617431) closed and 5VN3 (PMID: 28700571) open conformations. The α helix of Env (Met100–Leu116), proximal to the threefold symmetry axis, was used for superposition. Distances between selected C α atoms (Val114 of NbGFP, Fig. 2a, Supplementary Fig. 3) were measured, and structures were visualised using the PyMOL Molecular Graphics System, v. 3.0 (Schrödinger, LLC). Glycans, the nanobody His6-tag, and unresolved N- and C-terminal regions are not shown for clarity.

Graph-based classification of Env labelling configurations

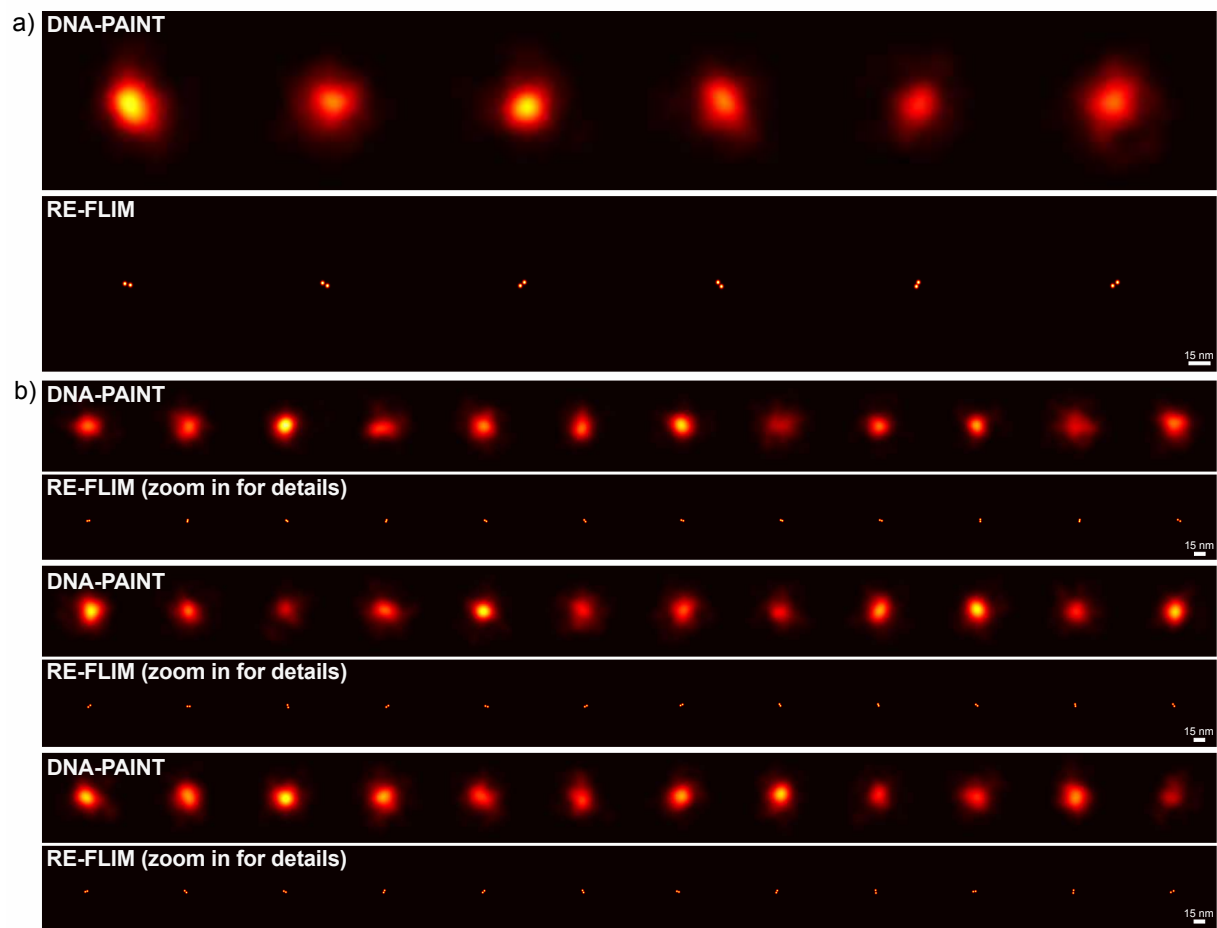
Env-associated cluster centres obtained from RE-FLIM cluster analysis were used to construct an undirected graph in which each centre was represented as a node, and an

