Multiplexed lifetime imaging of single molecules with a gated single-photon camera

Nathan Ronceray[1*], Salim Bennani[1], Marianna Mitsioni[1,2], Nicole Siegel[3], Maria Josefina Marcaida[2], Claudio Bruschini[4], Edoardo Charbon[4], Rahul Roy[5], Matteo Dal Peraro[2], Guillermo P. Acuna[3], Aleksandra Radenovic[1*]

[1]Institute of Bioengineering, School of Engineering, EPFL, Lausanne, Switzerland.
[2]Institute of Bioengineering, School of Life Sciences, EPFL, Lausanne, Switzerland.
[3]Department of Physics, University of Fribourg, Switzerland.
[4]AQUA, EPFL, Neuchâtel, Switzerland.
[5]Department of Chemical Engineering and Department of Bioengineering, Indian Institute of Science Bangalore, India.

*nathan.ronceray@epfl.ch; aleksandra.radenovic@epfl.ch;

Abstract

Fluorescence lifetime imaging microscopy (FLIM) is a powerful tool to discriminate fluorescent molecules or probe their nanoscale environment. Traditionally, FLIM uses time-correlated single-photon counting (TCSPC), which is precise but intrinsically low-throughput due to its dependence on point detectors. Although time-gated cameras have demonstrated the potential for high-throughput FLIM in bright samples with dense labeling, their use in single-molecule microscopy has not been explored extensively. Here, we report fast and accurate single-molecule FLIM with a commercial time-gated single-photon camera. Our optimized acquisition scheme achieves single-molecule lifetime measurements with a precision only about three times less than TCSPC, while allowing for the simultaneous multiplexing of over 3000 molecules. With this approach, we demonstrate parallelized lifetime measurements of large numbers of labeled pore-forming proteins on supported lipid bilayers, and multiplexed temporal single-molecule Förster resonance energy transfer measurements at 5-25 Hz. This method holds considerable promise for advancing multi-target single-molecule localization microscopy and biopolymer sequencing.

Introduction

Fluorescence lifetime measurements report the nanosecond-scale delay between an excitation laser pulse and the fluorescence emitted by a molecule. The decay of fluorescence following excitation typically follows an exponential distribution, characterized by a time constant known as the fluorescence excited state lifetime, τ. Similar to the emission spectrum, the lifetime contains unique information about the emitter's electronic structure, with common dye lifetimes ranging from 0.3 to 5 ns[1,2]. Furthermore, fluorescence lifetimes are sensitive to the surrounding environment, particularly through solvent effects and energy transfer processes[1]. Lifetime measurements enable the monitoring of biochemical reactions[3] and can be used to determine (sub)nanometer-scale distances via Förster resonance energy transfer (FRET), allowing the detection of molecular interactions or conformational changes in proteins[4]. Fluorescence lifetimes can also encode information about the axial localization of emitters relative to conductive substrates[5,6]. Typically, FLIM relies on TCSPC integrated on a confocal microscope with a point detector that records photon arrival times. Super-resolved variants of scanning confocal microscopy have been demonstrated with lifetime-resolved MINFLUX[7,8] and RASTMIN[9], but none of these techniques multiplexes many targets, making them low-throughput despite their unmatched localization precision.

Conversely, wide-field FLIM enables assigning lifetime values to multiple targets using parallel detection with a detector array, offering a much higher throughput. Wide-field TCSPC has been investigated[10] even at the single-molecule level but sensors enabling it suffer from constraints on sample brightness and a very low photon detection efficiency (PDE)[11,12]. Rather than recording photon arrival times, wide-field FLIM can be achieved by acquiring time-delayed images on a camera, which allows acquiring only specific temporal windows of the fluorescence decay from the sample. Although this delay can be obtained spatially[13,14], it is typically achieved by using a time gate that collects photons exclusively within a specific time window after the excitation. In most implementations, this time gate can be moved relative to the excitation pulse, allowing accurate sampling of the fluorescence decay. Gate positioning with picosecond-scale resolution can be achieved using intensified charge-coupled device cameras[15,16] or by using a Pockels cell in the emission path[17,18]. The latter approach proved very efficient at fast recording of neural activity[19], but showed imprecise results in the context of single-molecule imaging[17,18].

Single-photon avalanche diode (SPAD) arrays have emerged as readout-noise-free detectors with a good PDE[20]. Although the majority of fluorescence lifetime studies based on SPAD arrays utilized small asynchronous arrays (ranging from 20 to 400 detectors) with TCSPC capabilities[21–24], wide-field FLIM has also been demonstrated using synchronous time-gated SPAD cameras with nearly a million pixels[25]. In such SPAD cameras, the gating capability is monolithically integrated in the sensor, unlike intensifier or Pockels cells-based systems, thus offering better robustness and excellent temporal resolution.

Considerable efforts have been dedicated to establishing a palette of techniques for separating multi-exponential decays from a diffraction-limited spot[1,2] including the case of gated SPAD cameras[26]. Such techniques are generally photon-greedy and thus poorly suited for single-molecule lifetime measurements. However, single-molecule fluorescence decays tend to be simpler as a result of their well-defined electronic properties and local environment. Indeed, single-molecule decays are generally mono-exponential, unlike those of molecular ensembles where two or more exponential components can be found due to the presence of multiple dye populations in different local environments within the same imaged volume[11,27]. Although they have simpler decay shapes, single molecules present constraints due to their limited brightness and low photon budget. Hence, it is not surprising that single-molecule lifetime imaging has not been thoroughly investigated with gated SPAD cameras. In this work, we use the SPAD512^2 camera[25,28] which has a PDE of 30-40% when using microlens arrays, thus enabling single-molecule imaging[29]. With this sensor, we revisit a gated imaging scheme introduced 35 years ago[30] and demonstrate its potential for single-molecule fluorescence lifetime imaging microscopy (smFLIM).

Results

Single molecule (sm)FLIM scheme and setup

We designed a fluorescence microscopy setup using the SPAD camera for imaging molecules with total internal reflection illumination (Fig. 1a, details in Methods). The time gate is synchronized with the $\sim$ 50 ps pulsed supercontinuum light source used for wide-field illumination at a repetition rate of 26 MHz. The gate profile can be approximated by a square function with a rise time of $\sim$ 200 ps and a controllable gate width W in the range of $6-12$ ns (Fig. S10a). Controlling the relative delay of the gate opening with respect to the excitation pulse, we implement the so-called rapid lifetime determination scheme[30] consisting of alternating two gate positions. The first gate, positioned at the very beginning of the decay, collects nearly all the photons from the sample, and the second gate is offset by a delay T to reject the early emitted photons. We show in Figure 1b typical images of molecules with the full fluorescence signal in the first gate and a dimmer version of the image in the second gate. Mathematically, this 2-gate scheme enables retrieving the fluorescence lifetimes of molecules exhibiting monoexponential decay. Indeed, a molecule with unknown lifetime τ emitting a signal of S photons in the first gate, imaged with a background B_o will yield $N_o = S + B_o$ photons from the first gate and $N_1 = Se^{-T/\tau} + B_1$ from the second gate (Fig. 1c). The fluorescence lifetime τ is thus obtained as:

$$\tau = \frac{T}{\ln \frac{N_o - B_o}{N_1 - B_1}} \tag{1}$$

The background B_o and B_1 measured in the first and second gate may be different in the eventuality of fluorescent component of the background. In the following, we apply this scheme to highly multiplexed single-molecule lifetime measurements. While the 2-gate scheme employed here cannot outperform standard TCSPC-based FLIM approaches in terms of photon efficiency, it enables massive multiplexing by measuring a large number of lifetimes at once from a single field of view.

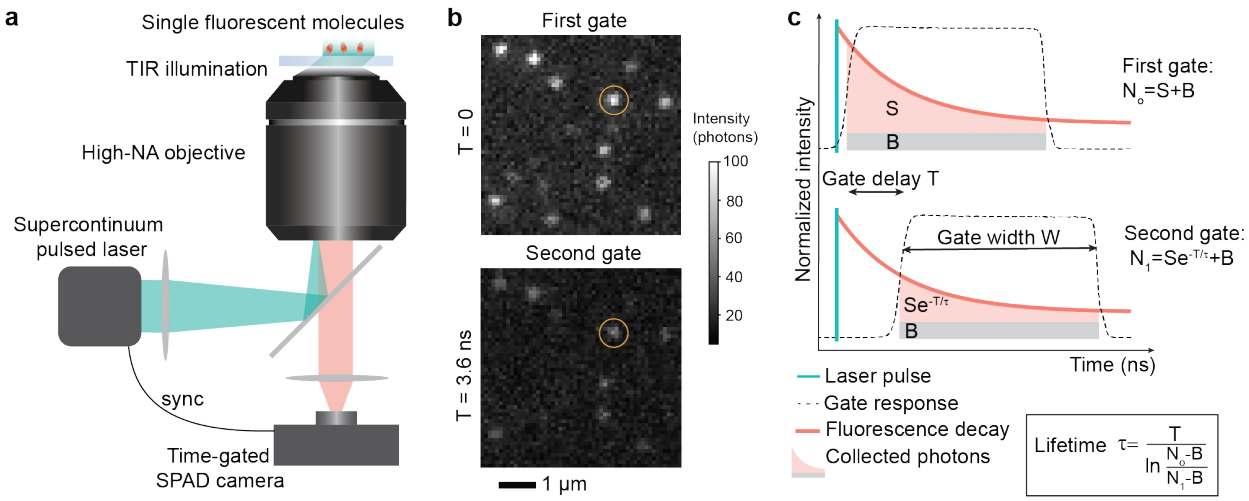

Fig. 1: **Single molecule FLIM setup and acquisition scheme**. **a**, Sketch of the experimental setup where a picosecond-pulsed green laser beam undergoes total internal reflection at the sample coverslip to excite single fluorescent molecules close to its surface. The emitted fluorescence is collected to form an image on the camera chip, which is synchronized with the excitation laser. **b**, Typical images of single molecules obtained from 20 ms integration time for the first gate capturing the full fluorescence decay (top) and the second gate capturing only late photons reaching the camera after a gate delay T. The same molecule is shown by yellow circles in the two cases. **c**, Sketch of the time-gated imaging scheme that enables retrieving single-molecule fluorescence lifetimes. We assumed $B_o = B_1 = B$ for simplicity on this sketch but in the general case we use $B_o \neq B_1 = B$. The gate response is mapped in more details in Figure S10.

High-throughput smFLIM on bacterial pore-forming proteins on lipid membranes

Our setup enables the imaging of thousands of individual molecules within a 51×51 µm^2 field of view, with a 100 nm pixel size at the sample. To demonstrate the high-throughput capability of our system, we imaged labeled aerolysin proteins embedded in supported lipid bilayers (Fig. 2b). Aerolysin is a β-barrel pore-forming toxin that assembles into oligomeric structures, forming transmembrane nanopores widely used in biosensing[31]. In our experiments, a mixture of labeled and unlabeled aerolysin monomers are initially present in the incubation solution and progressively integrate into the membrane, forming stable singly labeled heptameric assemblies within the lipid bilayer. This approach allows for control over the molecular density by adjusting the solution concentration and the incubation time. Most of our images were acquired at a density of roughly one labeled aerolysin pore complex per µm^2 (Fig. 2a). Detailed sample preparation methods are provided in the Methods section. The intensity traces of the first gate $N_o(t)$ and the second gate $N_1(t)$ were extracted for each of the $\sim$ 3000 fixed molecules in the field of view, allowing the application of stringent selection criteria to keep the pure single-molecule traces. For this, we used a python-based analysis pipeline relying on molecule detection (yellow squares in Fig. 2a) followed by filtering based on the identification of single-step photobleaching (red squares, see Methods) to ensure that the detected spot corresponds to a single molecule. Molecular lifetimes were then extracted by applying equation 1 to the time-averaged first and second gate signals. This enabled obtaining well-sampled lifetime histograms in under a minute, considerably speeding up the acquisition of lifetime statistics compared with standard confocal approaches. We show in Figure 2c histograms for proteins labeled with several dyes (LD555, Cy3B, and AF488) exhibiting different lifetimes and wavelengths. The measured lifetime values are presented in Table S1.

