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Article

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Generalized photoactivatable fluorescent probes enable background-suppressed 3D single-molecule imaging in hydrogels

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Abstract

Compared with the broad range of regular fluorophores, photoactivatable (PA) fluorophores remain limited in choice, partly due to their commercial availability, complex synthesis routes, and in some case suboptimal photophysical and photochemical properties. Here, we present a type of generalized and modular PA fluorescent probes using DNA as scaffolds. These probes are readily accessible, flexible with fluorophore cores and attachment moieties, and have an improved performance than some existing PA fluorophores. With the probes, we implement 3D single-molecule imaging in hydrogels, overcoming the “concentration barrier” and observing single enzymatic reactions and DNA hybridization events in real time. These versatile PA probes could facilitate a wider adoption of many single-molecule imaging techniques.

Keywords

photoactivatable fluorescent probes; confocal photoactivation, diffusion, and excitation (PhADE); 3D single-molecule imaging; hydrogels; concentration barrier

Introduction

Photoactivatable (PA) probes are indispensable reagents for many single-molecule and super-resolution imaging techniques, where precise and efficient spatiotemporal control of fluorescence emission is the key to achieve the full capability of these methods.¹⁻⁸ For instance, PA probes have been used to overcome the long-standing “concentration barrier” in single-molecule fluorescence imaging.^{9, 10} Single-molecule imaging techniques typically require nanomolar or lower concentrations of fluorescently labeled species in solution, because at higher concentrations, the background fluorescence from diffusing molecules overwhelms single-molecule signals, resulting in a reduced signal-to-noise ratio (SNR).¹⁰ This concentration range is insufficient for weak and transient biomolecular interactions with dissociation constants in the high-nanomolar to micromolar range.⁹ Existing strategies to overcome this barrier can be broadly divided into two categories. The first group relies on spatial separation, in which the effective excitation volume is reduced to minimize background fluorescence. Representative techniques include total internal reflection fluorescence (TIRF) microscopy,¹¹ zero-mode waveguides (ZMWs),¹² self-assembled nanoantennas,¹³ convex-lens induced confinement (CLiC) microscopy,¹⁴ single-molecule photoactivation Förster resonance energy transfer (PAFRET),¹⁵ and stimulated emission depletion (STED) microscopy,¹⁶ most of which confine fluorescence detection to surface-proximal geometries or require sophisticated instruments. In contrast, temporal separation techniques, as exemplified by the photoactivation, diffusion, and excitation (PhADE) strategy,¹ exploit PA probes to ensure that only bound molecules emit fluorescence, while unbound molecules diffuse away to avoid contributing to the background fluorescence. Although initially demonstrated with TIRF microscopy in 2D imaging, PhADE, in principle, can be extended to 3D imaging if combined with confocal or light-sheet microscopy.

In addition, PA probes have become central to modern super-resolution microscopy, such as (fluorescence) photoactivated localization microscopy [(f)PALM],^{2, 3} single-particle tracking PALM (sptPALM),⁴ patterned activation nonlinear structured illumination microscopy (PA NL-SIM),⁵ and minimal photon FLUX (MINFLUX).⁶ Commonly in these techniques, only a sparse subset of PA probes are activated at any given time, so that they can be localized or tracked beyond the diffraction limit. In comparison, the application of PA probes in DNA point accumulation for imaging in nanoscale topography (DNA-PAINT)¹⁷ is relatively less explored.¹⁸ Recently, DNA-PAINT has been combined with hydrogel-based imprinting and clearing.¹⁹ However, inefficient hybridization within dense hydrogel matrices necessitates the use of substantially higher imager strand concentrations, leading to elevated background fluorescence that might affect localization accuracy. In this context, PA DNA-PAINT,¹⁸ or alternatively FRET DNA-PAINT²⁰ and fluorogenic DNA-PAINT,²¹⁻²³ together with the use of buffer conditions that facilitate hybridization,²⁴ could offer a compelling solution by enabling high concentrations of imager strands and improving hybridization efficiency without introducing a high background fluorescence.

Consequently, the development of high-performance PA probes has been of keen interest in the field. PA probes are broadly categorized into fluorescent proteins and synthetic fluorophores. While fluorescent proteins can be genetically encoded,^{25, 26} synthetic fluorophores are generally brighter and more photostable,^{18, 27-32} making them particularly attractive for demanding imaging applications. Among the synthetic PA probes, rhodamine-based fluorophores represent one of the most extensively explored classes, offering a color palette covering the visible spectrum through structural tuning.^{28, 29, 31} However, rhodamine-based fluorophores, including the PA JF series, can

exhibit certain photophysical artifacts, such as blinking,³³ which may complicate quantitative image analysis, necessitating the development of other classes of synthetic PA probes. A modular design strategy has been developed in which a fluorophore is covalently linked to a quencher through a photolabile linker, allowing fluorescence “off-on” control on various fluorophore cores.^{27, 31, 32} Though powerful and versatile, most synthetic PA probes require relatively complex organic synthesis routes, making them hard to access unless commercially available, and limiting their wider applications in biophysical methods that rely on photoactivation.

In this study, we generated a type of modular, easily accessible, and versatile PA probes, named the fluorophore quencher DNA (FQD) probes. Similar to other DNA-scaffolded fluorescent probes (e.g., FluoroCubes),³⁴ the fluorescence, quenching, and attachment moieties of FQD are organized through DNA structures, and therefore are functionally decoupled, so that each part can be individually changed. Our photoactivation experiments both in bulk or at the single-molecule level show that FQD has an excellent on-off intensity ratio and can be switched on with mild UV illumination at an efficiency surpassing some commercially available PA fluorophore. With FQD, we implemented the PhADE method in combination with confocal microscopy (confocal PhADE) and demonstrated the 3D real-time observation of single rolling circle amplification (RCA) reactions and DNA hybridization events in hydrogels, even in the presence of high concentrations of labeled probes. We anticipate that FQD together with the methods developed around it could be broadly adopted for understanding important biological mechanisms at the single-molecule level.

Results

We designed two types of FQD that are bimolecular (FQD₂) and unimolecular (FQD₁) in structures. FQD₂ is comprised of two complementary oligo strands (**Fig. 1a**), in which one strand carries the fluorophore, e.g., Alexa Fluor 488, Alexa Fluor 594, and Alexa Fluor 647 (AF488, AF594, and AF647, respectively), and the other carries the photocleavable linker (PC Linker) and the quencher, e.g., Black Hole Quencher 2 (BHQ2). Native polyacrylamide gel electrophoresis analysis confirmed the successful annealing of several different FQD₂ variants (**Supplementary Fig. 1a**). The close proximity between the fluorophore and quencher upon DNA annealing leads to the non-fluorescent “off” state of FQD₂. The current FQD₂ uses a 29-base-pair (bp) DNA scaffold, yielding a molecular weight around 20 kDa, similar to that of many protein-labeling tags. To achieve the minimal size of FQD, we also designed the unimolecular FQD₁ (**Fig. 1a**), whose fluorophore, PC Linker, and quencher are separated only by one-nucleotide (nt) spacers (i.e., effective molecular weight about 2.5 kDa). To measure the photoactivation kinetics of FQD, the fluorescence spectra of FQD₁ and FQD₂ were obtained before and after different time points of UV illumination (~0.03 W/cm²) and compared to those of PA JF549 and PA JF646 treated under the same condition (**Supplementary Figs. 1b-g**). After UV illumination, a gradual recovery of fluorescence was observed for all PA probes, and FQD became fully activated at 5–10 min, whereas PA JF549 and PA JF646 required a longer illumination of > 60 min and 30 min, respectively, indicating a higher efficiency of photoactivation for FQD (**Supplementary Fig. 1h**). We calculated the on-off intensity ratios of different PA probes and found that all the FQD probes exhibited a > 100-fold intensity increase after photoactivation (e.g., FQD₂488 113.3-fold, FQD₂594 279.5-fold, FQD₁594 351.3-fold, FQD₂647 194.1-fold, PA JF549 50.6-fold, and PA JF646 103.9-fold, **Supplementary Fig. 1h**).