edge was drawn between any two centres separated by less than a defined linking radius. Connected components of the resulting graph were classified as trimer (three centres, fully pairwise-connected, forming a closed triangle), dimer-like (two centres joined by a single edge), or monomer-like (an isolated, unconnected centre). Connected components comprising three centres that were not fully pairwise-connected (i.e., open chains, in which one of the three possible edges exceeded the linking radius) were excluded from the trimer classification. Connected components of four or more centres were searched for closed triangles: where the triangles found were mutually disjoint, each was classified as trimer and the remaining centres of that component were left unassigned, whereas components in which a centre belonged to more than one triangle, or which contained more than ten centres, were excluded. This step was included because a genuine trimer with an unrelated centre falling within the linking radius would otherwise be discarded as part of a larger component, preferentially in regions of higher density. The same rule applied to CSR simulations recovered no additional triangles, confirming that the step does not generate spurious classifications.

The linking radius was set to 25 nm, corresponding to the expected inter-protomer spacing of the closed Env conformation (15.3 nm) plus twice the combined pairwise localisation uncertainty ($2 \times \sqrt{2} \times \sigma_{\text{RE-FLIM}} \approx 8.5$ nm, for $\sigma_{\text{RE-FLIM}} = 3$ nm), rounded up from 23.8 nm. Assuming Gaussian localisation error in each coordinate, this captures ~98% of true neighbouring protomer pairs. Classification specificity was confirmed against density-matched complete spatial randomness (CSR) simulations analysed through the identical pipeline: at a 25 nm linking radius, CSR yielded on average 1 closed triangles per simulated dataset, compared with 62 in the data (Supplementary Table 3). Within-group distances were quantified by pooling all pairwise separations from dimer-like and trimer groups. Every separation is bounded by the linking radius by construction, so within-group distance distributions were compared against density-matched CSR simulations at matched linking radii throughout (Supplementary Fig. 6, Supplementary Table 3).

Code availability

Picasso software is available via GitHub at <https://github.com/jungmannlab/picasso>. Event-based analysis code is accessible at <https://github.com/Simoncelli-lab/photonize>.



516

517 **Extended Data 1. RE-FLIM resolves 3.2 nm separations across the full field of view.**

518 Representative 3.2 nm-separated docking-strand pairs from DNA origami structures

519 imaged by wide-field TIRF fluorescence lifetime microscopy. **(a)** Magnified view of six