We verified that the gate delay value did not cause any systematic bias in the estimation of the lifetime distribution (Fig. S1a). We then estimated the throughput improvement of our scheme over sequential scanning with a point detector. Considering that all molecules are excited together, the acquisition must be long enough to capture all traces. We find that with a median photobleaching time of approximately one second, acquiring images for 10 seconds ensures that all photobleaching events are captured (Fig. S1b). Thus, while point scanning would require 3000 seconds to sequentially image the photobleaching of 3000 molecules, our wide-field scheme takes only 10 seconds. This 300-fold acceleration of single-molecule FLIM measurements opens the door for screening large populations and identifying potentially rare events among fluorescent traces. While these results demonstrate the potential of our scheme for *static* lifetime measurements, they rely on large photon budgets on the order of 10^4 (see Fig. S1c) which are not limited by shot noise.

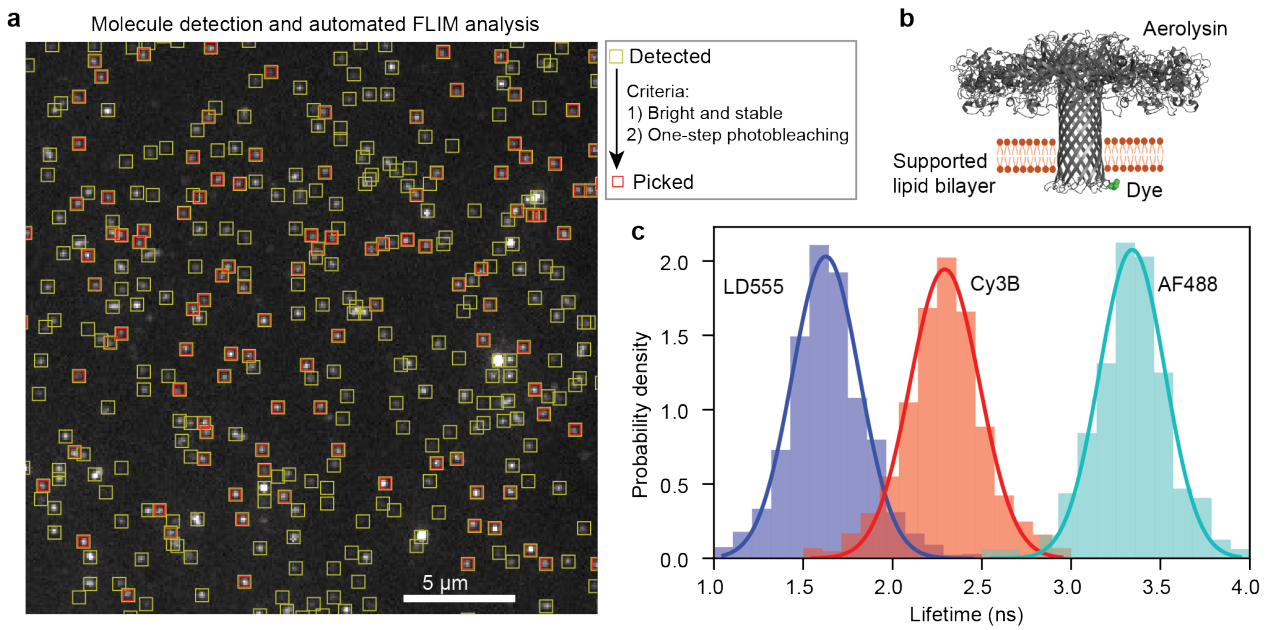

Fig. 2: **Highly multiplexed single-molecule FLIM of labeled membrane proteins**. **a**, Image of Cy3B-labeled aerolysin proteins in a supported lipid bilayer, showing detected molecules (yellow square) and picked molecules (red square) based on their suitability for lifetime assignment. **b**, Structure of the pore-forming aerolysin protein and position of the label. **c** Lifetime distributions obtained by highly multiplexed smFLIM from 5 fields of view for LD555 ($n = 1643$), Cy3B ($n = 2380$) and AF488 ($n = 301$) dyes, respectively. For each distribution the total acquisition time is less than one minute. The gate delay is 2.5 ns for LD555 and Cy3B and 3 ns for AF488.

Optimizing smFLIM

We now turn to the photon efficiency of our approach with the goal of quantifying its potential for *dynamic* lifetime measurements. Due to the low brightness levels characteristic of single-molecule imaging, optimizing the acquisition scheme is essential. For the ability to distinguish different lifetimes in a given sample, it is important to minimize the spread of the measured lifetime values of a given molecule because it will establish the achievable temporal resolution of lifetime measurements. This spread originates mainly from the shot-noise-induced error in the lifetime estimate. The standard metric to quantify the spread of lifetimes obtained through FLIM techniques is the F-value[20,32] that is defined as:

$$F = \sqrt{N}\,\frac{\Delta\tau}{\tau} \tag{2}$$

Where N is the number of available photons used to report a lifetime estimate ($N = 2 \times N_o$ in our scheme), τ and $\Delta\tau$ are the mean and standard deviation of the lifetime estimate distribution, respectively. For example, with $N = 1000$ available photons, the relative error on the lifetime is $\Delta\tau/\tau = F/\sqrt{1000} \approx F \times 3\%$. TCSPC theoretically achieves $F = 1$ in background-free conditions with a large number of channels[33], which means perfect photon efficiency of lifetime measurements, but SPAD array-based TCSPC was limited to $F \geq 1.5$ so far[20]. Gated imaging cannot reach perfect photon efficiency as it relies on rejecting photons. Our 2-gate scheme minimizes photon rejection compared with multi-gate schemes, but still rejects a fraction$(N_o - N_1)/N = (1 - e^{-T/\tau}) \times S/N$ of the available photons.

To evaluate the F-value of our scheme, we imaged fluorescent beads and systematically varied the main parameters of our system: the normalized gate delay $u = T/\tau_{\text{ref}}$ and the signal-to-background ratio (SBR) S/B (Fig. 3a). For the beads, the ground-truth fluorescence lifetime τ_{ref} was measured through a multi-gate approach (details in Figure S2). We then examine the variations of the lifetime statistics as obtained from equation 1 depending on the normalized gate delay u and the SBR, and evaluate F-values through equation 2 (Fig. 3b). The optimal gate delay depends strongly on the SBR: for a large SBR (blue curve), the optimal value of T is slightly above 2τ, in accordance with previous studies relying on Monte Carlo simulations[34,35]. The case of low SBR (red curve) differs in the optimum: the optimal value of T is slightly above τ. The results are in full agreement with the analytical calculation obtained by Heeg[36], which we adapted and extended in the Supplementary Information and in Figure S7. In Figure 3c, the optimal gate delay (top) and resulting F-value (bottom) are presented. The region highlighted in gray corresponds to the regime relevant to single-molecule imaging, in which the strong variation of the optimal gate delay shows the importance of carefully optimizing imaging parameters for such conditions. The resulting F

values in this region are in the range of $2-6$, indicating moderate loss of photon efficiency compared to TCSPC. To obtain a given relative lifetime uncertainty $\Delta\tau/\tau$ the integration time should be set such that $F/\sqrt{2\times N_o}$ is small enough.

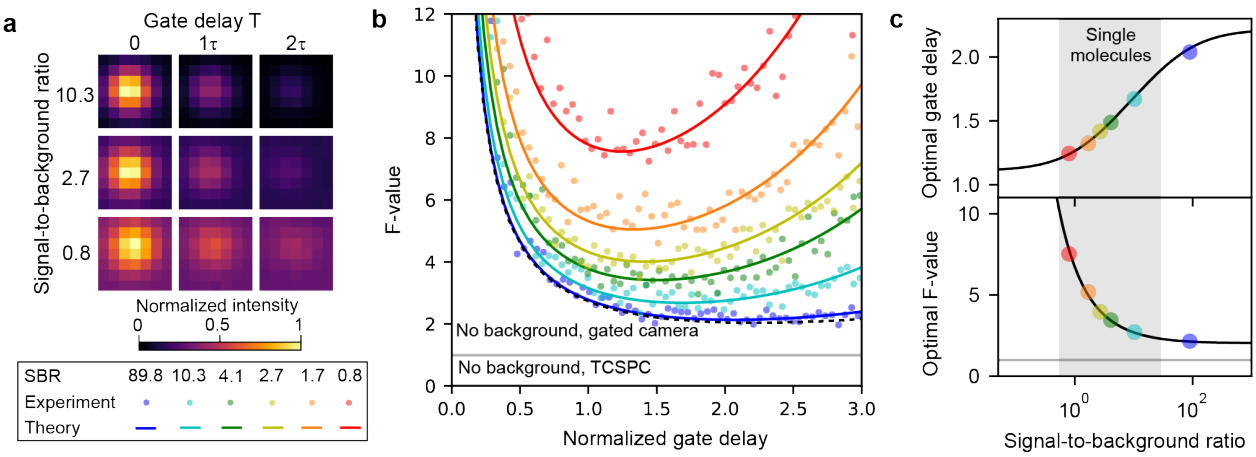

Fig. 3: **Optimization of the acquisition scheme**. **a**, Experimentally varying parameters using fluorescent bead imaging with controlled gate delay T and controlled background resulting in different SBR values. **b**, Experimental F-value are compared with the theoretically expected formula $F(u,b)=\sqrt{2(1+b)(1+b+e^{u}+be^{2u})}/u$ where $b=1/\mathrm{SBR}$ and $u=T/\tau$ is the normalized gate delay. **c**, Optimal gate delay (top) and minimum F-value at this optimal delay value (bottom) as a function of the emitter's SBR. Solid lines are numerically evaluated from deriving the minimum of the F-value formula, and markers indicate experiments.

Single-molecule FRET and dynamic smFLIM measurements

Having shown the multiplexing power of our scheme and identified the optimal operation scheme, we now demonstrate its applicability to monitoring lifetime changes in real time, as exemplified by single-molecule FRET efficiency changes[37]. We imaged DNA origami labeled with a donor dye (Cy3B) and an acceptor dye (Alexa 647) within the FRET range ($d\approx 5.7$ nm, details in Methods). We used a 600 nm shortpass filter in the emission path to image donor molecules only, and quantified FRET through their lifetimes. The sample contained origamis with only a donor and some with a donor-acceptor pair, resulting in distinct lifetime values τ_{D} and τ_{DA} (Fig. 4a-b). Extending the optimization presented in Figure 3 to the case of resolving two lifetimes, we found that the optimal gate delay value is in general close to the longest of the lifetimes in conditions used here (Fig. S8). We identified many occurrences ($n=7029$) of traces exhibiting single-step donor photobleaching, corresponding to the criterion used in Figure 2a. As expected, single-step photobleaching lifetimes were found to contain two populations with stable lifetimes around 2.1 ns and 3.4 ns, respectively.

We examined time-resolved lifetime traces $\tau(t)$ obtained by applying equation 1 to the time-dependent first and second gate signal $N_o(t)$ and $N_1(t)$ (Fig. 4c-d). We find that single-molecule traces sampled at 5 Hz enable clear lifetime discrimination of the emitter types as shown in the inset histogram, but at 25 Hz, shot noise fluctuations prevent clear resolution of molecules through their lifetimes. We found an important number ($n=413$) of intensity traces showing a step-wise increase due to acceptor photobleaching followed by a complete extinction due to donor photobleaching. As shown in the example trace in Figure 4d), a clear lifetime stepwise change can be resolved at 5 Hz sampling. We present in Figure 4d the full histograms of single-step donor photobleaching lifetimes (top) and acceptor-donor photobleaching traces (bottom), where precise lifetime estimates were obtained by applying equation 1 to segment-wise time averages of N_o and N_1. Additional example traces are presented in Figure S4 and Figure S5. The top inset of Figure 4d shows the distribution of single-molecule F-values obtained by applying equation 2 to experimentally observed lifetime fluctuations shown in the traces of Figure 4b-c. We find F-values in the range of $2-6$ in accordance with SBR conditions.