To evaluate the performance of FQD in single-molecule imaging experiments, biotinylated FQD₂488 and FQD₂594 were immobilized on a surface using NeutrAvidin and biotinylated bovine

serum albumin (BSA-biotin) and imaged with a confocal microscope (**Supplementary Fig. 2a**). The number of single-molecule spots increased after 405-nm illumination (**Supplementary Fig. 2b**), indicating the successful photoactivation of FQD₂₄₈₈ and FQD₂₅₉₄. The intensity trajectories of these molecules showed single activation and photobleaching steps, consistent with single fluorophores being observed (**Supplementary Figs. 2c,d**). To estimate the 405-nm laser intensity required for FQD activation, we titrated the input laser power setting on the microscope, and measured the percentage of photoactivated molecules after different illumination periods. At the 40–100% input laser powers (approximately 0.6 kW/cm² at 100% and for details see Methods), most FQD₂₄₈₈ and FQD₂₅₉₄ molecules were switched on within 5 frames of activation (with each frame corresponding to a single-molecule photoactivation time of 4.78 ms, **Supplementary Figs. 2e-h** and for details see Methods).

To further explore 3D single-molecule imaging, the Acrydite-labeled Oligo A in FQD₂₅₉₄ was formulated with acrylamide and bis-acrylamide monomers; upon mixing with the initiator ammonium persulfate (APS) and the catalyst tetramethylethylenediamine (TEMED), Acrydite-Oligo A-AF594 became cross-linked into a hydrogel (**Figs. 1b,c**). The PC Linker and BHQ2-containing Oligo B was then diffused into the hydrogel, resulting in the disappearance of single-molecule spots following the in situ hybridization between the two oligo strands (**Figs. 1b,d**). After 405-nm illumination, a recovery of the Acrydite-FQD₂₅₉₄ spots was observed (**Figs. 1b,e**), indicating the successful photoactivation of Acrydite-FQD₂₅₉₄ in 3D hydrogels. The signals of photoactivated Acrydite-FQD₂₅₉₄ in 3D hydrogels were collected at varying imaging depths, revealing a slight decrease in the SNR at an increasing depth. However, distinct single-molecule signals could still be detected at 13 μm (**Figs. 1f,g**), substantially surpassing the imaging depth achievable with TIRF and highly inclined and laminated optical sheet (HILO) microscopy.³⁵ Single-molecule photoactivation and observation of Acrydite-FQD₂₅₉₄ was further confirmed through the intensity trajectory analysis (**Fig. 1h**), and a 20% input laser power was as sufficient for photoactivation as higher input laser powers (**Figs. 1i,j**). Based on the results above, we validated the feasibility of single-molecule photoactivation and confocal imaging of FQD both on 2D surfaces and in 3D hydrogels.

Next, we tested if FQD can be used in confocal PhADE experiments (for details see Methods) to break the “concentration barrier”. A high concentration of diffusing FQD₂₅₉₄ (1 μM) was added in the solution to interfere with the detection of immobilized FQD₂₅₉₄ on the surface (**Supplementary Fig. 3a**). During a PhADE cycle, both the immobilized and diffusing FQD₂₅₉₄ molecules in the imaging volume were initially photoactivated; however, the non-immobilized FQD₂₅₉₄ subsequently diffused out of the imaging volume, leading to a gradual increase in the SNR of the immobilized FQD₂₅₉₄ molecules, so that the single-molecule signals were eventually detected (**Supplementary Figs. 3b,c**). Consistent with PhADE being a diffusion-dependent process, the decay of background fluorescence was hampered at an increased solution viscosity (25% vs. 0% glycerol, **Supplementary Fig. 3d**). This effect was further supported by the diffusion coefficients of FQD₂₅₉₄ determined with fluorescence correlation spectroscopy (FCS), i.e., $113.498 \pm 4.818 \mu\text{m}^2/\text{s}$ and $37.835 \pm 2.502 \mu\text{m}^2/\text{s}$ in 0% and 25% glycerol, respectively (**Supplementary Figs. 3e,f**).

So far, the PhADE strategy has only been implemented with 2D imaging on surfaces. We applied the confocal PhADE method to image single Acrydite-FQD₂₅₉₄ molecules tethered in 3D hydrogels in the presence of diffusing FQD₂₅₉₄ (1 μM , **Fig. 2a**), and our results demonstrated a single PhADE cycle happening in 3D (at an imaging depth of 4 μm), where the recovery of SNR and the detection of single-molecule signals were achieved for the immobilized FQD₂₅₉₄ molecules

(Figs. 2b,c and Supplementary Fig. 4). Next, multiple PhADE cycles were strung for continuous observation (Fig. 2d), and the 1–2 frames at the end of each PhADE cycle were selected and concatenated post-data acquisition to generate a time-lapse dataset for downstream analysis (Fig. 2e), providing an effective time resolution equal to the duration of one PhADE cycle. The decay of background fluorescence followed a double-exponential model in hydrogels with time constants similar to that obtained on surfaces in aqueous solution (0.6 s and 4.0 s in hydrogels vs. 4.4 s on surfaces, Fig. 2d and Supplementary Fig. 3d). Based on the results above, we found that the confocal PhADE method could be implemented for breaking the “concentration barrier” in single-molecule fluorescence imaging both in 2D and 3D systems, which showed a minimal “dead time” thanks to the fast diffusion and dissipation process.

To demonstrate the utility of 3D confocal PhADE, we applied this method to monitor the growth process of individual RCA products in hydrogels. To study RCA reactions in the hydrogel, we hybridized the Acrydite-labeled RCA primer with the circular RCA template, and copolymerized the complex with the hydrogel. For monitoring RCA reactions in real time, we designed a hybridization probe complementary to the RCA product and labeled the probe with FQD₂594 (RCA probe-FQD₂594). RCA reactions were initiated by adding phi29 DNA Polymerase and dNTPs in the buffer that soaks the hydrogel. As the reaction proceeded, each amplification cycle generated a binding site for RCA probe-FQD₂594, resulting in a progressive increase in fluorescence intensity (Fig. 2f). At the 2.5 μ M probe concentration, individual RCA reactions could barely be imaged with the regular AF594-labeled hybridization probe (RCA probe-AF594) because of an overwhelming background fluorescence (Fig. 2g). In comparison, clear visualization and tracking of single RCA reactions were allowed with RCA probe-FQD₂594 when imaged with the 3D confocal PhADE method under the identical probe concentration and imaging conditions (Fig. 2h and Supplementary Fig. 5). This approach enabled real-time visualization of individual RCA reactions even at 10 μ m above the imaging surface. We extracted the fluorescence intensity trajectories and analyzed the growth curves for individual RCA reactions (Fig. 2i). Most trajectories displayed a biphasic profile, with an initial steep phase followed by a slower, more gradual one (Supplementary Fig. 6). To understand how these trajectories reflect the underlying RCA reaction kinetics, we simulated the reaction and photoactivation processes with different parameters. The first phase was found to reflect contributions from both the reaction and photoactivation processes, while the slope of the second phase was more closely correlated with the reaction kinetics (Supplementary Fig. 6e).

To further test if 3D confocal PhADE can detect signals from single fluorophores, we utilized DNA hybridization in hydrogels as the model system. A challenge of performing DNA-PAINT in hydrogel-embedded samples is the high background fluorescence generated by the high concentrations of imager strands needed to achieve sufficient hybridization kinetics.¹⁹ To measure the DNA hybridization kinetics in hydrogels, we anchored the Acrydite-AF488-docking strands during polymerization, and added the complementary FQD₁594-imager strands via diffusion (Fig. 3a). A 3D confocal reconstruction revealed the spatial distribution of the Acrydite-AF488-docking strands throughout the hydrogel matrix (Fig. 3b). We acquired single-molecule images from different color channels, and the dual-color co-localization analysis confirmed the specific interaction between the FQD₁594-imager strands and the immobilized Acrydite-AF488-docking strands (Fig. 3c). Notably, at an imager strand concentration of 100 nM, conventional confocal microscopy of the imager strands labeled with regular AF594 failed to visualize single-molecule

spots due to the high background fluorescence (**Fig. 3d**, top). In contrast, 3D confocal PhADE detected single-molecule signals of the FQD₁₅₉₄-imager strands at the identical probe concentration and imaging conditions, displaying distinct unoccupied and hybridized states in the representative fluorescence intensity trajectory (**Fig. 3d**, bottom). Even at an imager strand concentration of 200 nM, clear single-molecule steps could be observed (**Supplementary Fig. 7a**), indicating that 1 frame of 0.52 s is sufficient for diffusion and background fluorescence dissipation in hydrogels at this imager strand concentration.