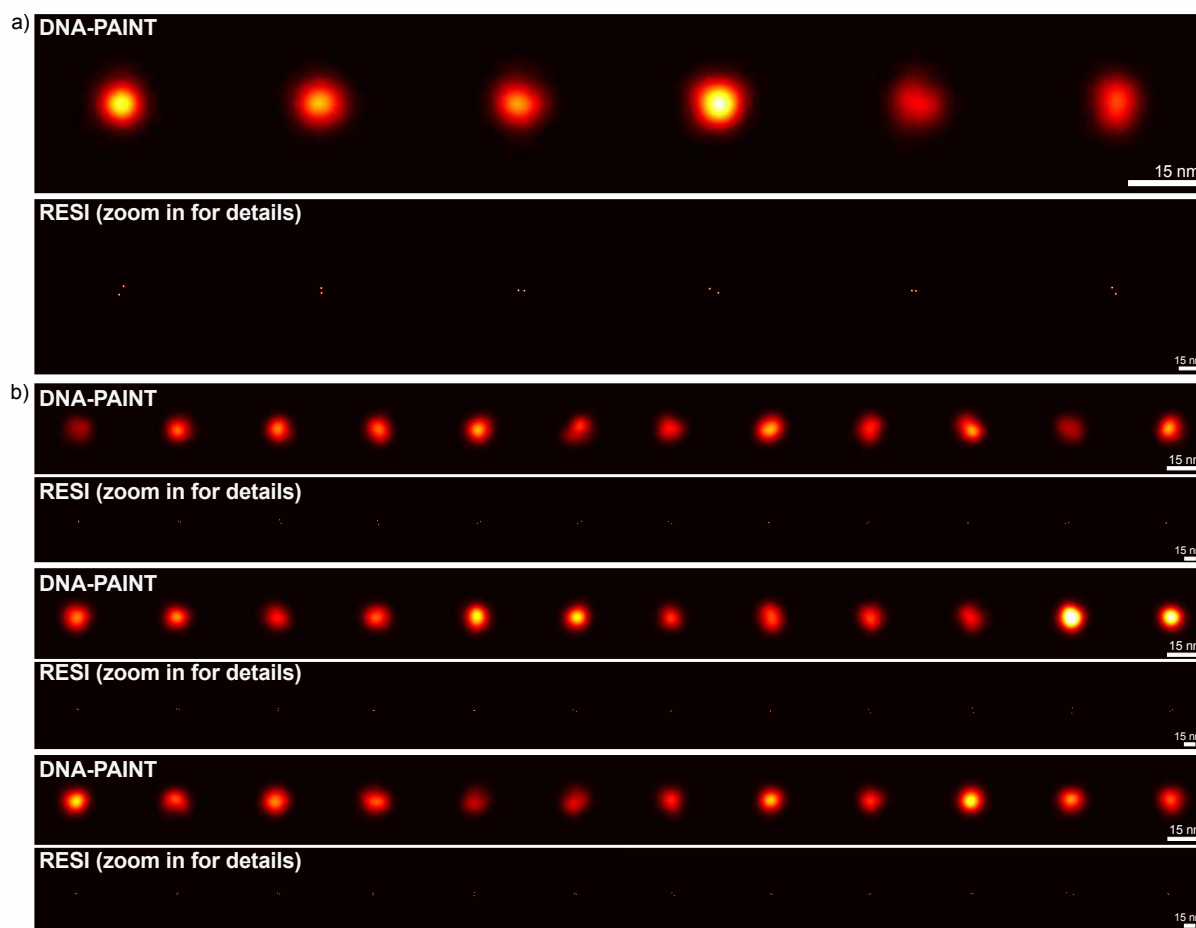
520 representative docking-strand pairs shown at DNA-PAINT resolution (upper row) and RE-