Acceptor-donor photobleaching traces as in Figure 4c enable retrieving the FRET and non-FRET lifetime values of the same molecule, yielding the single-molecule FRET efficiency[38,39] through $E=1-\tau_{\mathrm{DA}}/\tau_{\mathrm{D}}$. We show in Figure 4e the analysis of acceptor-donor photobleaching traces to examine the FRET efficiency and compare to intensity-based lifetime measurements. We find good agreement between the lifetime-based readout of the FRET efficiency (horizontal axis) intensity-based value[38] $E=1-N_{\mathrm{DA}}/N_{\mathrm{D}}$ obtained from photons in the first gate in the presence (N_{DA}) and in the absence (N_{D}) of acceptor. Lifetime-based FRET measurements are generally

preferred over their intensity counterpart, being less prone to errors[40]. This is exemplified by the much sharper lifetime distribution compared to the intensities in Figure S3d. We further demonstrate the rejection of intensity-induced artifacts through examples of two-step photobleaching traces exhibiting no lifetime changes (Fig. S6). This demonstrates the robustness and accuracy of the smFLIM scheme.

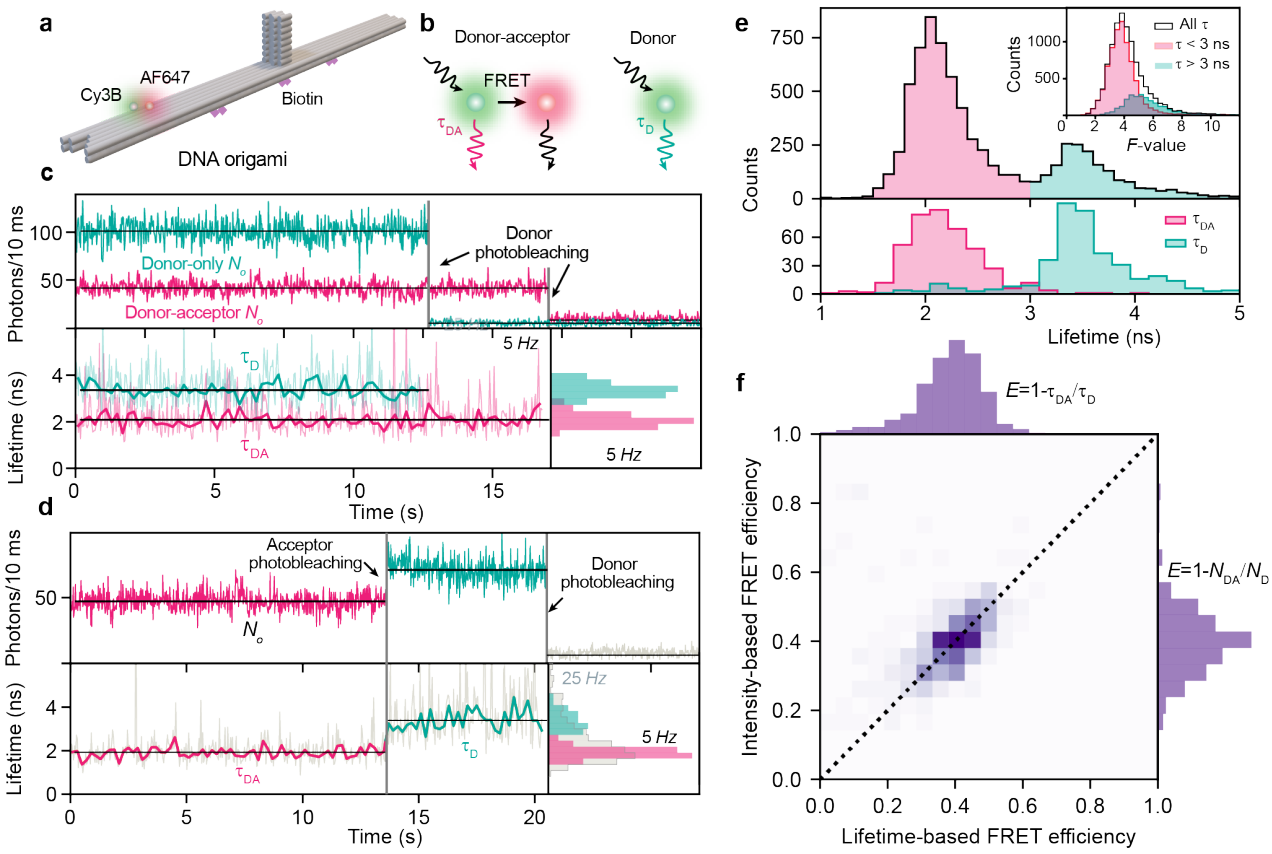

Fig. 4: **Single-molecule FRET and dynamic smFLIM**. **a**, Sketch of the DNA origami design used in this experiment. **b**, Sketch of the emission process of a donor-acceptor pair in the FRET range (5.7 nm distance) where the donor has a lifetime τ_{DA} (left) and donor alone with lifetime τ_{D} (right). **c**, Top: representative intensity traces with single-step photobleaching events. Bottom: corresponding lifetime traces sampled at 5 Hz show a short donor lifetime corresponding to the FRET case, and a long donor lifetime in the absence of FRET. **d**, Representative intensity trace with successive acceptor and donor photobleaching, which enables a readout of the FRET and non-FRET donor lifetimes for the same molecule. Light traces in **c-d** sampled at 25 Hz are too noisy for a clear readout but 5 Hz sampling enables to reduce shot noise fluctuations. **e**, Lifetime distributions obtained from all events corresponding to **c** (top, $n = 7029$) and to **d** (bottom, $n = 413$). The top inset shows experimental F-value in the range $2 - 6$. **f**, Single-molecule FRET histograms obtained through lifetime (horizontal) and intensity (vertical) show good agreement.

Discussion

We demonstrated wide-field single-molecule lifetime imaging that enables multiplexed time-resolved measurements for monitoring dynamic lifetime changes. Our approach enables screening large numbers of molecules and identification of subpopulations exhibiting distinct molecular characteristics. We chose single-molecule FRET for the demonstration but our scheme is ideally suited for other lifetime-changing phenomena and particularly non-radiative energy transfer to bulk metal or 2D materials[5,6,41]. The latter has recently been demonstrated to enable real-time structural biology of enzymes at work through sub-nanometer axial localization[42]. Another important avenue for smFLIM is molecular discrimination for sequencing by synthesis applications[43,44]. These applications rely on the ability to resolve two dye molecules that transiently bind to an enzyme, which can be done using spectral information. Unlike lifetime decays, spectra have non-universal shapes and thus need high-resolution signal to be quantitatively measured. Ratiometric imaging[45] offers an efficient simplification as employed in some high-throughput sequencing systems. For example, a dual view imaging system is used together with a dual-color

excitation scheme, which enables resolving four different probes. Unlike these widespread approaches, our wide-field single-molecule FLIM scheme offers an optimal throughput for separating populations of molecules without splitting the single-molecule photon budget over different channels. Our approach also reduces the optics requirements which makes it highly suitable for integrated platforms. In this work, we demonstrated that 2-3 distinct lifetime species can clearly be discriminated (Fig. 2c and 4d), which opens the door for lifetime-based molecule discrimination as recently used in an integrated protein sequencer[46].

Furthermore, our scheme is applicable to wide-field super-resolution imaging approaches such as PALM, STORM and DNA-PAINT[47]. The latter would be a natural application of our scheme as binding times can be controlled precisely to yield ~ 1000 photons from 100 ms-long binding events and would yield sub-10% relative error in lifetime considering experimental F-values. This opens the door for lifetime-based target multiplexing in super-resolution imaging as demonstrated through confocal FLIM and TCSPC approaches[12,24,48,49], as well as lifetime-based super-resolved sensing with environment-sensitive probes[50,51].

Outlook

This work establishes gated single-photon cameras as a promising tool for highly multiplexed single-molecule lifetime measurements relying on an optimized scheme enabling time-resolved measurements. The use of this approach should benefit lifetime-based assays of biomolecules for structural biology, diagnostic assays, biopolymer sequencing and single-molecule super-resolution microscopy. SPAD cameras have zero readout noise, which makes them theoretically ideal for high-speed imaging, but two drawbacks remain: warm pixels (Fig. S9) and the lower PDE compared with EMCCD and sCMOS cameras. Nevertheless, we have demonstrated their suitability for single-molecule lifetime imaging, a task that neither EMCCD nor sCMOS cameras could achieve reliably thus far. Compared to confocal TCSPC, our method has a dramatically higher throughput at the cost of a reduced temporal resolution[42].

Still, we can anticipate several technological improvements in the near future which will improve the detector sensitivity, PDE and decrease the instrument noise, ultimately pushing the bandwidth of our scheme. Improvements in the fill factor and cooling of SPAD cameras are expected to increase the PDE and suppress the warm pixel issue. Newer variants of the SPAD camera that achieve 100% temporal aperture could realize the same scheme without photon rejection[52], thus decreasing the F-values and increasing the temporal resolution by up to 40%. With these combined improvements, we expect our approach to facilitate reliable wide-field single-molecule lifetime measurements at video-rate speeds in the future.

Methods

Microscope details

Our setup is a home-built objective-type TIRF microscope based on a commercial inverted microscope body (Olympus IX-71).

Illumination was performed with a supercontinuum laser (NKT Photonics SuperK Fianium FIU-15) equipped with a pulse picker to divide the native repetition rate of 78 MHz. A repetition rate of 26 MHz was used. The supercontinuum laser was fiber-coupled to a tunable filter (NKT Photonics SuperK VARIA) which enabled selecting a spectral excitation window of 10 nm or more, centered at the desired wavelength. The unpolarized laser beam coming out of the tunable filter was fiber-coupled to the SuperK collimator, after which the laser beam propagated in free space. A 20× beam expander (Thorlabs GBE20-A) was used in combination with another 2× beam expander (GBE02-A) to flatten the collimated light, which was then focused by an achromatic doublet lens (Thorlabs AC508-300-A-ML) to fill the back focal plane of the high-NA objective (Olympus UPlanSApo100×, NA=1.50). The measured power at the back focal plane was around 5 mW for typical illumination conditions.

The microscope filter wheel is equipped with dichroic mirror/emission filter sets (Semrock Di03-R488-t3-25x36/BLP01-488R-25, Di03-R561-t3-25x36/LP02-561RU-25) that enabled filtering out the excitation light and directing emitted light at the camera. For smFRET experiments, a 600 nm shortpass filter (Thorlabs FESH0600) was placed before the camera to selectively image the donor.

SPAD camera operation

The camera used in this work is the SPAD512^2 from Pi Imaging Technology, which is the commercial version of the SwissSPAD2 camera developed by the AQUA group at EPFL[28]. It is a 512×512 SPAD array in which the single-photon-sensitive pixels of the camera are operated synchronously. The camera thus outputs binary images, at a maximum speed of $97,700$ frames-per-second (fps). $2^q - 1$ binary images can be binned to reconstruct q-bit

images akin to conventional EMCCD or sCMOS images, with the difference that no analog-to-digital conversion and no amplification are required, since photons are converted to digital codes directly. Optionally, the camera's time gate can be used to reject photons based on their time delay with a reference signal. It can be precisely positioned by $\sim$ 18 ps steps. It is equipped with a microlens array for a $\sim$ 5-fold increase of the native fill factor of 10.5%[25]. The camera chip was positioned at the primary image plane of the microscope. The built-in optional 1.6× magnification of the microscope was used to achieve a pixel size at the image of 100 nm. An operating voltage $V_{\text{ex}} = 4$ V was used for all measurements to minimize noise (at the cost of a slightly reduced PDE[28]). The cooling fans of the camera were used for all measurements. Images were acquired in the 8-bit mode with 20 ms exposure times for proteins in supported lipid bilayer, and 6-bit mode with 10 ms exposure time for DNA origami imaging. Further information on the camera is available at https://piimaging.com/doc-spad512s.

Data analysis

Acquisition and pre-processing

The camera was operated through the software provided by Pi Imaging technology. 6-bit or 8-bit images were saved in the PNG extension. For processing, images were analyzed using python. The photon counts of pixels were corrected by *(i)* applying the dark count rate mask detailed in Figure S9 to exclude warm pixels and *(ii)* performing pile-up correction.