By monitoring these individual events in real time at varying imager strand concentrations, the hybridization kinetic parameters within the hydrogel were characterized. The dissociation rate (k_{off}) was calculated from the mean bound dwelltime (τ_b) to be approximately 0.06 s^{-1} , independent of the imager strand concentration (**Fig. 3e**), whereas the apparent association rate (k_{on}) was derived from the mean unbound dwelltime (τ_d), exhibiting a dependence on the imager strand concentration. Interestingly, as the imager strand concentration increased, the observed k_{on} increased and reached a plateau at 50 nM (**Fig. 3f**, see Discussion). The centroid localization analysis was then performed on the single-molecule spots observed in the 3D confocal PhADE images. We clustered all the localization events in a 2D histogram and determined the single-molecule localization precision to be about 35 nm (full width at half maximum, FWHM = $\sim 2.35\sigma$) in the xy-direction (**Figs. 3g,h**), similar to the resolution of related DNA-PAINT techniques.^{19, 36} Moreover, the localization precision did not deteriorate at higher imager strand concentrations (up to 200 nM), demonstrating the effective background suppression of 3D confocal PhADE (**Supplementary Fig. 7b**).

Discussion

In this study, we synthesized a series of FQD probes with different colors, attachment groups, and scaffold structures. Our results showed that the FQD probes could be photoactivated under modest UV illumination with an efficiency beyond that of a commercial PA fluorophore (**Supplementary Fig. 1**). Regardless of the fluorophores and DNA structures tested, all FQD probes exhibited a high on-off intensity ratio (> 100 -fold) and nearly uniform photoactivation kinetics, speaking to the advantage of its modular architecture. In comparison, a BODIPY-based and 2,6-dinitrobenzyl-caged PA fluorophore exhibits deteriorating on-off intensity ratios at the yellow and red fluorescence wavelength.³² Moreover, single-molecule photoactivation and imaging of FQD both on 2D surfaces (**Supplementary Fig. 2**) and in 3D hydrogels (**Fig. 1**) were demonstrated with a confocal microscope. While the SNR slightly decreased as the imaging depth increased in hydrogels, we were able to perform decent single-molecule imaging at 13 μm from the surface (**Figs. 1f,g**). FQD can be ordered with the fluorophore and quencher-of-choice, or as the unlabeled amino form, which can be easily conjugated in small batches in house (for details see Methods), making them accessible and flexible for various application scenarios.

Thanks to the unique advantages of FQD, we utilized the probes to expand the capabilities of several single-molecule imaging techniques. For instance, combining PhADE with confocal microscopy, we were able to overcome the “concentration barrier” in single-molecule fluorescence imaging both in 2D (**Supplementary Fig. 3**) and 3D systems (**Fig. 2**), where the target single molecules were tethered in hydrogels. Single-molecule in vitro imaging experiments typically depends on functionalized and passivated surfaces for tethering target molecules for long-term observation.³⁷⁻⁴⁰ Here, the hydrogel matrix is employed to immobilize the two single-molecule

reaction systems, i.e., RCA and DNA hybridization, for continuous monitoring, offering a practical alternative that could circumvent the need for the relatively lengthy surface preparation steps. The non-specific binding level of DNA is minimal in hydrogels, as we did not observe single-molecule signals at an imager strand concentration of 100 nM when the docking strand is absent (**Supplementary Fig. 7c**).

One of the drawbacks of the current confocal PhADE method is the limited imaging speed. For instance, as a trade-off for a relatively large field of view in the RCA experiments, we shortened the pixel dwelltime to 9.75 μ s but only achieved a modest time resolution of 4.1 s, including 1.3 s of photoactivation and 2.6 s of diffusion and excitation (**Figs. 2g,h**). However, for a typical phi29 DNA Polymerase (50–100 nt/s), it takes about 1–1.5 s to amplify a 70-nt template, rendering the time resolution inadequate for observing step-wise intensity changes. In addition, while the shortened pixel dwelltime prevents detector saturation at high RCA probe-AF594 concentrations, it also reduces the SNR and makes it difficult to discern single-fluorophore intensity changes out of a large random noise from the RCA reaction spots containing tens of hybridized probes. The overall imaging speed can be increased by adopting faster imaging modalities, such as spinning-disk confocal microscopy.³⁶ With further improvement in imaging speed, 3D confocal PhADE could be potentially of assistance to applications such as in situ sequencing (ISS),⁴¹⁻⁴⁴ where individual polymerase reactions in cell samples are imaged in the presence of high concentrations of labeled nucleotides.

Cell and tissue imprinting in hydrogels, combined with optical clearing, reduces cellular auto-fluorescence while improving light penetration and imaging depth,⁴⁵⁻⁴⁸ rendering favorable sample conditions for advanced 3D super-resolution imaging techniques.⁴⁹⁻⁵¹ Here, using the confocal PhADE method, we demonstrated that the FQD probes could facilitate the direct monitoring of DNA hybridization processes in a model hydrogel system, observing the binding and unbinding kinetics between the docking and imager strands and achieving super-resolution localizations at elevated imager strand concentrations (**Fig. 3**). Although the apparent k_{on} rate saturated above 50 nM (**Fig. 3f**), it is mainly due to the existence of an unlabeled or photobleached portion of the imager strands and the long bound dwelltimes of the 10-nt complementary probes, i.e., the bound dwelltimes of the non-fluorescent imager strands are misinterpreted as unbound dwelltimes (**Supplementary Fig. 7d**). This artifact can be mitigated if a faster-dissociation imager strand is used in combination with a faster imaging modality (**Supplementary Fig. 7e**).

In summary, these FQD probes are versatile and substantially extend the current capabilities of single-molecule imaging methods. To further enhance the performance of FQD, it could be integrated with the DNA-scaffolded FluoroCubes,^{34, 52} which can also be treated and quenched as a single functional unit. Moreover, incorporating alternative oligonucleotide chemistries, such as LNA and PNA, could improve the intracellular stability of FQD and thereby allow for its application in live-cell and in vivo imaging.

Methods

Materials

Sample chambers were ordered from Cellvis (C18SB-1.5H). HEPES solution (Sigma-Aldrich, 83264), Tris-HCl (pH 7.4, Beyotime, ST786), Tris-HCl (pH 8.0, Beyotime, ST786), PBS (pH 7.4, Beyotime, ST476), potassium chloride (Aladdin, P112133), magnesium chloride (Sigma-Aldrich, 442615), EDTA (ThermoFisher, AM9260G), glucose (Sigma-Aldrich, G7528), (+)-6-hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid (Trolox, Sigma-Aldrich, 238813), glucose oxidase (Sigma-Aldrich, G2133), catalase (Sigma-Aldrich, C9322), Alconox (Sigma-Aldrich, Z742914), potassium hydroxide (Sigma-Aldrich, 484016), BSA (Sigma-Aldrich, A1933), biotin-PEG3 NHS ester (MCE, 130291), ultrafiltration spin columns (10 kDa, Beyotime, FUF051), Tween 20 (Sigma-Aldrich, T8787), NeutrAvidin (Thermo Scientific, 31000), triethoxyvinylsilane (Sigma-Aldrich, 679275), ethanol (Aladdin, E111993), acetic acid (Aladdin, A298827), 40% acrylamide-bisacrylamide (19:1) (Beyotime, ST004B), APS (Sigma-Aldrich, A3678), TEMED (Sigma-Aldrich, T7024), glycerol (Sigma-Aldrich, G5516), AF594 NHS ester (Lumiprobe, 2825), AF647 NHS ester (MCE, HY-D1119), PA JF549 NHS ester (MCE, HY-133536), PA JF646 NHS ester (MCE, HY-133535), NA-Green (Beyotime, D0133), DMSO (Sigma-Aldrich, 276855), sodium bicarbonate (Aladdin, M0113576), Oligo Clean & Concentrator (OCC, Zymo Research, D4060), CircLigase II (Lucigen, CL9021K), phi29 DNA Polymerase (Beyotime, D7053S), DL500 DNA Marker (Takara, 3590Q).

Buffers

Buffer A was 25 mM HEPES, pH 7.6, 150 mM KCl, ultrapure H₂O.

Buffer B was 50 mM HEPES pH 7.6, 150 mM KCl, 0.8% glucose, 4 mM Trolox, ultrapure H₂O, and 1X Gloxy (165 U/mL glucose oxidase and 2170 U/mL catalase) was added before imaging.⁵³

Buffer C was 5 mM Tris-HCl pH 8, 75 mM MgCl₂, 0.05% Tween-20, 1 mM EDTA, 0.8% glucose, 4 mM Trolox, ultrapure H₂O, and 1X Gloxy was added before imaging.⁵³

Buffer D was 1 U phi29 DNA Polymerase, 0.25 mM dNTPs, 1X RCA reaction buffer, 0.8% glucose, 4 mM Trolox, ultrapure H₂O, and 1X Gloxy was added before imaging.⁵³

Buffer E was 25 mM HEPES, 150 mM KCl, 0.2% Tween 20, ultrapure H₂O.