521 FLIM resolution (lower row). **(b)** Additional docking-strand pairs sampled from across the

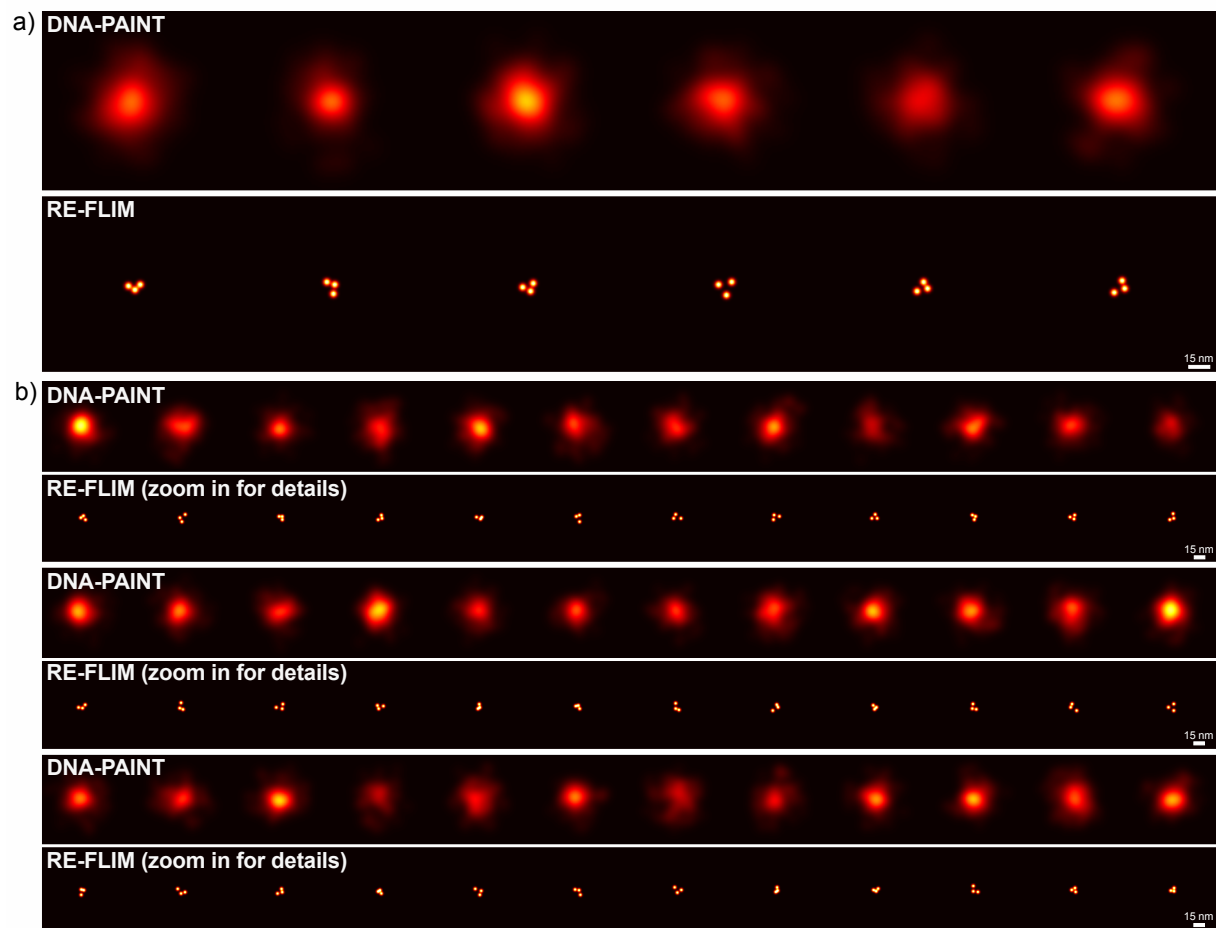
522 field of view, shown at DNA-PAINT resolution (upper rows) and RE-FLIM resolution (lower

523 rows), demonstrating uniform resolving performance across the imaging area. Scale bars,

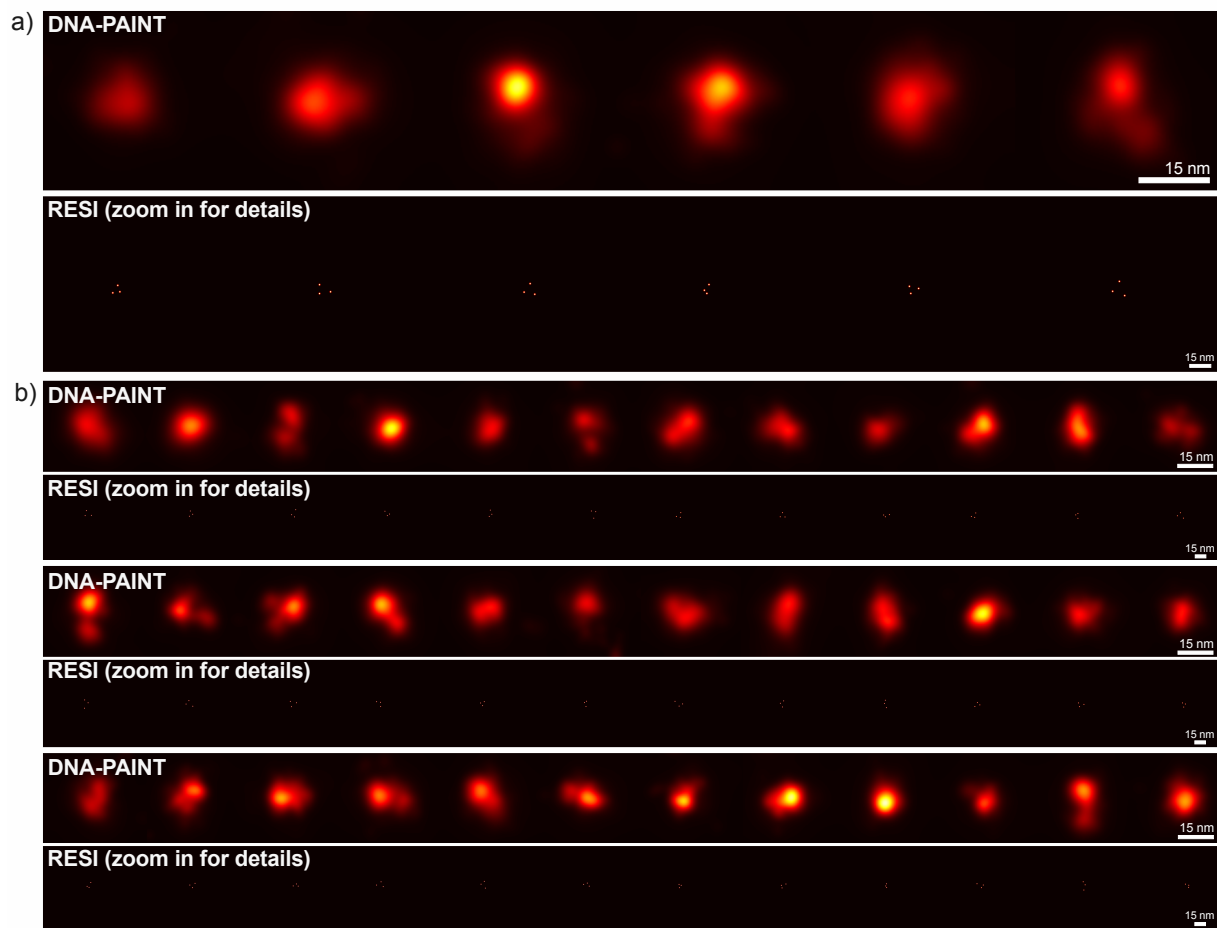
524 15 nm.