Pile-up correction

Pile-up correction compensates for possible multiple photon arrivals during integration on the single-photon detectors. For this, the measured counts N_{measured} were transformed into the actual value N used for the analysis in this work using the formula, valid for q-bit imaging:

$$N = -\log\left(1 - \frac{N_{\text{measured}}}{2^q - 1}\right) \times (2^q - 1) \tag{3}$$

In practice, we only used partial filling of the camera dynamic range (<25 photons per pixel per frame for 8-bit images, which is below 10% of the saturation of the pixel), ensuring that the above correction would not distort data much, and would not contribute extra error in lifetime assignments.

Molecule detection and intensity trace extraction

Molecules were detected through the Laplacian of Gaussian approach applied to the average image from the first 10 frames following illumination. We consistently used the following parameters: $\sigma_{\min} = 1$, $\sigma_{\max} = 5$, a threshold of 1 and overlap of 0.9 as they worked reliably on our images with a pixel size of 100 nm. The time traces of the molecules were defined as the sum of the corrected photon counts of pixels within a box around the blob center position. We chose the box size to be 5×5 pixels (0.25µm^2) which did not crop emitter spots significantly while minimizing overlap with neighbouring emitters.

Lifetime calculations

Following trace extraction, we obtain emitter-wise first gate $N_o(t)$ and second gate $N_1(t)$ time series. We developed two approaches for lifetime extraction: *(i)* a simple, conservative single-lifetime calculation approach for static lifetime measurements, and *(ii)* a more refined, time-resolved lifetime trace calculation. We convolved the trace with a step function and applied a peak finding algorithm to locate intensity change steps. We used a 10-frame duration convolution kernel, and a minimum peak intensity of 15 corrected photons which offered a good compromise between time sensitivity and noise, as shown in Figure S5.

For static lifetime calculations, we only kept stable traces with a single photobleaching step. For the histograms presented in Figure 2c, we applied stringent selection criteria illustrated by the large rejection rate in Figure 2a. The criteria for the time traces are the following: a brightness cutoff to remove the dimmest emitters, a stability criterion to remove high-fluctuation traces, and a single-step photobleaching criterion to ensure that single molecules were imaged. We set the brightness threshold to 50 corrected photons per 20 ms gated frame. Our analysis also identifies the photobleaching time t_{OFF}. We chose the intensity stability criterion that ON-state fluctuation of $N_o(t)$ during the interval $[0, t_{\text{OFF}}]$ should not exceed twice the shot noise limit.

For dynamic lifetime calculations, we keep all traces and identify their ascending and descending steps by finding negative and positive peaks in their step-convolved signal. The series of times of ascending and descending steps $\{t_{\text{up}}\}$ and $\{t_{\text{down}}\}$ provided a way to classify traces according to their temporal dynamics. We further identified the background level B as the lowest observed level following the last photobleaching step. This enabled us to apply equation 1 in a straightforward way to the time trace, which yields a lifetime value per molecule per gate sequence (N_o, N_1) (green dots in all plots of Figures S4,S5,S6). As these values were subject to considerable shot-noise-induced fluctuations when sampled at the imaging rate of 50 Hz, we could time-average them to produce more precise results at 25 Hz or 5 Hz (blue dots in the plots). Refining on this approach, we could use the stable

segments of the trace identified through convolution and peak finding and provide precise segment-averaged lifetime estimates (black horizontal lines in lifetime plots).

Sample preparation

Aerolysin purification and labeling

The clone of the full-length wild-type aerolysin protein (in the pET22b vector with a C-terminal hexa-histidine-tag (His-tag)) was kindly provided by the Van der Goot laboratory at EPFL. The mutant L249C was generated by Genscript using the wild-type plasmid as a template. Both plasmids were transformed separately into BL21 (DE3 Plys) E. coli cells by heat shock. Cells were grown to an optical density of 0.6 to 0.7 at 600 nm in LB media. Protein expression was induced by the addition of 1 mM IPTG and subsequent growth overnight at 18 °C. Cell pellets were resuspended in lysis buffer (500 mM NaCl, 50 mM tris, pH 7,4), mixed with cOmplete Protease Inhibitor Cocktail, and then lysed by sonication on ice. The turbonuclease solution was added to the resulting suspension and then centrifuged (20,000 g for 30 minutes at 4 °C). After syringe filtration over a 0.45 µm filter, the supernatant was applied to a HisTrap HP column previously equilibrated with lysis buffer. The proteins were eluted as monomers with a gradient of 30 column volumes of elution buffer (500 mM NaCl, 50 mM tris, 500 mM imidazole, pH 7,4). Aerolysin-containing fractions were then buffer-exchanged to the final buffer (20 mM tris, 150 mM NaCl, pH 7.4) using a HiPrep desalting column. The purification of the selected fractions was confirmed by SDS-polyacrylamide gel electrophoresis. The proteins were concentrated to 0.5 mg/mL and stored at -20 °C.

The dyes used for labeling were each dissolved in anhydrous DMSO at a final concentration of 1 mM concentration to ensure stability and stored at -80°C. All steps involving the dyes were performed under light-protected conditions to prevent photodegradation. For the labeling process, mutant L249C was mixed with 10 molar excess of TCEP in a reaction vessel from which air was removed and replaced with an inert gas to prevent oxidation. This mixture was allowed to react for 20 minutes at room temperature. The dye of choice was then added to the reaction mixture at a 10 molar excess compared to the protein concentration. The reaction was left to proceed overnight at 4°C. The protein was purified using 10 kDa cutoff Amicon® Ultra Centrifugal Filter to effectively eliminate the free dye from the sample. A total of five spins (10,000 g for 1 minute each) were performed, with the final buffer added after each spin to ensure the thorough removal of the free dye.

The aerolysin activation process to undergo conformational changes and enable heptamerization includes incubation with trypsin in agarose at a ratio of 1:4 (trypsin:pore, v/v). To ensure that the oligomers consist of one labeled monomer and six unlabeled ones, the labeled monomers of the L249C mutant were mixed with aerolysin wild-type monomers in a molar ratio 1:10, followed by mixing with trypsin and incubating on a spin wheel for 2h at 44 °C. The final labeled proteins were obtained by spinning the sample at 11,000 g for 10 minutes and collecting the supernatant.

Supported lipid bilayer preparation and assembly

DPHPC lipids (0.35-0.4 mg) were initially dissolved in chloroform. The chloroform was removed by subjecting the lipid solution to vacuum evaporation in a desiccator for 1 hour, resulting in a dried lipid film. The film was rehydrated with 1 mL of PBS (pH 7.4) and subjected to brief sonication for 5 minutes to aid in the dissolution of the lipids. The resulting lipid suspension was incubated overnight at 37°C on a heating plate. After incubation, the solution was vortexed until a milky appearance was achieved, followed by further sonication for 1 hour until the solution became clear. To stabilize the vesicles, 1 µL of a 3M calcium chloride stock solution was added to the lipid suspension to reach a final concentration of 3 mM. The vesicle size can be verified using dynamic light scattering. For the layer deposition, coverslips were first cleaned with Hellmanex III detergent to ensure a pristine surface. The deposition chambers, including the coverslip and cylinder, were then plasma-cleaned to enhance the adhesion of the lipid bilayers. The lipid solution was deposited onto the cleaned chamber and incubated in a humidified chamber for at least 90 minutes. Following incubation, the chamber was washed 5-6 times with PBS to remove any unbound lipids and ensure a well-defined lipid bilayer. The proteins were added to the solution in a final concentration of 1 nM and incubated for 10min, or longer to reach the desired density of single molecules, before washing again with PBS for another 5 times to reduce the background 10 minutes, or longer, to reach the desired density of single molecules, before washing again with PBS for another 5 times to reduce background noise during the measurements.

Origami design and assembly

DNA origami synthesis

The DNA origami design employed in our studies was designed using CaDNAno6. The structure is available at https://nanobase.org/structure/146 and details can be found in a previous publication[53]. A 7249-nucleotide long

scaffold extracted from the M13mp18 bacteriophage (Bayou Biolabs LLC) was folded into the desired shape using 243 staples in 1× TAE (40 mM Tris, 10 mM acetate, 1 mM EDTA), 12 mM $MgCl_2$, pH 8 buffer. It was mixed in a 10-fold excess of staples (purchased from IDT) over the scaffold, and 100-fold for the functional staples (fluorophores and biotin, purchased from Biomers GmbH) shown in Table S2. The mixture was heated to 70 °C and cooled to 25 °C at a rate of 1 °C every 20 min. The DNA origami structures were later purified by 1% agarose (LE Agarose, Biozym Scientific GmbH) gel electrophoresis at 70 V for 2 h and stored at 4 °C.

DNA origami sample preparation

Coverslips were sonicated in 1% Hellmanex III detergent in deionized water for 10 min, blow-dried with nitrogen and subsequently O_2 plasma cleaned for 90 seconds. A 100 µm cylindrical glass chamber was then glued to the coverslip using Kwik-kast (World Precision Instruments). A 10 mg/ml BSA-Biotin solution in 1×PBS was then incubated on the surface for 30 min followed by 30 min of 10 mg/ml Neutravidin in 1×PBS to create attachment sites all over the surface of the coverslip. Origamis at a concentration of 50 pM in 1× TAE 12mM $MgCl_2$ are added to the surface and their immobilization onto the glass coverslip is monitored during the incubation using a wide-field fluorescence setup. Washing is performed 3 times after each incubation step with 1× TAE 12 mM $MgCl_2$.

Author contributions

N.R. and A.R. conceived the project; N.R., A.R., S.B., G.A. and R.R. designed experiments; N.R., S.B. and M.M. performed experiments. N.R. analyzed data with input from S.B. ; N.R. performed the theoretical analysis and simulations with input from S.B. ; M.M., M.J.M. and M.D.P. established the protocol for protein construct preparation and control. M.M. purified proteins under the supervision of M.J.M., M.D.P. and A.R. ; N.S. designed and prepared the DNA origami under the supervision of G.A. ; M.M., S.B. and N.R. prepared samples; A.R. supervised the project; N.R. wrote the manuscript with input from S.B., R.R., G.A., C.B., E.C. and A.R; All authors discussed the results and commented on the manuscript.

Competing interests

Claudio Bruschini and Edoardo Charbon are co-founders of Pi Imaging Technology. Edoardo Charbon is also co-founder of Novoviz which has not been involved in this work.

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Supplementary Information

Multiplexed lifetime imaging of single molecules with a gated single-photon camera

Nathan Ronceray, Salim Bennani, Marianna Mitsioni, Nicole Siegel, Maria Josefina Marcaida, Claudio Bruschini, Edoardo Charbon, Rahul Roy, Matteo Dal Peraro, Guillermo P. Acuna and Aleksandra Radenovic

Sample	Dye	Lifetime (ns)	Lifetime standard deviation (ns)
Aerolysin on supported lipid bilayer	LD555	1.63	0.19
Aerolysin on supported lipid bilayer	Cy3B	2.29	0.19
Aerolysin on supported lipid bilayer	AF488	3.34	0.18
DNA origami (FRET)	Cy3B	2.09	0.25
DNA origami (no FRET)	Cy3B	3.45	0.24

Table S1: **Lifetime values reported in this work**, as obtained from gaussian fitting of the lifetime distributions. We find a notable difference between Cy3B in the aerolysin-lipid system and in the DNA origami system. This could be explained by the very different environment of the dye in the two experiments. For example, when bound to single-stranded DNA in DNA-PAINT experiments, an intermediate value of 2.8 ns was found[12].

Staple Name	Sequence (5'→ 3')
Biotin 1	ACAGGAAGATTGTCCCCCTTATTCACCCTCATTTGTTTC - **Biotin**
Biotin 2	GTTGATAGATATAAGCATAAGTATAGC - **Biotin**
Biotin 3	AGAGTACTCACGCTAACCTTTAATTGC - **Biotin**
Biotin 4	CACTAAAACACTCACGAACTAACACTAAAGT - **Biotin**
Biotin 5	TCACGACGTTGGGCGCTTTGGTAAAAC - **Biotin**
Biotin 6	CAGAGATAGCGATAGTGAATAACATAA - **Biotin**
FRET acceptor	ATT ATC ACC GGA AAT GTT AGC AAA CGT AGA A - **Alexa647**
FRET donor	AAT ATT GAC GTC ACC GTG CGT AGA TTT TCA GG - **Cy3B**

Table S2: **List of staples for the DNA origami design**. The preparation details are provided in the Methods section.