Oligonucleotide preparation

Oligo A-AF488/594, Acrydite-Oligo A-amino, Oligo B, Oligo B-biotin, Oligo C-amino, P-Oligo D, Oligo E-Acrydite, FQD₁594-imager strand, Acrydite-AF488-docking strand (10 nt), Acrydite-docking strand (8 nt), AF488-docking strand-biotin and other oligos were purchased from Sangon Biotech.

Acrydite-Oligo A-AF594 was labeled in house. Specifically, Acrydite-Oligo A-amino was mixed with AF594 NHS ester in NaHCO₃ buffer at a 50-fold molar excess for 3 h at room temperature. Acrydite-Oligo A-AF594 was then purified with OCC. The labeling efficiency was determined by UV-absorbance using NanoDrop.

Oligo C-AF594 and Oligo C-AF647 were labeled in house. Specifically, Oligo C-amino was mixed with AF594 and AF647 NHS ester in NaHCO₃ buffer at a 50-fold molar excess for 3 h at room temperature, respectively. Oligo C-AF594 and Oligo C-AF647 were then purified with OCC. The labeling efficiency was determined by UV-absorbance using NanoDrop.

FQD₂488/594 (FQD₂488/594-biotin) was synthesized by hybridizing Oligo A-AF488/AF594 with Oligo B (Oligo B-biotin). Acrydite-FQD₂594 was synthesized by hybridizing Acrydite-Oligo A-AF594 with Oligo B. RCA probe-FQD₂594 and FQD₂647 were synthesized by hybridizing Oligo C-

AF594 and Oligo C-AF647 with Oligo B, respectively. Acrydite-AF488-docking strand-biotin (8 nt) was synthesized by hybridizing Acrydite-docking strand (8 nt) with AF488-docking strand-biotin.

P-Oligo D was ligated into a circular form using the CirLigase II kit. The circularized Oligo D was purified with OCC. The Acrydite-labeled RCA primer (Oligo E-Acrydite, 10 μ M) and the circular RCA template were mixed for hybridization in Buffer A, and the hybridized RCA initiation complex was purified with OCC.

DNA hybridization was carried out by heating samples to 95 °C for 5 min, followed by slow cooling to 30 °C in approximately 10 min in Buffer A. Native polyacrylamide gel electrophoresis analysis (10%) was used to examine DNA hybridization. All oligo samples were stored at -20 °C.

Bulk photoactivation assays and fluorescence spectrometry

The samples were diluted in buffer A at a concentration of 200 nM and illuminated with a UV LED lamp at a fixed angle and distance for the specified time periods. The UV illumination intensity was measured with a power meter with the lamp held at the same fixed angle and distance. All fluorescence spectra were measured on a Carry Eclipse (Agilent) fluorometer. For FQD₂488, the excitation wavelength was 480 nm and the emission wavelength window was 500–600 nm. For FQD₂594 and FQD₁594, the excitation wavelength was 580 nm and the emission wavelength window was 600–700 nm. For FQD₂647 and PA JF646, the excitation wavelength was 640 nm and the emission wavelength window was 660–760 nm. For PA JF549, the excitation wavelength was 540 nm and the emission wavelength window was 560–660 nm. The excitation and emission slit width was 10 nm, and the scan speed was 600 nm/min. A photomultiplier tube was used as the detector.

Imaging surface preparation

The potassium hydroxide-treated imaging surface was prepared by the sequential sonication steps as follows: glass slides and coverslips were first sonicated in 0.5% (w/v) Alconox solution for 20 min, then in ultrapure H₂O for 5 min, and in 2 M KOH solution for 20 min. The slides were then thoroughly washed with ultrapure H₂O and dried with nitrogen gas. The treated slides and coverslips were assembled into imaging channels before experiments.

For the triethoxyvinylsilane-modified imaging surface, imaging chambers were cleaned and KOH-treated following the same procedure described above. The chambers were then subjected to oxygen plasma treatment (80 W, 5 min), followed by overnight incubation at room temperature in 0.3% acetic acid, 0.2% triethoxyvinylsilane and 95% ethanol. Finally, the imaging chambers were rinsed with absolute ethanol and dried with nitrogen gas.

Confocal microscopy

The single-molecule imaging experiments were performed on a Leica Stellaris 8 STED microscope or a Leica TCS SP8 STED microscope. Specifically, the PA DNA-PAINT experiments were carried out in the fluorescence recovery after photobleaching (FRAP) mode on a Leica Stellaris 8 STED microscope with a 405-nm laser, a white light laser (WLL) source, and an HC PL APO CS2 100 $\times$ oil-immersion objective (NA = 1.4). Other single-molecule imaging experiments were performed with a Leica TCS SP8 STED microscope that is equipped with a 405-nm laser, a WLL source, and an HC PL APO CS2 100 $\times$ oil-immersion objective (NA = 1.4). All confocal PhADE experiments were performed in the FRAP mode.

AF488, AF594, and NA-Green were excited with the 488-nm, 580-nm, and 500-nm lasers, respectively, from the WLL source. Fluorescence emission was collected in the photon-counting mode (gate time 0.3–6.0 ns) with wavelength windows of 500–600 nm (for AF488), 600–700 nm (for AF594), and 515–615 nm (for NA-Green) using a HyD detector. The laser power, scanning speed, image size and z-depth, and pixel size were specified in the figure captions for each experiment.

The photoactivation time per molecule was estimated from the 405-nm illumination duration during confocal scanning of 7×7 pixels. For example, based on the pixel dwelltime of 97.65 μ s and pixel size of 53.84 nm, the calculated photoactivation time per molecule was 4.78 ms per frame (Leica TCS SP8 STED microscope). For the pixel dwelltime of 102.38 μ s and pixel size of 51.53 nm, the calculated photoactivation time per molecule was 5.01 ms per frame (Leica Stellaris 8 STED microscope). The effective diffusion time in a PhADE cycle was derived from the time interval between the two consecutive confocal scans of the same molecule.

The 405-nm laser intensity at each input laser power setting was measured with a power meter held after the objective. The 405-nm laser power density was estimated with an assumed focal spot size of 350 × 350 nm².

2D confocal PhADE

BSA-biotin was custom synthesized with BSA and biotin-PEG3 NHS ester. Specifically, biotin-PEG3 NHS ester was added to BSA in a large molar excess and the mixture was incubated at room temperature overnight. The excess biotin-PEG3 NHS ester was removed with ultrafiltration spin columns.

50 μ L of 0.2 mg/mL BSA-biotin was introduced to an imaging channel and incubated for 20 min, followed by a wash with 100 μ L of Buffer E. Next, 50 μ L of 0.1 mg/mL NeutrAvidin was added and incubated for 20 min, and the channel was washed with 100 μ L of Buffer A. 100 μ L of 1 nM FQD₂488/594-biotin was then added and incubated for 5 min, followed by another wash with Buffer A. Finally, 1 μ M FQD₂488/594 was introduced to the channel in Buffer B containing 1X Gloxy.

2D confocal PhADE was carried on a confocal microscope (see confocal microscopy). This process includes the photoactivation (405 nm) and excitation (488 nm or 594 nm) steps. For each experiment, the detailed imaging parameters are shown in the corresponding figure captions.

3D confocal PhADE

A pre-hydrogel solution (5 μ L) was first prepared by mixing 2% acrylamide-bisacrylamide (19:1), Acrydite-Oligo A-AF594 (6 nM), and ultrapure H₂O. Subsequently, 0.1% APS and 1X TEMED were added sequentially. The mixture was gently pipetted up and down three times and immediately added into a triethoxyvinylsilane-functionalized imaging chamber. All the preparation steps were performed on ice until polymerization. After polymerization, the chamber was washed three times with 300 μ L of 1X PBS buffer (2 min per wash). Next, 100 μ L of Oligo B (5 nM) was added onto the hydrogel and allowed to hybridize overnight at 4 °C. Following hybridization, the hydrogel was washed three times with 300 μ L of 1X PBS buffer (2 min per wash). Finally, 1 μ M FQD₂594 was introduced to the chamber in Buffer B containing 1X Gloxy.

3D confocal PhADE was carried on a confocal microscope (see confocal microscopy). This process includes the photoactivation (405 nm) and excitation (594 nm) steps. For each experiment, the detailed imaging parameters are shown in the corresponding figure captions.