Extended Data 2. RESI resolves 3.2 nm separations across the full field of view consistent with RE-FLIM. Representative 3.2 nm-separated docking-strand pairs from DNA origami structures imaged by TIRF microscopy using an sCMOS camera. **(a)** Magnified view of representative docking-strand pairs shown at DNA-PAINT resolution (upper row) and RESI resolution (lower row). **(b)** Additional docking-strand pairs sampled from across the field of view, shown at DNA-PAINT resolution (upper rows) and RESI resolution (lower rows). Scale bars, 15 nm.



Extended Data 3. RE-FLIM resolves ~5.5 nm trimeric arrangements across the full field of view. Representative $5.0 \times 5.6 \times 5.6$ nm spaced docking-strand trimers from DNA origami structures imaged by wide-field TIRF fluorescence lifetime microscopy. (a) Magnified views of representative docking-strand trimers shown at DNA-PAINT resolution (upper row) and RE-FLIM resolution (lower row). RE-FLIM resolves the three vertices of each designed trimer, whereas DNA-PAINT does not resolve the sub-6 nm intra-trimer arrangement. (b) Additional docking-strand trimers sampled from across the field of view, shown at DNA-PAINT resolution (upper rows) and RE-FLIM resolution (lower rows), demonstrating consistent recovery of the designed trimer geometry throughout the imaging area. Scale bars, 15 nm.



544

545 **Extended Data 4. RESI resolves ~5.5 nm trimeric arrangements across the full**
 546 **field of view consistent with RE-FLIM.** Representative $\sim 5.0 \times 5.6 \times 5.6$ nm spaced
 547 docking-strand trimers from DNA origami structures imaged by TIRF microscopy using
 548 an sCMOS camera. (a) Magnified views of representative docking-strand trimers shown
 549 at DNA-PAINT resolution (upper row) and RESI resolution (lower row). (b) Additional
 550 docking-strand trimers sampled from across the field of view, shown at DNA-PAINT
 551 resolution (upper rows) and RESI resolution (lower rows). Scale bars, 15 nm.

552

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [ExtendedData1.pdf](#)
- [ExtendedData2.pdf](#)
- [ExtendedData3.pdf](#)
- [ExtendedData4.pdf](#)
- [SupplementaryInformationGentilietal.pdf](#)