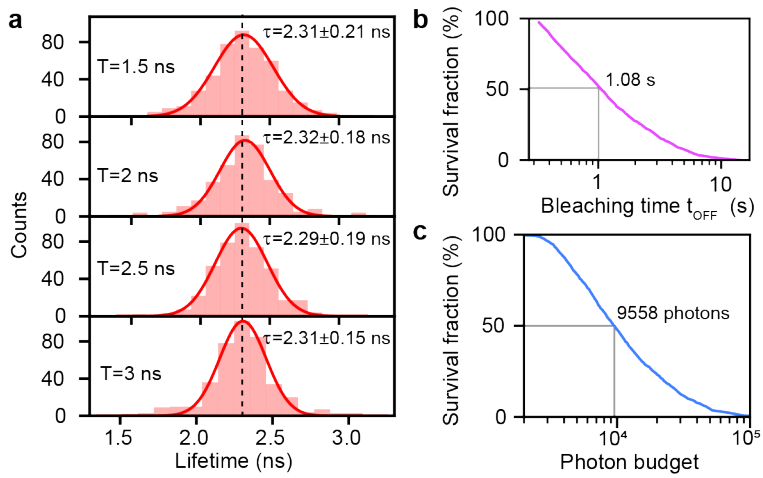

Fig. S1: **Extended data on Cy3B-labeled aerolysin in supported lipid bilayer**. **a**, Comparison of single-molecule lifetime distributions with different gate delay T. We observe no significant gate delay-induced lifetime bias (dashed line). The distributions contain fewer counts than in Figure 2c as they were taken from a single field of view. **b**, Bleaching time t_{OFF} survival fraction distribution, showing a median bleaching time of 1.08 s. **c**, Photon budget survival fraction distribution, where the photon budget was obtained by summing all available photons before photobleaching, calculated as $2 \times N_o \times t_{\mathrm{OFF}}/\Delta t$ where Δt is the frame integration time. The median photon budget of $\sim$ 9600 photons is indicated.

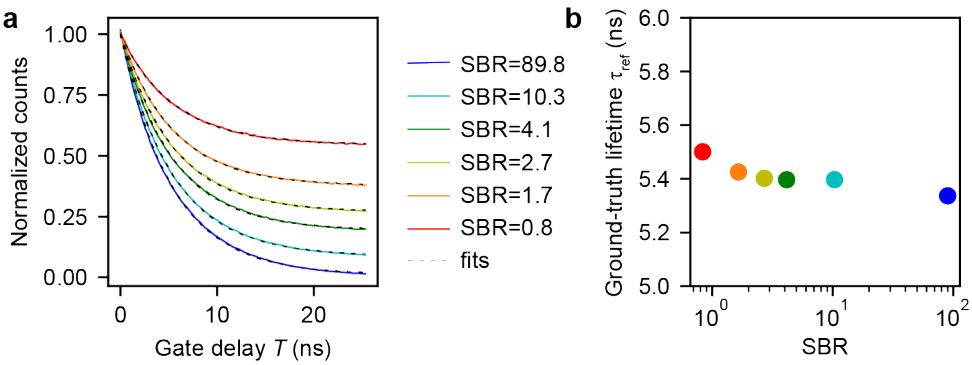

Fig. S2: **Determination of reference lifetimes of the fluorescent beads.** Fluorescent beads (PS-Speck™ Microscope Point Source Kit, ThermoFisher Scientific) were imaged at 1% of the power used for single molecules with 3 ms exposure time in 8-bit mode. **a**, The decay was measured using 100 gate delay positions spaced by 256 ps. An average decay was obtained from 90 repetitions, over which no noticeable photobleaching occurred. The reference lifetime τ_{ref} was obtained from least-square fitting of the curves to a single exponential with background. **b**, As we found that the background induced mild bias on the lifetime readout, we used the measured reference lifetime value for calculation of the F-value curves in Figure 3b. Error bars are smaller than the markers.

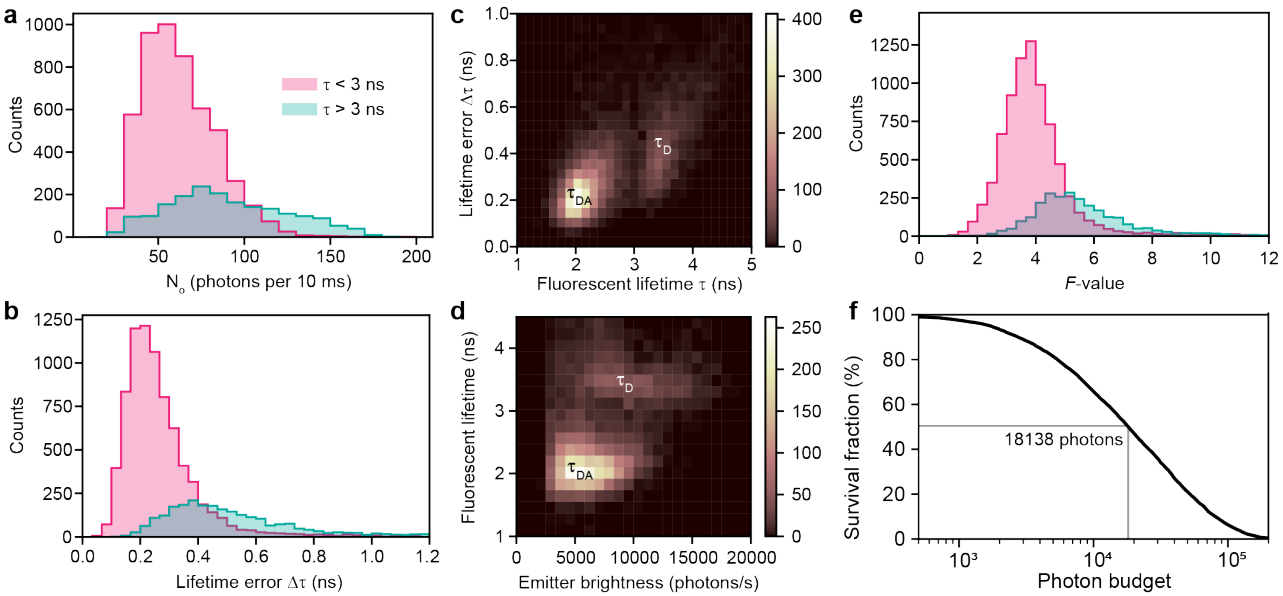

Fig. S3: **smFRET extended data underlying Figure 4d. a**, Histogram of N_o values obtained with 10 ms integration time. **b**, Histogram of lifetime errors for 200 ms integration time, calculated as the standard deviation of the 5 Hz sampling trace for traces longer than 600 ms. **c**, Lifetime-lifetime error 2D histogram revealing the two emitter populations. **d**, Brightness-lifetime 2D histogram. The brightness in photons per second is simply $N_o \times 100$. **e**, F-value histogram obtained with the lifetime distribution in Figure 4d and the standard deviation in **b**. Equation 2 was applied taking $N = 2 \times 10\, N_o$. **f**, Photon budget cumulative distribution function, limited by photobleaching. The median value of ~ 18100 photons is indicated.

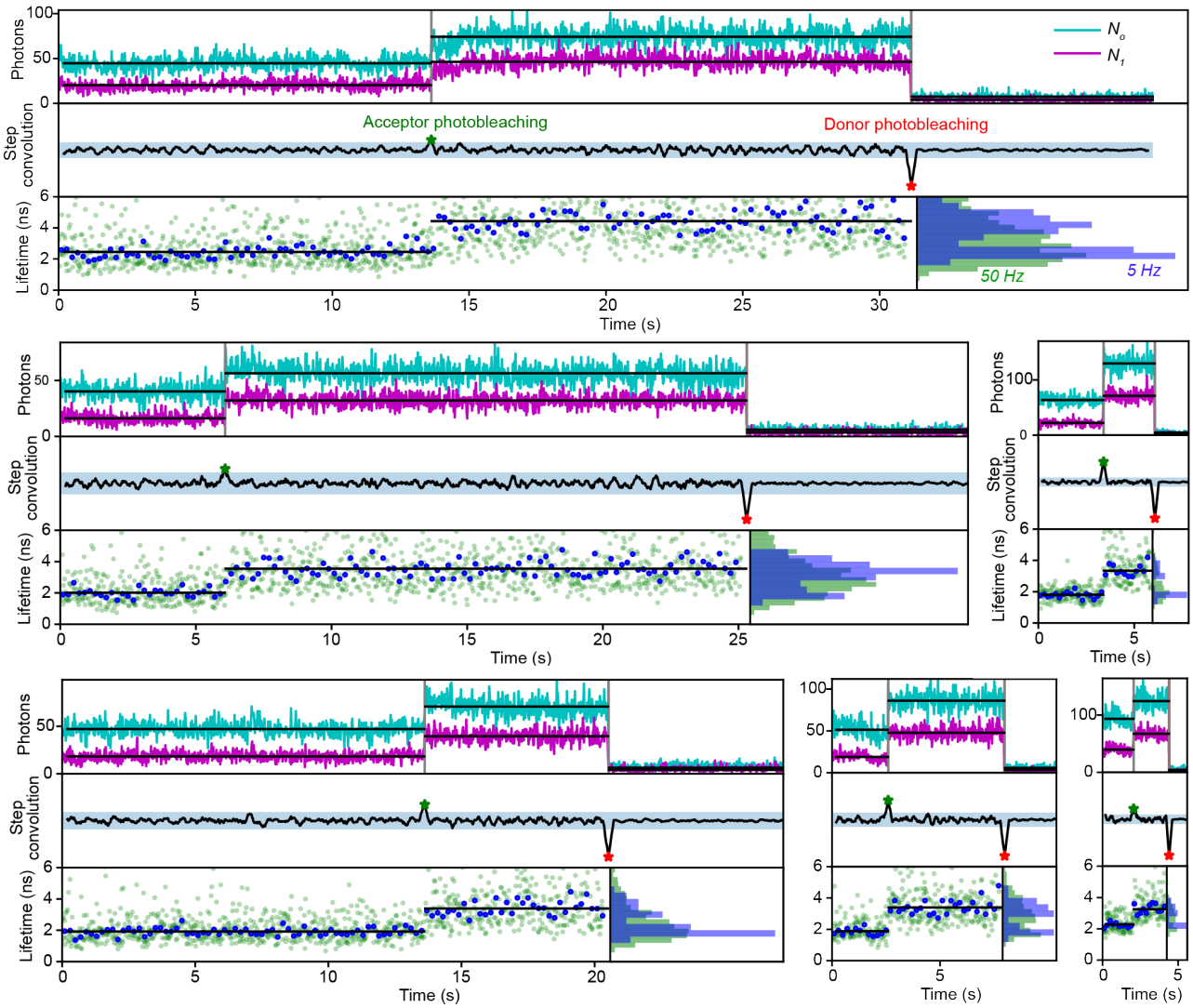

Fig. S4: **Donor-acceptor photobleaching representative traces**. Example traces with the raw intensity in the first and second gate (top), its step convolution used for step finding (middle) and the lifetime trace at the native frame rate of 50 Hz and upon ten-fold time binning to obtain a rate of 5 Hz. The trace presented in Figure 4c is reproduced at the bottom left. The highlighted region in the step convolution shows the peak amplitude threshold described in the Methods.