3D confocal PhADE imaging of individual RCA reactions

A pre-hydrogel solution (5 μ L) was first prepared by mixing 3% acrylamide-bisacrylamide (19:1), the RCA initiation complex (125 nM), and ultrapure H₂O. Subsequently, 0.2% APS and 10X TEMED were added sequentially. The mixture was gently pipetted up and down three times and immediately added into a triethoxyvinylsilane-functionalized imaging chamber. All the preparation steps were performed on ice until polymerization. After polymerization, the chamber was washed three times with 300 μ L of 1X PBS buffer (2 min per wash). Next, 1X RCA reaction buffer (from phi29 DNA Polymerase, Beyotime, D7053S) containing RCA probe-FQD₂594 or RCA probe-AF594 (2.5 μ M) were added into the hydrogel that was already copolymerized with the RCA initiation complex and incubated on ice for 10 min. The RCA reaction mixture was prepared on ice by combining phi29 DNA Polymerase (1 U), dNTPs (250 μ M), 1X RCA reaction buffer, the oxygen-scavenging system components (0.8% glucose, 4 mM Trolox), and 1X Gloxy. The real-time imaging of RCA reactions was carried out by the 3D confocal PhADE imaging method (see confocal microscopy and 3D confocal PhADE).

Simulation of the reaction and photoactivation processes

The time duration for each RCA cycle was simulated by a Gamma random number function in MatLab (steps = 70 nt, time per step = 1/phi29 DNA Polymerase speed). Since the RCA probe was at a high concentration, its binding was considered instantaneous. At each photoactivation frame, the number of newly photoactivated probes was simulated by a normal random number function in MatLab (mean = the number of dark probes $\times$ photoactivation efficiency, standard deviation = $\sqrt{\text{mean}}$). The number of total photoactivated probes was the cumulative sum of the number of newly photoactivated probes, without considering photobleaching. Various combinations of phi29 DNA Polymerase speed and photoactivation efficiency were simulated.

3D PA DNA-PAINT imaging

A pre-hydrogel solution (5 μ L) was first prepared by mixing 2% acrylamide-bisacrylamide (19:1), the Acrydite-AF488-docking strand (6 nM), and ultrapure H₂O. Subsequently, 0.1% APS and 1X TEMED were added sequentially. The mixture was gently pipetted up and down three times and immediately added into a triethoxyvinylsilane-functionalized imaging chamber. All the preparation steps were performed on ice until polymerization. After polymerization, the chamber was washed three times with 300 μ L of 1X PBS buffer (2 min per wash). Next, the FQD₁594-imager strands (20, 50, 100, 200 nM) in Buffer C were incubated with the hydrogel for 1 h before 1X Gloxy was added. The PA DNA-PAINT imaging was carried out by the 3D confocal PhADE imaging method (see confocal microscopy and 3D confocal PhADE).

Simulation of the apparent association rate

The Gillespie method was performed to simulate τ_b and τ_d dwelltimes, given the specified dissociation rate (k_{off}) and association rate (k_{on}). To mimic the effect of non-fluorescent imager strands, a portion of the bound events, specified by the labeling efficiency, were randomly selected and merged with the neighboring unbound events. The apparent dissociation rate and association rate were calculated from the mean τ_b and τ_d dwelltimes, respectively, after considering that a portion of bound events were misinterpreted as unbound events.

Data analysis

All images were processed by custom-made scripts in MatLab. For background subtraction, a background image for each image was generated with a 7×7-pixel tiling-window median value, interpolation and smoothing algorithm. For single-molecule selection, signal spots were selected with a local maximum method with three exclusion criteria, i.e., if a spot was 1) too large, 2) too close to another spot, or 3) too close to the edge of the image, it was excluded for analysis. For single-molecule intensity extraction, the 7×7-pixel intensities centered around each selected single molecule were background-subtracted and summed with a Gaussian-weighted method (FWHM ~3 pixels). The noise and background values were calculated as the standard deviation and average of pixel intensities, respectively, from imaging regions where no spots were detected.

Single-molecule centroid localization analysis for PA DNA-PAINT was performed using ThunderSTORM in ImageJ and custom-made scripts in MatLab. Specifically, drift correction with the cross-correlation or fiducial marker method was applied to the raw localization data to obtain the drift-corrected super-resolution 2D histograms. The FWHM values of individual localization event clusters were calculated by 2D Gaussian fitting implemented in MatLab.

Table 1 The Oligo list.

Names	Sequences (5' to 3') and modifications
Oligo A strands	Oligo A-AF488/594 CTCTATTTTGACTATATTTGACCTTAATC-AF488/594 Acrydite-Oligo A-amino Acrydite-CTCTATTTTGACTATATTTGACCTTAATC-NH ₂
Oligo B strands	Oligo B BHQ2-GA/iPCLink/TTAAGGTCAAATATAGTCAAAATAGAG Oligo B-biotin BHQ2-GA/iPCLink/TTAAGGTCAAATATAGTCAAAATAGAG-biotin
Oligo C strands	Oligo C-amino CACCATAACATACCACTTTGATCTCTATTTTGACTATATTTGACCTTAATC-NH ₂
Oligo D strands	P-Oligo D P-CCTATCTACATCACCCCAATCACCATAACATACCACTTTGACAATAACACACTCTC AACACCACCTACCA
Oligo E strands	Oligo E-Acrydite GGTGATGTAGATAGGTGGTAGGTGGTGTT-Acrydite
imager strands	FQD₁594-imager strand BHQ2/T/iPCLink/T/iAF594dT/TTCTAGATGTAT
docking strands	Acrydite-AF488-docking strand (10 nt) Acrydite-ATCCC/iAF488dT/ATCTTATACATCTAG Acrydite-docking strand (8 nt) Acrydite-CTTTTCTTCTTTCTTTCTTTCTCCTCCTCATACTCT AF488-docking strand-biotin AAGAAAGAAAAGAAGAAAAGT/iAF488dT/TT-biotin

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Author contributions

H.T.: Methodology, Investigation, Formal analysis, Writing–Original Draft, Writing–Review & Editing;

S.L.: Methodology, Investigation, Formal analysis, Writing–Original Draft, Writing–Review & Editing;

X.Z.: Investigation;

X.X.: Writing–Original Draft, Writing–Review & Editing, Supervision, Funding acquisition;

B.H.: Conceptualization, Methodology, Writing–Original Draft, Writing–Review & Editing, Supervision, Funding acquisition

Conflict of interests

The authors declare no competing financial interest.

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Fig. 1 3D single-molecule imaging of Acrydite-FQD₂594 immobilized within a hydrogel matrix. **a**, Structures of FQD₁ and FQD₂ probes. FQD₂ represents the bimolecular construct annealed of two complementary oligo strands, whereas FQD₁ denotes the unimolecular construct made of a single oligo strand. **b**, Preparation workflow of anchoring Acrydite-FQD₂594 in a polyacrylamide hydrogel during the polymerization process. **c-e**, Representative volumetric 3D confocal reconstructions of FQD₂594 molecules revealing that Acrydite-Oligo A-AF594 is immobilized in the hydrogel matrix (**c**); subsequent hybridization with the PC Linker and BHQ2-modified Oligo B yields FQD₂594 in the non-fluorescent "off" state (**d**); upon photoactivation, the PC Linker undergoes cleavage and BHQ2 hence dissociates, converting FQD₂594 to the fluorescent "on" state (**e**). Images were acquired using the Z-Stack mode. The image size was $6.84 \times 6.84 \times 2.5 \mu\text{m}^3$, with a Z-step size of $0.5 \mu\text{m}$ covering a Z-range of $2.0\text{--}4.5 \mu\text{m}$ (relative to the coverslip surface at $0 \mu\text{m}$). Both hydrogel polymerization and oligo hybridization were conducted in 1X PBS buffer (pH 7.4). **f**, Confocal images of FQD₂594 at varying Z depths. The image size was $6.84 \times 6.84 \mu\text{m}^2$. **g**, Statistics of the SNR of FQD₂594 molecules across different imaging depths. 3 random imaging regions ($6.84 \times 6.84 \mu\text{m}^2$) with 75–98 molecules were analyzed per Z depth. **h**, Representative single-molecule images of FQD₂594 before (BP) and after (AP) photoactivation, and intensity trajectories corresponding to the specific spots (I, II) annotated in the left images. The imaging depth was $2 \mu\text{m}$, and the image size was $3.42 \times 3.42 \mu\text{m}^2$. **i**, Quantification of the average spot counts per imaging area before hybridization (BH), after hybridization/before photoactivation (AH/BP), and after photoactivation (AP). 5 random imaging regions (Z depth at $2 \mu\text{m}$, $13.76 \times 13.76 \mu\text{m}^2$) were analyzed per condition. **j**, Quantification of the average spot counts of photoactivated FQD₂594 per imaging area at different 405-nm laser powers. 5 random imaging regions (Z depth at $2 \mu\text{m}$, $13.76 \times 13.76 \mu\text{m}^2$) were analyzed per condition. For the above confocal experiments, a scanning speed of $97.65 \mu\text{s/pixel}$ and a pixel size of $53.84 \times 53.84 \text{ nm}^2$ were used. Photoactivation of 1 frame (equivalent to a single-molecule activation time of 4.78 ms per frame) was performed using a 20% 405-nm laser power unless otherwise specified. Error bars, the standard deviation (SD). Scale bars, $1 \mu\text{m}$. All the single-molecule images were obtained in Buffer B, and 1X Gloxy was added before imaging.

Fig. 2 3D confocal PhADE imaging. **a**, Principle of 3D confocal PhADE imaging. Acrydite-FQD₂594 is immobilized, while diffusing FQD₂594 is infused into the hydrogel. Upon photoactivation (405 nm), both the immobilized Acrydite-FQD₂594 and the diffusing FQD₂594 near the focal spot are converted to the “on” state. During excitation (580 nm), the non-immobilized FQD₂594 diffuses out of the imaging volume, leading to a time-dependent increase in the SNR of the immobilized Acrydite-FQD₂594. **b**, 3D confocal PhADE imaging of Acrydite-FQD₂594 immobilized in a hydrogel matrix with 1 μ M diffusing FQD₂594 (one PhADE cycle). The imaging depth was 4 μ m, and the image size was $3.42 \times 3.42 \mu\text{m}^2$. Each image represents an average of five raw frames, corresponding to the time periods (A1–A8) annotated in **c**. **c**, Time-dependent SNR for the immobilized Acrydite-FQD₂594 in a single PhADE cycle. “Point A” denotes the specific spot marked in **b**; “Average” represents the mean SNR over all identified spots ($n = 9$). For the confocal experiments in **a-c**, a scanning speed of 97.65 $\mu\text{s}/\text{pixel}$ and a pixel size of $53.84 \times 53.84 \text{ nm}^2$ were used. Photoactivation of 2 frames (equivalent to a single-molecule activation time of 4.78 ms per frame) was performed using a 20% 405-nm laser power in **a-c**. **d**, Intensity decay curves of background fluorescence in continuous PhADE cycles at 1 μ M FQD₂594. The double-exponential decay time constants were 0.6 s and 4.0 s, respectively. For the confocal experiments in **d**, a scanning speed of 97.65 $\mu\text{s}/\text{pixel}$ and a pixel size of $53.84 \times 53.84 \text{ nm}^2$ were used. Photoactivation of 2 frames (equivalent to a single-molecule activation time of 4.78 ms per frame) was performed using a 20% 405-nm laser power in **d**. The duration of diffusion and excitation was 20 frames. **e**, Workflow and representative images of 3D confocal PhADE imaging (continuous PhADE cycles). **f**, Workflow of observing individual RCA reactions in the hydrogel with 3D confocal PhADE imaging. The RCA initiation complex, consisting of the Acrydite-labeled RCA primer and the circular RCA template, is copolymerized into the hydrogel network, followed by the infusion of RCA probe-FQD₂594. The RCA reaction is initiated by the addition of phi29 DNA Polymerase and dNTPs. As the reaction proceeds, each amplification cycle generates a binding site for RCA probe-FQD₂594, resulting in a progressive increase in fluorescence intensity. **g**, 3D confocal imaging of individual RCA reactions (without PhADE) at 2.5 μ M RCA probe-AF594. The imaging depth was 10 μ m, and the image size was $13.76 \times 13.76 \mu\text{m}^2$. Each image represents an average of two raw frames. **h**, 3D confocal PhADE imaging of individual RCA reactions at 2.5 μ M RCA probe-FQD₂594 (continuous PhADE cycles). The imaging depth was 10 μ m, and the image size was $13.76 \times 13.76 \mu\text{m}^2$. Each image represents an average of two raw frames. **i**, Fluorescence intensity trajectories representing the growth curves of individual RCA reactions. The addition of phi29 DNA Polymerase and dNTPs was at $t = 0$ s. 13 consecutive movies were obtained from different regions, with each movie consisting of 25 PhADE cycles. For the confocal experiments in **f-i**, a scanning speed of 9.75 $\mu\text{s}/\text{pixel}$ and a pixel size of $53.84 \times 53.84 \text{ nm}^2$ were used. Photoactivation of 1 frame (equivalent to a single-molecule activation time of 4.78 ms per frame) was performed using a 20% 405-nm laser power in **f-i**. The duration of diffusion and excitation was 2 frames (approximately 2.6 s). Scale bars, 1 μ m. All the single-molecule images in **a-e** were obtained in Buffer B, and 1X Gloxy was added before imaging. All the single-molecule results in **f-i** were obtained in Buffer D, and 1X Gloxy was added before imaging.

Fig. 3 3D PA DNA-PAINT imaging. **a**, Workflow of 3D PA DNA-PAINT imaging in a hydrogel matrix. The FQD₁₅₉₄-labeled imager strands interact with the Acrydite-AF488-labeled docking strands in the hydrogel. **b**, A representative volumetric 3D confocal reconstruction revealing that the Acrydite-AF488-docking strands are immobilized in the hydrogel matrix. Images were acquired using the Z-Stack mode. The image size was $6.84 \times 6.84 \times 5 \mu\text{m}^3$, with a Z-step size of $1 \mu\text{m}$ covering a Z-range of $2.0\text{--}7.0 \mu\text{m}$ (relative to the coverslip surface at $0 \mu\text{m}$). **c**, Co-localization analysis between the AF488 channel (left) and the FQD₁₅₉₄ channel (middle), and the co-localized pixel positions (right). The imaging depth was $4 \mu\text{m}$, and the image size was $2.42 \times 2.42 \mu\text{m}^2$. **d**, Representative intensity trajectories with the unbound or dissociated state (τ_d) and the bound state (τ_b). Comparison between the confocal mode (top) and the confocal PhADE mode (bottom). **e**, Rate of dissociation ($k_{\text{off}} = 1/\tau_b$) as a function of the FQD₁₅₉₄-imager strand concentration. **f**, Rate of binding ($k_{\text{on}} = 1/\tau_d$) as a function of the FQD₁₅₉₄-imager strand concentration. The mean dwelltimes were derived from 3 random groups with 92–259 and 70–194 events in **e,f**, respectively. **g**, A representative diffraction-limited single-molecule spot (left) and its corresponding super-resolution localization cluster (right). **h**, Cross-section profiles in **g**. For the above confocal experiments, a scanning speed of $102.38 \mu\text{s}/\text{pixel}$ and a pixel size of $51.53 \times 51.53 \text{ nm}^2$ were used. Photoactivation of 1 frame (equivalent to a single-molecule activation time of 5.01 ms per frame) was performed using a 20% 405-nm laser power. The duration of diffusion and excitation was 1 frame (approximately 0.52 s). Error bars, the standard deviation (SD). Scale bars, $0.5 \mu\text{m}$. All the single-molecule results were obtained in Buffer C, and 1X Gloxy was added before imaging.