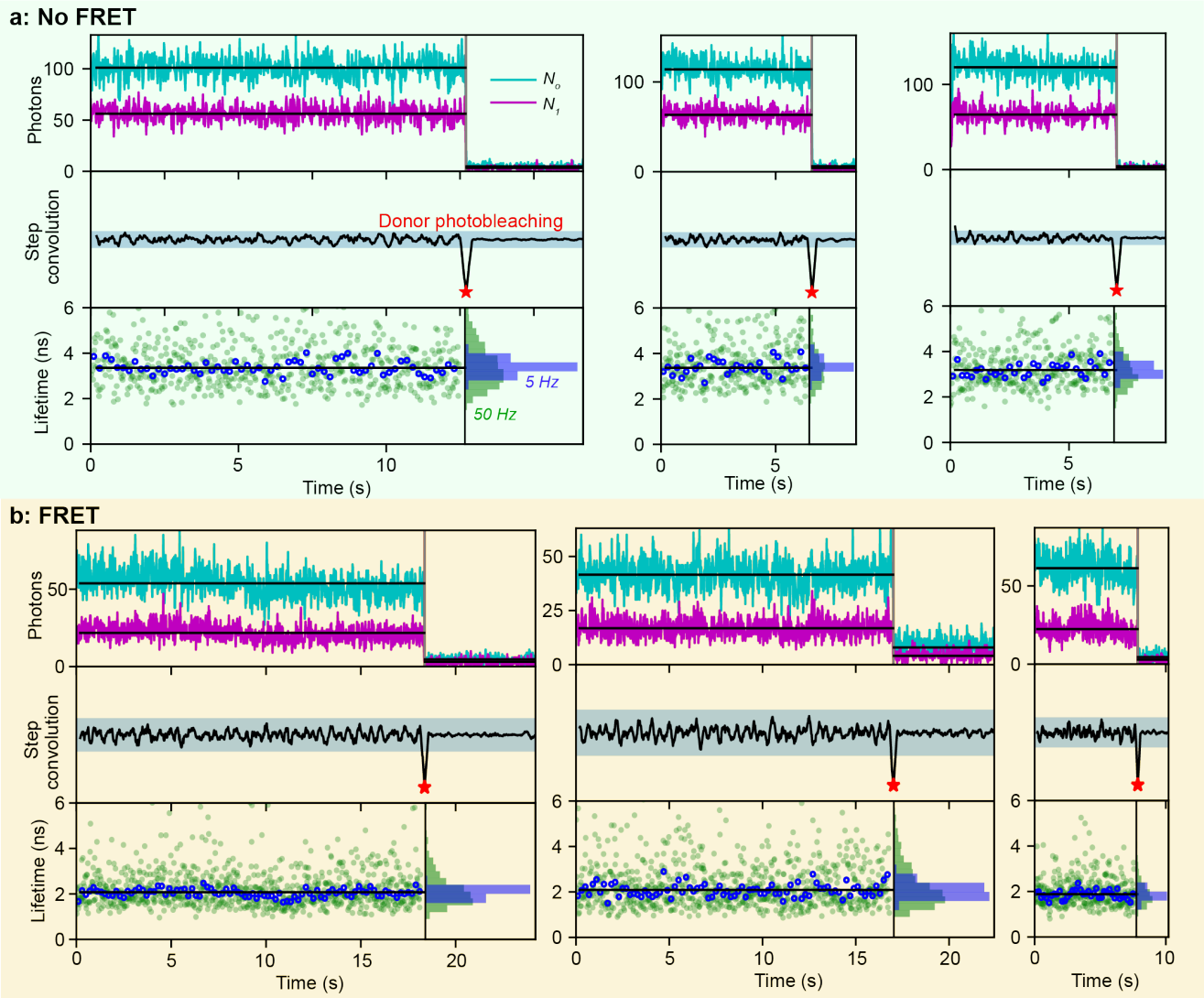

Fig. S5: **One-step donor photobleaching traces**. Six example traces of one-step photobleaching traces in the no-FRET (top) and FRET (bottom) cases. Each trace graph layout is the same as in Figure S4. The left traces correspond to the ones provided in Figure 4b.

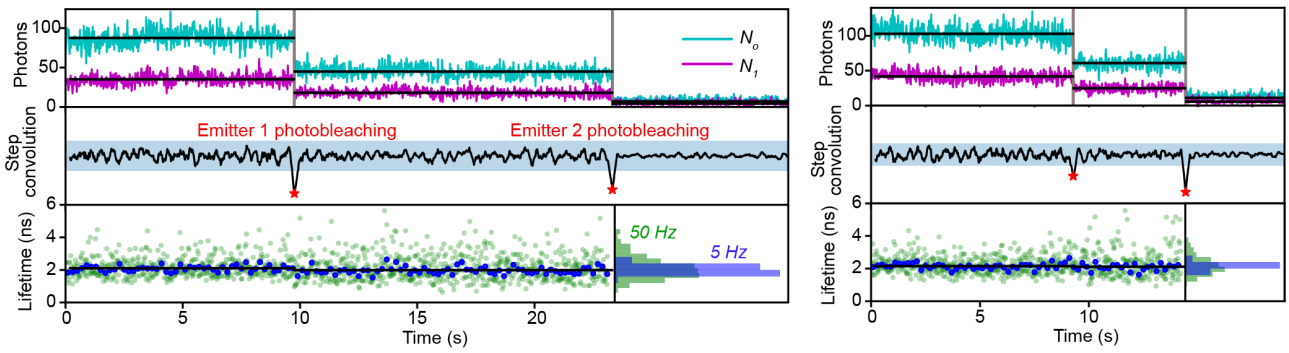

Fig. S6: **Two-step donor photobleaching without lifetime change**. Two example traces of two-step donor photobleaching without lifetime change, showing the absence of intensity-induced bias. Each trace graph layout is the same as in Figure S4.

Derivation of the optimal gate delay

We define the optimal gate delay as the one that minimizes the uncertainty of the lifetime measurement with a given photon budget N. The quantity that quantifies the photon cost of the lifetime measurement is the F-factor defined by $\Delta\tau/\tau = F/\sqrt{N}$. It is affected by the background level B as well as the choice of the gate delay T. It should be noted that some studies use a different definition of the F-value, where N is taken as the number of detected photons rather than the number of available photons, resulting in a metric that does not truly reflect the performance of gated imaging schemes[25]. With the typical values of $N = 100 - 1000$ detected photons used in this work, the pixel-wise binomial statistics of photon counts of the SPAD camera[54] aggregated by emitter point spread function have gaussian-like statistics and verify $\Delta N \approx \sqrt{N}$.

We extend here the analytical calculation by Heeg[36]: assuming for simplicity a constant nonfluorescent background B, the first gate measurement with a fluorescent signal of S reads $N_o = S + B$, and the second gate measurement delayed by T reads $N_1 = Se^{-T/\tau} + B$. Therefore, the lifetime can be retrieved as:

$$\tau/T = 1/\ln\left(\frac{N_o - B}{N_1 - B}\right) = f(N_o, N_1) \tag{4}$$

We thus predict that the error on the τ/T estimate from N_o and N_1 will be given by:

$$\frac{\Delta\tau}{T} = \sqrt{\left(\frac{\partial f}{\partial N_o}\right)^2 \Delta N_o^2 + \left(\frac{\partial f}{\partial N_1}\right)^2 \Delta N_1^2} = \frac{1}{\ln^2(\frac{N_o}{N_1})}\sqrt{\left(\frac{\Delta N_o}{N_o}\right)^2 + \left(\frac{\Delta N_1}{N_1}\right)^2} \tag{5}$$

$$\frac{\Delta\tau}{T} = \frac{1}{\ln^2(\frac{N_o - B}{N_1 - B})}\sqrt{\left(\frac{\Delta N_o}{N_o - B}\right)^2 + \left(\frac{\Delta N_1}{N_1 - B}\right)^2} \tag{6}$$

Considering that $N_o \approx S + B$ and $N_1 \approx Se^{-T/\tau} + B$, we obtain:

$$\frac{\Delta\tau}{\tau} = \frac{1}{u}\sqrt{\left(\frac{\sqrt{S+B}}{S}\right)^2 + \left(\frac{\sqrt{Se^{-u} + B}}{Se^{-u}}\right)^2} \tag{7}$$

Where we introduced $u = T/\tau$. Finally, introducing $b = B/S$ (the inverse of the SBR), we obtain:

$$\frac{\Delta\tau}{\tau} = \frac{1}{\sqrt{S}}\sqrt{1 + b + e^u + be^{2u}}/u \tag{8}$$

The estimate of τ is therefore limited by shot noise (factor $1/\sqrt{S}$), with a prefactor that depends on the choice of the gate delay T with respect to the lifetime of the emitter τ. As the measurement of (N_o, N_1) involved a photon budget of $N = 2(S + B)$, we finally obtain:

$$F(u, b) = \sqrt{2(S+B)}\frac{\Delta\tau}{\tau} = \sqrt{2(1+b)(1 + b + e^u + be^{2u})}/u \tag{9}$$

The optimal normalized gate delay $u^*(b)$ is obtained through numerically minimizing $F(u, b)$ with respect to u.

Bias in lifetime estimate

When using equation 1 to estimate the lifetime of an emitter from two gated frames, there can be a bias in the few-photon regime: the distribution of repeated lifetime estimates from N photons $\tau(S, B, T)$ may not have a mean value corresponding to the ground-truth lifetime τ_{ref} obtained with $N = S + B \to \infty$ (written as τ for simplicity in all the above). These can be formalized by considering the average relative bias error $\epsilon(S, B, T) = \frac{\langle\tau(S,B,T)\rangle - \tau_{\text{ref}}}{\tau_{\text{ref}}}$. We can see that this quantify is given by:

$$\epsilon = \frac{\langle f(N_o, N_1)\rangle - f(\langle N_o\rangle, \langle N_1\rangle)}{f(\langle N_o\rangle, \langle N_1\rangle)} \tag{10}$$

A convexity analysis is required to evaluate the bias by first computing second order derivatives.

$$\frac{\partial^2 f}{\partial N_o^2} = \frac{1}{(N_o - B)^2(\log\frac{N_o - B}{N_1 - B})^2} + \frac{2}{(N_o - B)^2(\log\frac{N_o - B}{N_1 - B})^3} \tag{11}$$

$$\frac{\partial^2 f}{\partial N_1^2} = \frac{-1}{(N_1 - B)^2(\log \frac{N_o - B}{N_1 - B})^2} + \frac{2}{(N_1 - B)^2(\log \frac{N_o - B}{N_1 - B})^3} \tag{12}$$

The bias can be obtained through a second-order expansion of f around $(\langle N_o \rangle, \langle N_1 \rangle)$:

$$\epsilon = \Big(\frac{\partial^2 f}{\partial N_o^2}(\langle N_o \rangle, \langle N_1 \rangle)\frac{\Delta N_o^2}{2} + \frac{\partial^2 f}{\partial N_1^2}(\langle N_o \rangle, \langle N_1 \rangle)\frac{\Delta N_1^2}{2}\Big)/f(\langle N_o \rangle, \langle N_1 \rangle) \tag{13}$$

Using the expressions for the derivatives derived above and replacing variables by their mean values, we obtain:

$$\epsilon(S, u, b) = \frac{1}{2Su^2}\big((2+u)(1+b) + (2-u)e^{2u}(e^{-u} + b)\big) \tag{14}$$

As is standard for such statistical estimators, the bias is inversely proportional to the signal.