Figures

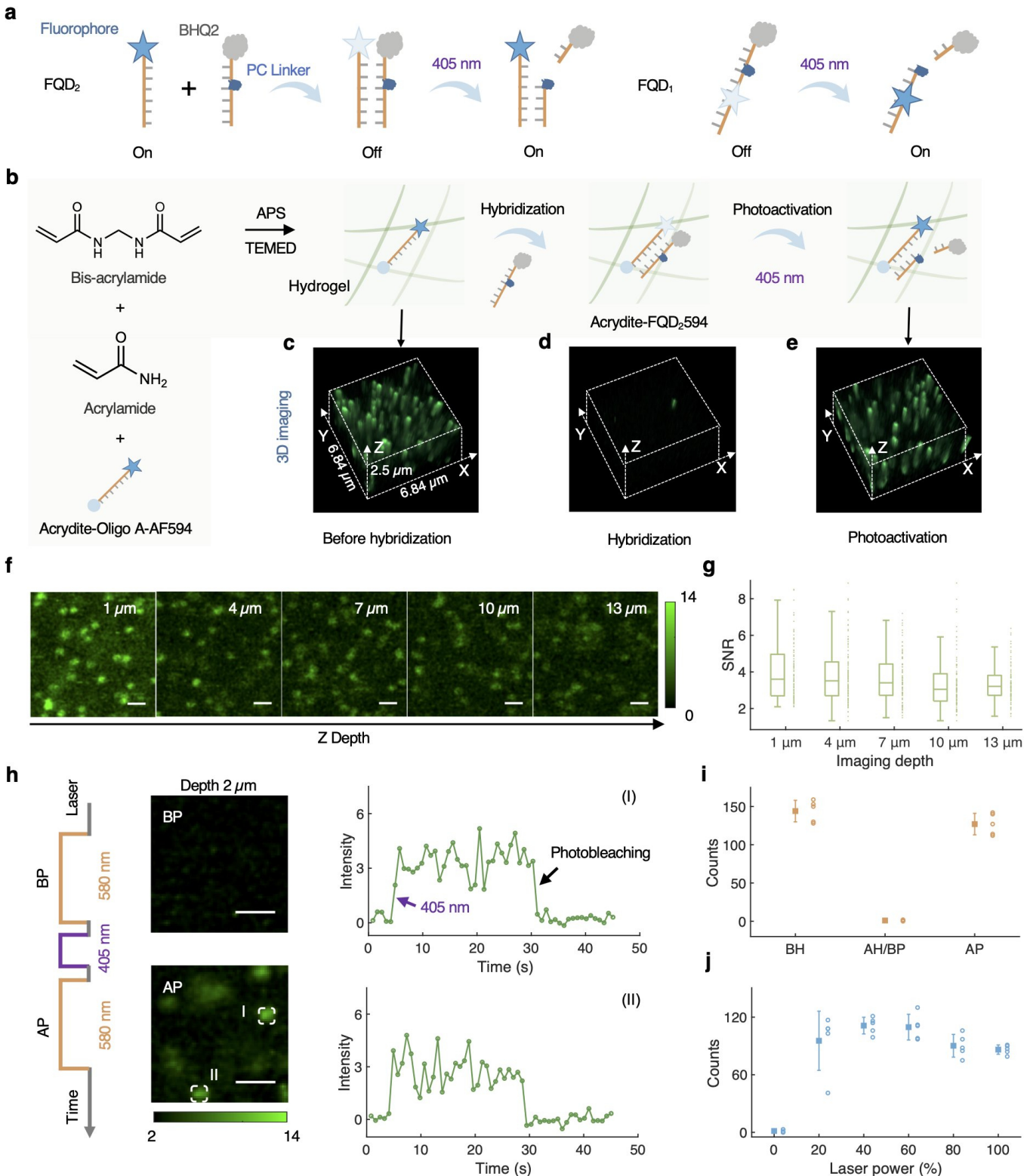


Figure 1

3D single-molecule imaging of Acrydite-FQD₂594 immobilized within a hydrogel matrix. **a**, Structures of FQD₁ and FQD₂ probes. FQD₂ represents the bimolecular construct annealed of two complementary

oligo strands, whereas FQD₁ denotes the unimolecular construct made of a single oligo strand. **b**, Preparation workflow of anchoring Acrydite-FQD₂594 in a polyacrylamide hydrogel during the polymerization process. **c-e**, Representative volumetric 3D confocal reconstructions of FQD₂594 molecules revealing that Acrydite-Oligo A-AF594 is immobilized in the hydrogel matrix (**c**); subsequent hybridization with the PC Linker and BHQ2-modified Oligo B yields FQD₂594 in the non-fluorescent “off” state (**d**); upon photoactivation, the PC Linker undergoes cleavage and BHQ2 hence dissociates, converting FQD₂594 to the fluorescent “on” state (**e**). Images were acquired using the Z-Stack mode. The image size was $6.84 \times 6.84 \times 2.5 \text{ mm}^3$, with a Z-step size of 0.5 mm covering a Z-range of 2.0–4.5 mm (relative to the coverslip surface at 0 mm). Both hydrogel polymerization and oligo hybridization were conducted in 1X PBS buffer (pH 7.4). **f**, Confocal images of FQD₂594 at varying Z depths. The image size was $6.84 \times 6.84 \text{ mm}^2$. **g**, Statistics of the SNR of FQD₂594 molecules across different imaging depths. 3 random imaging regions ($6.84 \times 6.84 \text{ mm}^2$) with 75–98 molecules were analyzed per Z depth. **h**, Representative single-molecule images of FQD₂594 before (BP) and after (AP) photoactivation, and intensity trajectories corresponding to the specific spots (I, II) annotated in the left images. The imaging depth was 2 μm , and the image size was $3.42 \times 3.42 \text{ mm}^2$. **i**, Quantification of the average spot counts per imaging area before hybridization (BH), after hybridization/before photoactivation (AH/BP), and after photoactivation (AP). 5 random imaging regions (Z depth at 2 μm , $13.76 \times 13.76 \text{ mm}^2$) were analyzed per condition. **j**, Quantification of the average spot counts of photoactivated FQD₂594 per imaging area at different 405-nm laser powers. 5 random imaging regions (Z depth at 2 μm , $13.76 \times 13.76 \text{ mm}^2$) were analyzed per condition. For the above confocal experiments, a scanning speed of 97.65 ms/pixel and a pixel size of $53.84 \times 53.84 \text{ nm}^2$ were used. Photoactivation of 1 frame (equivalent to a single-molecule activation time of 4.78 ms per frame) was performed using a 20% 405-nm laser power unless otherwise specified. Error bars, the standard deviation (SD). Scale bars, 1 mm. All the single-molecule images were obtained in Buffer B, and 1X Gloxy was added before imaging.

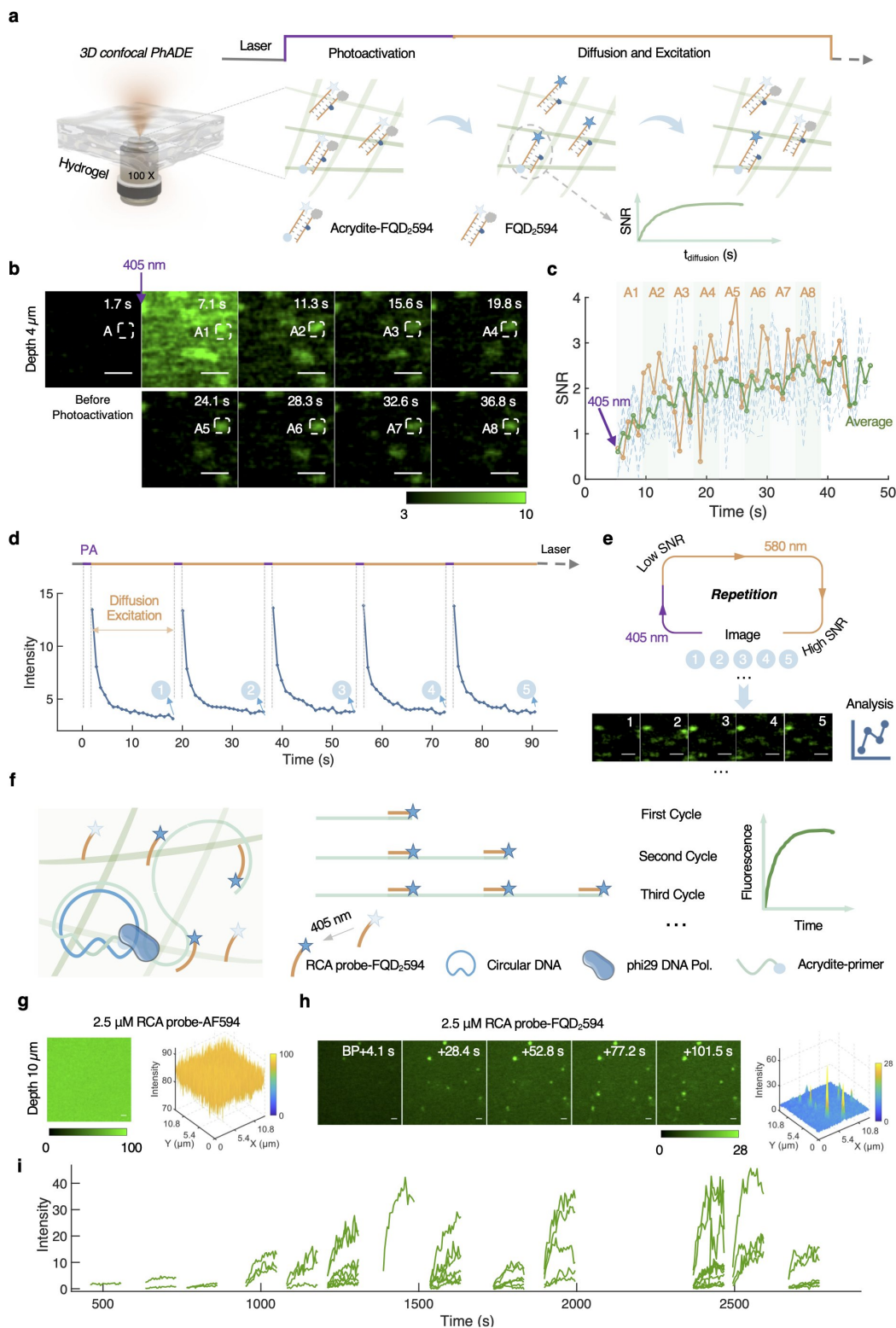


Figure 2

3D confocal PhADE imaging. **a**, Principle of 3D confocal PhADE imaging. Acrydite-FQD₂594 is immobilized, while diffusing FQD₂594 is infused into the hydrogel. Upon photoactivation (405 nm), both the immobilized Acrydite-FQD₂594 and the diffusing FQD₂594 near the focal spot are converted to the “on” state. During excitation (580 nm), the non-immobilized FQD₂594 diffuses out of the imaging volume,

leading to a time-dependent increase in the SNR of the immobilized Acrydite-FQD₂594. **b**, 3D confocal PhADE imaging of Acrydite-FQD₂594 immobilized in a hydrogel matrix with 1 mM diffusing FQD₂594 (one PhADE cycle). The imaging depth was 4 μm, and the image size was 3.42 × 3.42 mm². Each image represents an average of five raw frames, corresponding to the time periods (A1–A8) annotated in **c**. **c**, Time-dependent SNR for the immobilized Acrydite-FQD₂594 in a single PhADE cycle. "Point A" denotes the specific spot marked in **b**; "Average" represents the mean SNR over all identified spots (n = 9). For the confocal experiments in **a-c**, a scanning speed of 97.65 ms/pixel and a pixel size of 53.84 × 53.84 nm² were used. Photoactivation of 2 frames (equivalent to a single-molecule activation time of 4.78 ms per frame) was performed using a 20% 405-nm laser power in **a-c**. **d**, Intensity decay curves of background fluorescence in continuous PhADE cycles at 1 mM FQD₂594. The double-exponential decay time constants were 0.6 s and 4.0 s, respectively. For the confocal experiments in **d**, a scanning speed of 97.65 ms/pixel and a pixel size of 53.84 × 53.84 nm² were used. Photoactivation of 2 frames (equivalent to a single-molecule activation time of 4.78 ms per frame) was performed using a 20% 405-nm laser power in **d**. The duration of diffusion and excitation was 20 frames. **e**, Workflow and representative images of 3D confocal PhADE imaging (continuous PhADE cycles). **f**, Workflow of observing individual RCA reactions in the hydrogel with 3D confocal PhADE imaging. The RCA initiation complex, consisting of the Acrydite-labeled RCA primer and the circular RCA template, is copolymerized into the hydrogel network, followed by the infusion of RCA probe-FQD₂594. The RCA reaction is initiated by the addition of phi29 DNA Polymerase and dNTPs. As the reaction proceeds, each amplification cycle generates a binding site for RCA probe-FQD₂594, resulting in a progressive increase in fluorescence intensity. **g**, 3D confocal imaging of individual RCA reactions (without PhADE) at 2.5 mM RCA probe-AF594. The imaging depth was 10 μm, and the image size was 13.76 × 13.76 mm². Each image represents an average of two raw frames. **h**, 3D confocal PhADE imaging of individual RCA reactions at 2.5 mM RCA probe-FQD₂594 (continuous PhADE cycles). The imaging depth was 10 μm, and the image size was 13.76 × 13.76 mm². Each image represents an average of two raw frames. **i**, Fluorescence intensity trajectories representing the growth curves of individual RCA reactions. The addition of phi29 DNA Polymerase and dNTPs was at t = 0 s. 13 consecutive movies were obtained from different regions, with each movie consisting of 25 PhADE cycles. For the confocal experiments in **f-i**, a scanning speed of 9.75 ms/pixel and a pixel size of 53.84 × 53.84 nm² were used. Photoactivation of 1 frame (equivalent to a single-molecule activation time of 4.78 ms per frame) was performed using a 20% 405-nm laser power in **f-i**. The duration of diffusion and excitation was 2 frames (approximately 2.6 s). Scale bars, 1 mm. All the single-molecule images in **a-e** were obtained in Buffer B, and 1X Gloxy was added before imaging. All the single-molecule results in **f-i** were obtained in Buffer D, and 1X Gloxy was added before imaging.

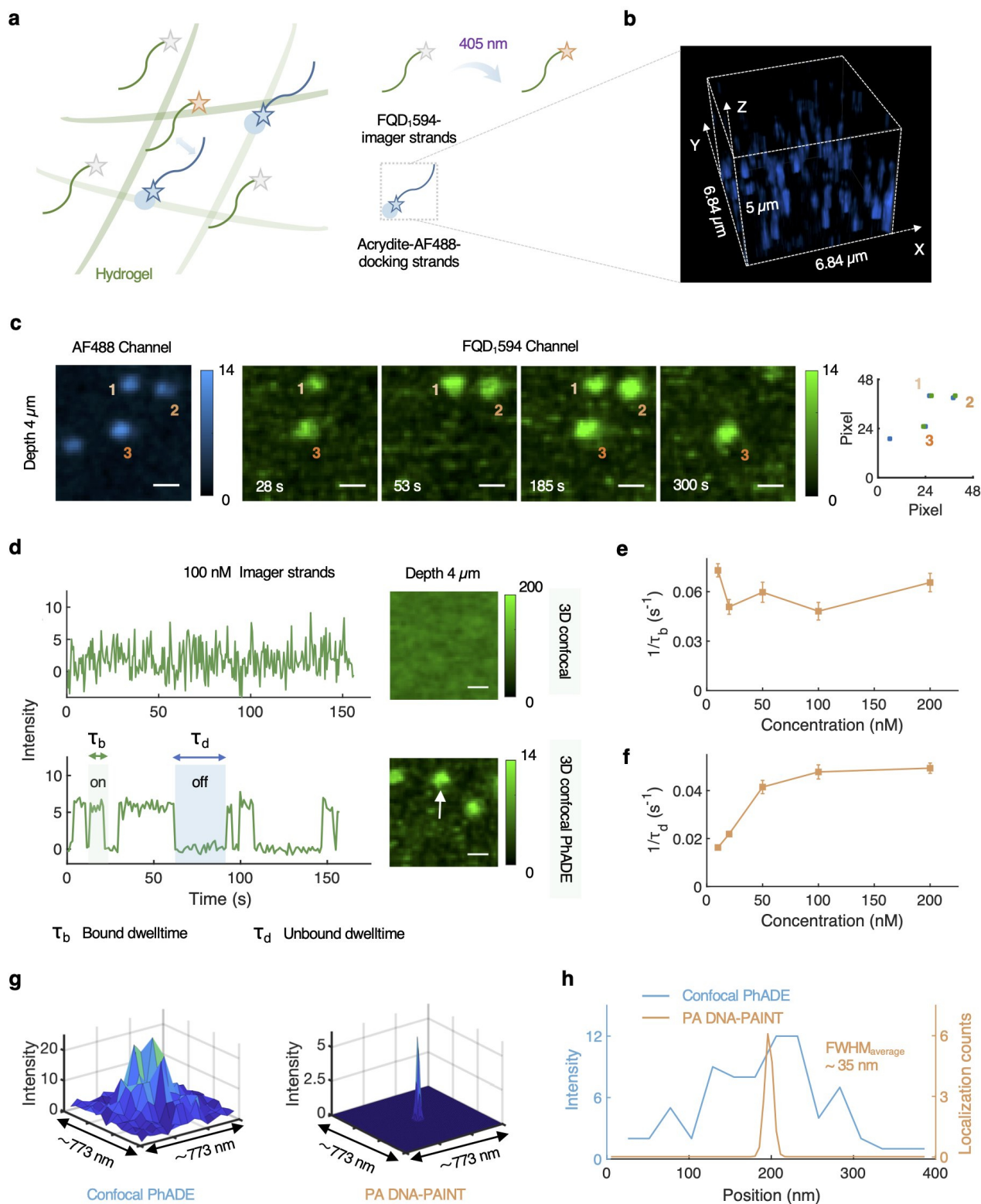


Figure 3

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