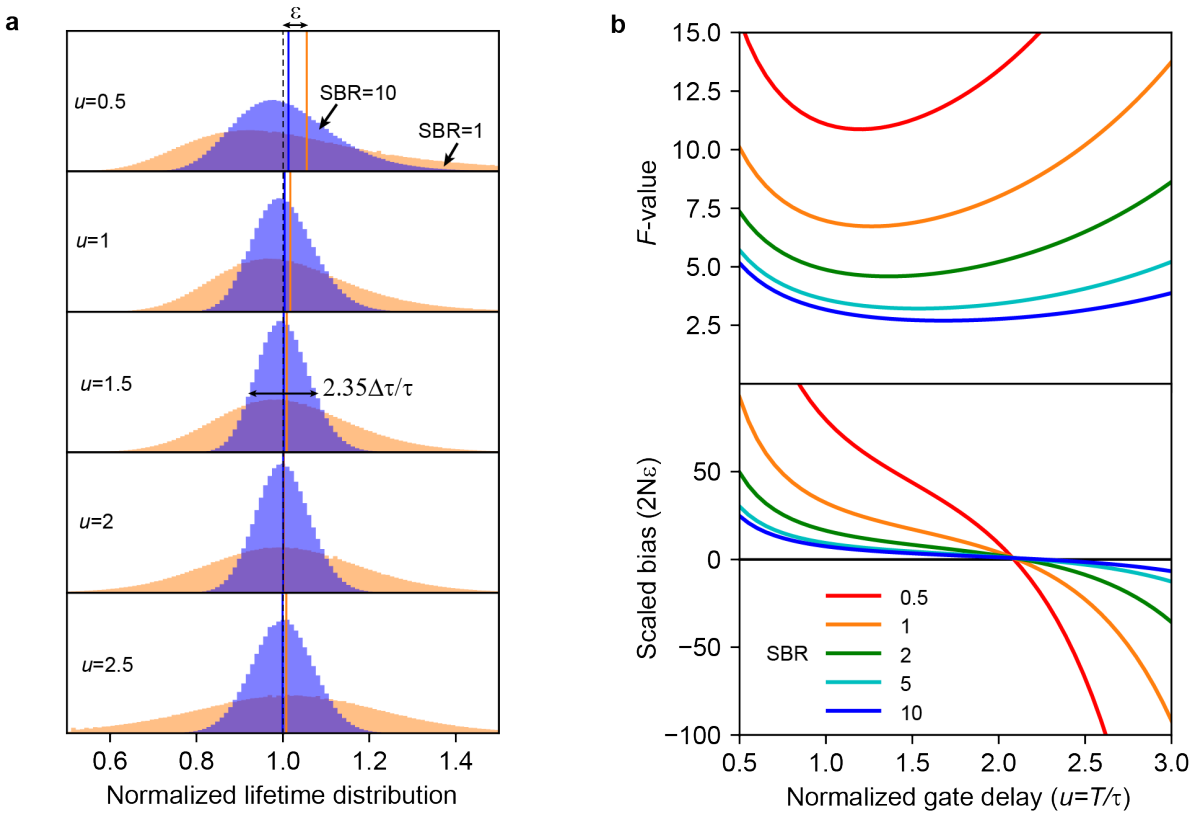

Fig. S7: **Variance and bias of the lifetime estimation: Monte Carlo simulations and analytical results**. **a,** Monte Carlo simulations of the lifetime estimate distributions for SBR of 1 (orange) and 10 (blue) with different normalized gate delays $u = 0.5, 1, 1.5, 2, 2.5$. The distribution was obtained by simulating 10^8 emitters with an average Poissonian photon budget of $2N = 2000$ photons per lifetime estimate. The arrows illustrate both the bias ϵ and the full width at half maximum of the distribution $\approx 2.35\Delta\tau/\tau$. **b**, Analytical F-value given by $F = \sqrt{2N}\Delta\tau/\tau$ and the scaled bias $2N\epsilon$ as a function of the normalized gate delay for different SBR values ranging from 0.5 to 10. The analytical calculations details above were checked for correctness using Monte Carlo simulations.

Resolving two lifetimes

A practical application scenario of single-molecule FLIM is to discriminate between two molecules or molecular states based on their lifetimes. We now consider the optimal scheme for resolving two lifetimes τ_{short} and τ_{long}. We define their ratio as $X = \tau_{\text{short}}/\tau_{\text{long}}$ and we aim to optimize the gate delay T to minimize fluctuations of X for the optimal separation of the two lifetimes. For this, we minimize the relative uncertainty on the lifetime ratio given by:

$$\frac{\Delta X}{X} = \sqrt{\frac{\Delta\tau_{\text{short}}^2}{\tau_{\text{short}}^2} + \frac{\Delta\tau_{\text{long}}^2}{\tau_{\text{long}}^2}} \tag{15}$$

2-lifetime case

Assuming that the long and short lifetime estimates are given the same photon budget N and have the same background, we obtain:

$$\frac{\Delta X}{X} = \frac{1}{\sqrt{N}}\sqrt{F^2(T/\tau_{\text{short}}, b) + F^2(T/\tau_{\text{long}}, b)} \tag{16}$$

Where F is the function defined in equation 9. Defining $u = T/\tau_{\text{long}}$, this becomes:

$$\frac{\Delta X}{X} = \frac{1}{\sqrt{N}}\sqrt{F^2(u/X, b) + F^2(u, b)} = G(u, b, X) \tag{17}$$

We solve $\frac{\partial G}{\partial u}(u, b, X) = 0$ to obtain the optimal gate position u^* as a function of X and b. The results are presented in Figure S8a.

FRET case

In this scenario, the long lifetime corresponds to the donor lifetime in the absence of FRET τ_{donor}, and the short lifetime corresponds to the donor lifetime in the presence of FRET τ_{FRET} such that the FRET efficiency is given by $E = 1 - \tau_{FRET}/\tau_{\text{donor}}$. The photon budget in this case can be assumed to be N for the donor alone and $(1 - E)N$ for the donor in the presence of FRET. A modified version of equation 17 is then obtained:

$$\frac{\Delta X}{X} = \frac{1}{\sqrt{N}}\sqrt{F^2(\frac{u}{1-E}, \frac{b}{1-E}) + F^2(u, b)} = G_{\text{FRET}}(u, b, E) \tag{18}$$

We solve $\frac{\partial G_{\text{FRET}}}{\partial u}(u, b, X) = 0$ to obtain the optimal gate position u^* as a function of E and b. The results are presented in Figure S8b. We find that in both scenarios, for well-separated lifetimes ($X = 0.5$ or equivalently $E = 0.5$) and realistic SBR of $3 - 10$, the optimal value of u is slightly above 1. In other words, to resolve two lifetimes, the optimal strategy is to use a gate delay T close to the longest lifetime τ_{long} for the 2-lifetime case or to the donor lifetime τ_{donor} in the FRET case. Pathological cases with very high FRET or very low FRET need specific strategies. These calculations can be adapted to cases where the two lifetimes are measured with different SBR and/or photon budgets.

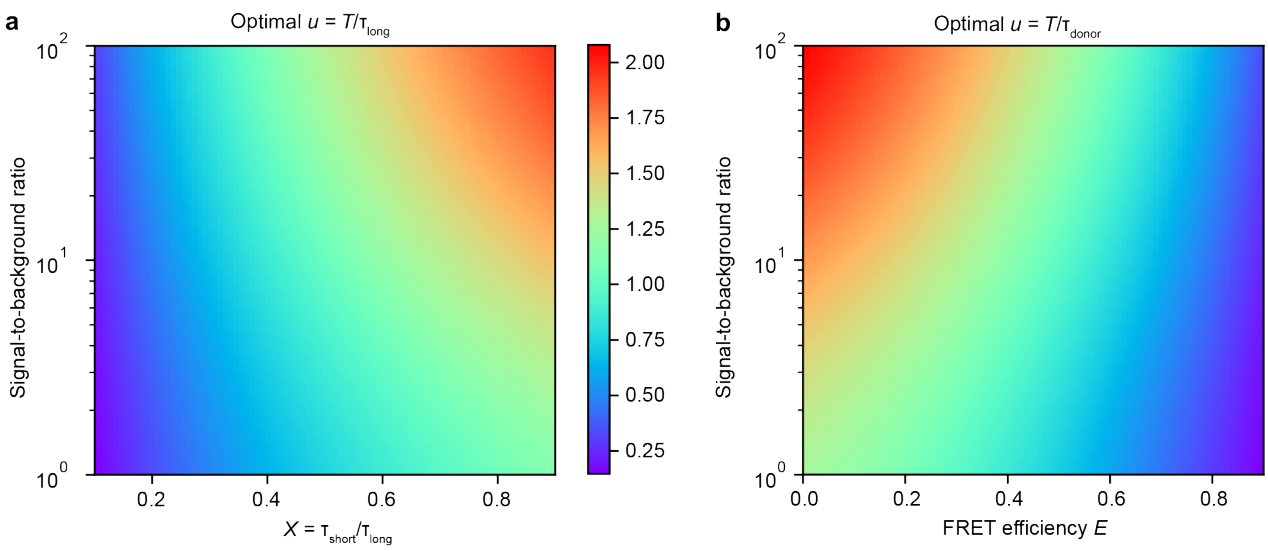

Fig. S8: **Resolving two lifetimes**. **a**, 2-lifetime case: optimal gate delay position relative to the long lifetime, as a function of the short-to-long lifetime ratio X and the SBR. **b**, FRET case: optimal gate delay position relative to the donor lifetime, as a function of the FRET effiency E and the SBR. For SBR values in the single-molecule range and $X \approx 0.4$ as in Figure 4, the optimal gate position is close to the long lifetime.

Camera characterization

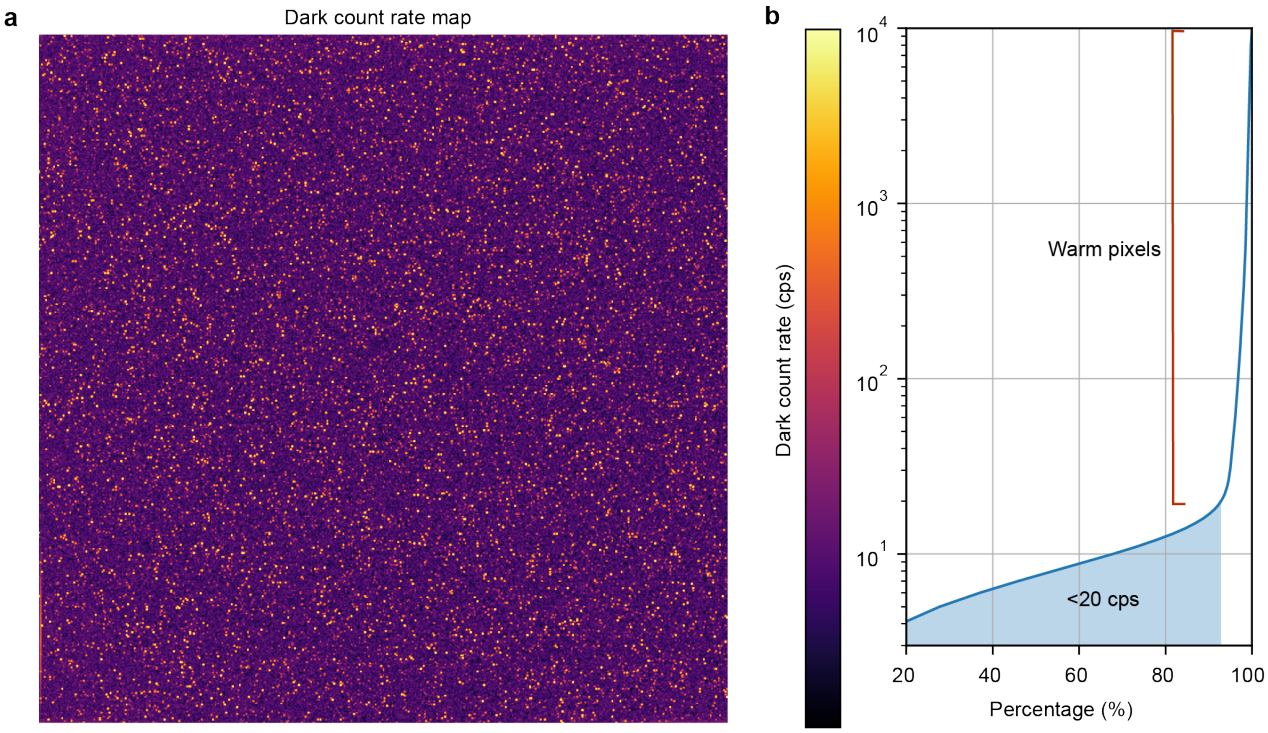

Fig. S9: **Dark count rate map and distribution**. **a**, Dark count rate measured by acquiring 10000 frames in the absence of illuminations. Note the logarithmic color scale. **b**, Cumulative distribution corresponding to the map, showing the 5% of warm pixels as the steep ascending part of the curve above 20 counts per second. These pixels were filtered out in the analysis by applying a mask to the images.

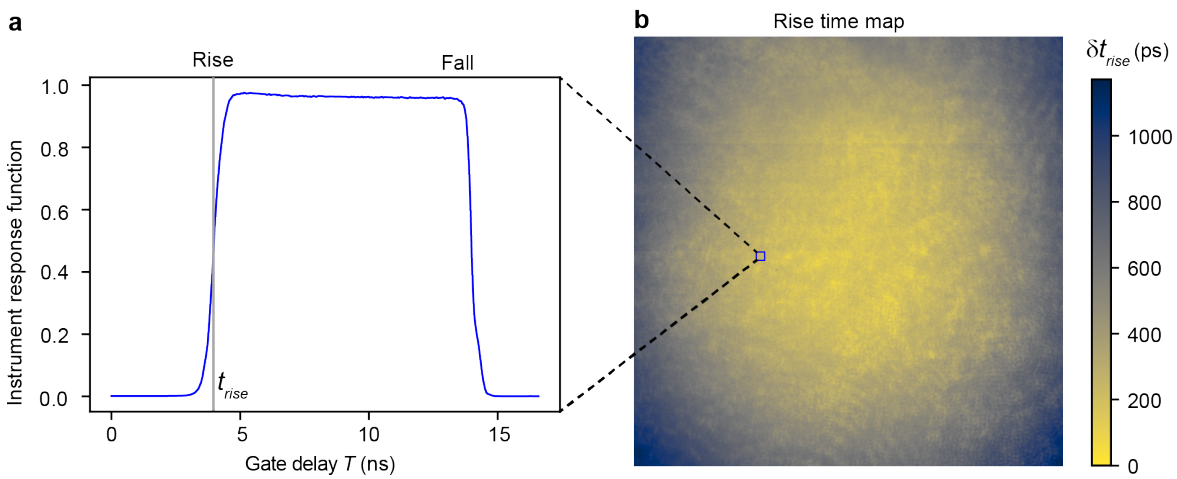

Fig. S10: **Instrument response function of the SPAD camera**. **a**, Instrument response function measured as the response to pulsed laser light reflected onto a coverslip, averaged over a 10×10 pixel region. **b**, Map of the opening time delays showing retardation of the chip edges by close to 1 ns, which was considered in the positioning of the first gate N_o. A more complete analysis or the gate properties was done in previous work[28].

References

[1] Berezin, M. Y. & Achilefu, S. Fluorescence lifetime measurements and biological imaging. *Chemical Reviews* **110**, 2641–2684 (2010).

[2] Datta, R., Heaster, T. M., Sharick, J. T., Gillette, A. A. & Skala, M. C. Fluorescence lifetime imaging microscopy: fundamentals and advances in instrumentation, analysis, and applications. *Journal of Biomedical Optics* **25**, 071203–071203 (2020).

[3] Sorokina, M., Koh, H.-R., Patel, S. S. & Ha, T. Fluorescent lifetime trajectories of a single fluorophore reveal reaction intermediates during transcription initiation. *Journal of the American Chemical Society* **131**, 9630–9631 (2009).

[4] Ha, T. *et al.* Fluorescence resonance energy transfer at the single-molecule level. *Nature Reviews Methods Primers* **4**, 21 (2024).

[5] Chizhik, A. I., Rother, J., Gregor, I., Janshoff, A. & Enderlein, J. Metal-induced energy transfer for live cell nanoscopy. *Nature Photonics* **8**, 124–127 (2014).

[6] Ghosh, A. *et al.* Graphene-based metal-induced energy transfer for sub-nanometre optical localization. *Nature Photonics* **13**, 860–865 (2019).

[7] Masullo, L. A. *et al.* Pulsed interleaved MINFLUX. *Nano Letters* **21**, 840–846 (2020).

[8] Cole, F. *et al.* Super-resolved FRET and co-tracking in pMINFLUX. *Nature Photonics* 1–7 (2024).

[9] Masullo, L. A. *et al.* An alternative to MINFLUX that enables nanometer resolution in a confocal microscope. *Light: Science & Applications* **11**, 199 (2022).

[10] Hirvonen, L. M. & Suhling, K. Wide-field TCSPC: methods and applications. *Measurement Science and Technology* **28**, 012003 (2016).

[11] Oleksiievets, N. *et al.* Wide-field fluorescence lifetime imaging of single molecules. *The Journal of Physical Chemistry A* **124**, 3494–3500 (2020).

[12] Oleksiievets, N. *et al.* Fluorescence lifetime DNA-PAINT for multiplexed super-resolution imaging of cells. *Communications Biology* **5**, 38 (2022).

[13] Agronskaia, A., Tertoolen, L. & Gerritsen, H. High frame rate fluorescence lifetime imaging. *Journal of Physics D-Applied Physics* **36**, 1655–1662 (2003).

[14] Kapitany, V., Zickus, V., Fatima, A., Carles, G. & Faccio, D. Single-shot time-folded fluorescence lifetime imaging. *Proceedings of the National Academy of Sciences* **120**, e2214617120 (2023).

[15] Wang, X. F., Uchida, T., Coleman, D. M. & Minami, S. A two-dimensional fluorescence lifetime imaging system using a gated image intensifier. *Applied Spectroscopy* **45**, 360–366 (1991).

[16] Periasamy, A. *et al.* Time-resolved fluorescence lifetime imaging microscopy using a picosecond pulsed tunable dye laser system. *Review of scientific instruments* **67**, 3722–3731 (1996).

[17] Bowman, A. J., Klopfer, B. B., Juffmann, T. & Kasevich, M. A. Electro-optic imaging enables efficient wide-field fluorescence lifetime microscopy. *Nature Communications* **10**, 4561 (2019).

[18] Bowman, A. J. & Kasevich, M. A. Resonant electro-optic imaging for microscopy at nanosecond resolution. *ACS Nano* **15**, 16043–16054 (2021).

[19] Bowman, A. J., Huang, C., Schnitzer, M. J. & Kasevich, M. A. Wide-field fluorescence lifetime imaging of neuron spiking and subthreshold activity in vivo. *Science* **380**, 1270–1275 (2023).

[20] Bruschini, C., Homulle, H., Antolovic, I. M., Burri, S. & Charbon, E. Single-photon avalanche diode imagers in biophotonics: review and outlook. *Light: Science & Applications* **8**, 87 (2019).

[21] Castello, M. *et al.* A robust and versatile platform for image scanning microscopy enabling super-resolution FLIM. *Nature Methods* **16**, 175–178 (2019).

[22] Blanquer, G. *et al.* Relocating single molecules in super-resolved fluorescence lifetime images near a plasmonic nanostructure. *ACS Photonics* **7**, 393–400 (2020).

[23] Bucci, A. *et al.* 4D single-particle tracking with asynchronous read-out single-photon avalanche diode array detector. *Nature Communications* **15**, 6188 (2024).

[24] Radmacher, N. *et al.* Doubling the resolution of fluorescence-lifetime single-molecule localization microscopy with image scanning microscopy. *Nature Photonics* 1–8 (2024).

[25] Ulku, A. *et al.* Wide-field time-gated SPAD imager for phasor-based FLIM applications. *Methods and applications in fluorescence* **8**, 024002 (2020).

[26] Michalet, X. Continuous and discrete phasor analysis of binned or time-gated periodic decays. *Biophysical Journal* **120**, 264a (2021).

[27] Niehörster, T. *et al.* Multi-target spectrally resolved fluorescence lifetime imaging microscopy. *Nature Methods* **13**, 257–262 (2016).

[28] Ulku, A. C. *et al.* A 512× 512 SPAD image sensor with integrated gating for widefield FLIM. *IEEE Journal of Selected Topics in Quantum Electronics* **25**, 1–12 (2018).

[29] Antolovic, I. M., Burri, S., Bruschini, C., Hoebe, R. A. & Charbon, E. SPAD imagers for super resolution localization microscopy enable analysis of fast fluorophore blinking. *Scientific Reports* **7**, 44108 (2017).

[30] Ballew, R. M. & Demas, J. An error analysis of the rapid lifetime determination method for the evaluation of single exponential decays. *Analytical Chemistry* **61**, 30–33 (1989).

[31] Mayer, S. F., Cao, C. & Dal Peraro, M. Biological nanopores for single-molecule sensing. *iScience* **25** (2022).

[32] Draaijer, A., Sanders, R. & Gerritsen, H. *Fluorescence lifetime imaging, a new tool in confocal microscopy* (Springer, 1995).

[33] Köllner, M. & Wolfrum, J. How many photons are necessary for fluorescence-lifetime measurements? *Chemical Physics Letters* **200**, 199–204 (1992).

[34] Sharman, K. K., Periasamy, A., Ashworth, H. & Demas, J. Error analysis of the rapid lifetime determination method for double-exponential decays and new windowing schemes. *Analytical Chemistry* **71**, 947–952 (1999).

[35] Chan, S. P., Fuller, Z., Demas, J. & DeGraff, B. Optimized gating scheme for rapid lifetime determinations of single-exponential luminescence lifetimes. *Analytical chemistry* **73**, 4486–4490 (2001).

[36] Heeg, B. Precision of mono-exponential decay estimates from rapid lifetime determination in the presence of signal photon and background noise. *Measurement Science and Technology* **25**, 105201 (2014).

[37] Edel, J. B., Eid, J. S. & Meller, A. Accurate single molecule FRET efficiency determination for surface immobilized DNA using maximum likelihood calculated lifetimes. *The Journal of Physical Chemistry B* **111**, 2986–2990 (2007).

[38] Lakowicz, J. R. *Principles of fluorescence spectroscopy* (Springer, 2006).

[39] Bohlen, J. *et al.* Plasmon-assisted förster resonance energy transfer at the single-molecule level in the moderate quenching regime. *Nanoscale* **11**, 7674–7681 (2019).

[40] Periasamy, A., Mazumder, N., Sun, Y., Christopher, K. G. & Day, R. N. FRET microscopy: basics, issues and advantages of FLIM-FRET imaging. *Advanced time-correlated single photon counting applications* 249–276 (2015).

[41] Richter, L., Szalai, A. M., Manzanares-Palenzuela, C. L., Kamińska, I. & Tinnefeld, P. Exploring the synergies of single-molecule fluorescence and 2D materials coupled by DNA. *Advanced Materials* **35**, 2303152 (2023).

[42] Szalai, A. M. *et al.* Real-time structural biology of DNA and DNA-protein complexes on an optical microscope. *bioRxiv* 2023–11 (2023).

[43] Braslavsky, I., Hebert, B., Kartalov, E. & Quake, S. R. Sequence information can be obtained from single DNA molecules. *Proceedings of the National Academy of Sciences* **100**, 3960–3964 (2003).

[44] Fuller, C. W. *et al.* The challenges of sequencing by synthesis. *Nature Biotechnology* **27**, 1013–1023 (2009).

[45] Demchenko, A. P. The concept of λ-ratiometry in fluorescence sensing and imaging. *Journal of Fluorescence* **20**, 1099–1128 (2010).

[46] Reed, B. D. *et al.* Real-time dynamic single-molecule protein sequencing on an integrated semiconductor device. *Science* **378**, 186–192 (2022).

[47] Lelek, M. *et al.* Single-molecule localization microscopy. *Nature Reviews Methods Primers* **1**, 39 (2021).

[48] Oleksiievets, N. *et al.* Single-molecule fluorescence lifetime imaging using wide-field and confocal-laser scanning microscopy: A comparative analysis. *Nano Letters* **22**, 6454–6461 (2022).

[49] Zaza, C. *et al.* Super-resolved FRET imaging by confocal fluorescence-lifetime single-molecule localization microscopy. *Small Methods* **7**, 2201565 (2023).

[50] Chen, T., Ghosh, A. & Enderlein, J. Cholesterol-induced nanoscale variations in the thickness of phospholipid membranes. *Nano Letters* **23**, 2421–2426 (2023).

[51] Jana, S. *et al.* Local water content in polymer gels measured with super-resolved fluorescence lifetime imaging. *Angewandte Chemie International Edition* e202318421 (2024).

[52] Wayne, M. *et al.* A 500× 500 dual-gate SPAD imager with 100% temporal aperture and 1 ns minimum gate length for FLIM and phasor imaging applications. *IEEE Transactions on Electron Devices* **69**, 2865–2872 (2022).

[53] Adamczyk, A. K. *et al.* DNA self-assembly of single molecules with deterministic position and orientation. *ACS Nano* **16**, 16924–16931 (2022).

[54] Houwink, Q. *et al.* Theoretical minimum uncertainty of single-molecule localizations using a single-photon avalanche diode array. *Optics Express* **29**, 39920–39929 (2021).