Direct object resolution by image subtraction: a new molecular ruler for nanometric measurements on complexed fluorophores

Electronic Supplementary Information

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S1. PSF Simulations

All point-spread function (PSF) simulations and processing thereof were performed using the open source software, ImageJ¹ (http://rsb.info.nih.gov/ij/). PSFs were generator using a plugin² based on a scalar based diffraction model³ with parameters defined by the numerical aperture (NA), refractive index of the immersion medium, wavelength light and the system magnification that defines the lateral resolution r (nm/pixel) of the image. To generate amplitudeto-distance (AD) conversion curve, two PSFs separated by a distance d nm were simulated by translating one PSF by $d \sin \theta / r$ pixels along x and $d \cos \theta / r$ along y, where θ is the direction of displacement. The two normalized PSFs were then subtracted to produce a 32bit image with a gray scale dynamic range covering both +ve and -ve values (**Fig. S1a-c**). The amplitude for different displacements of the PSF was calculated as the difference between the maximum intensity and minimum intensity in the difference image (**Fig. S1d**).

S2. Materials and Methods

dsDNA-QD constructs. A 100 base oligomer of DNA (ATDBio Ltd) with 5'biotinylation was designed to provide effective annealing with its compliment and reduced secondary structure and self-dimer formation. Formation of dsDNA from the sequence 5'-GCA CGA GCG ATA GTC TAT ACG TCT GCG CGT ATG TGA TCC TAG CGC GTA CTG GCG TAT CGA CAT CTG TAC ATG TAG ACA TCT ACT AGA ATT CGA CGA CCG A-3' with its compliment was estimated to have a free energy ~ -188 kcal/mol, a value typically >10 fold lower than any internal hairpin, self-complimentary structure or other hetero-dimeric structure other than a full 100 base paired dsDNA.

Complementary DNA oligos (50 μ M) were mixed 1:1 in an annealing buffer (10nM tris(hydroxymethyl)aminomethane (154563-5G, Sigma Aldrich), 50mM NaCl (S7653-250G, Sigma Aldrich), 1mM Ethylenediaminetetraacetic acid (ED-100G, Sigma Aldrich) and heated for 5 minutes at 60°C. The sample was left to cool down to room temperature and cooled further at 6°C for 20 min prior to use. Excess dsDNA was stored at -20°C. Streptavidin functonalised, ZnS capped CdSe quantum dots (QDs) with peak emission at 585 nm (QD585 M10111P, Life Tech.) were diluted to 200 pM and mixed with the 100bp dsDNA in a tetraborate solution (50 mM). A QD:dsDNA coupling ratio of approx. 2 was found to provide good numbers of dimers (**Fig. S2**), without significant aggregation. The mixture was incubated for at least after 20 minutes prior to imaging on a custom-built total internal reflection fluorescence microscope (TIRF) microscope.

TIRF imaging. Typically, a 50 µL aliquot of the dsDNA-coupled QD sample was placed on a coverslip mounted on the microscope stage. Coverslips were cleaned prior to mounting by passing through a flame and ozonating for at least 5 mins. The sample was excited by a kryptonargon ion gas laser (165, Spectra Physics) operating at 488 nm, which was coupled through a 60X 1.45NA objective lens of an inverted microscope (Nikon TE2000) via a dichroic beamsplitter (FF547/651-Di01, Semrock). The laser was focused off-axis at the back focal plane of the objective by a 200 mm lens to achieve total internal reflection at the coverslip/sample interface. Fluorescence collected by the objective and passed through the dichroic was further filtered using a single-bandpass filter (FF01-593/40, Semrock) prior to imaging on EMCCD camera (Evolve 512, Photometrics). The camera was coupled to imaging port via a 7X zoom lens (Edmund Optics) allowing variable magnification up to approximately 500X. Movies were recorded as 512 x 512 pixel image stacks of up to 2500 frames with exposures of 100 ms per frame, using the μ Manager⁴ acquisition interface for ImageJ¹.

Image processing. Images were processed in ImageJ using bespoke macros. In an initial step all frames within an image stack were realigned to correct of stage drift (see Section S3) during acquisition. For an image stack $A = \{1..n\}$, of a *n* frames, the set of difference images $B = \{1..n(n-1)/2\}$ was initially produced by subtracting the *j*th frame, A_j from the *i*th frame, A_i for all *i* and j > i in the raw data set A. Potential QD-dimers were identified as dipole-like objects in the difference image with discernible intensity gradients across the diffraction-limited profile. Each object was isolated in a region of interest (typically 16 x 16 pixels ROI, but dependent on magnification) and background subtraction (intensity minimum) and intensity normalization was performed frame-by-frame prior to repeating the subtraction process to obtain the difference amplitude. In general, intensity maxima and minima in the difference image were obtained from the intensity mean in a 2 x 2 ROI containing the absolute maximum and separately the minimum intensity. In this case, the amplitude-to-separation curve was produced in an equivalent manner. The set of difference images, B, was further used to identify and hence separate frames in the raw

image stack A into subsets in which one [10] or other [01] QD is switched-on. Each QD was then localised in all frames of each set by fitting the 2D Gaussian⁵

$$I(x, y) = \exp\left[-\left(x - x_{1(2)}\right)^2 / 2s^2 - \left(y - y_{1(2)}\right)^2 / 2s^2\right] + I_0$$
 S1

to the PSF of each QD 1 and (2) separately using Marquardt-Levenberg, non-linear curve fitting in OriginPro 8 (OriginLab Coorp). The distance between QDs was determined from the vectorial distance $\sqrt{(x_1 - x_2)^2 + (y_1 - y_2)^2}$. In the analysis of the effects of signal strength on DORIS derived QD distances and separations determined from 2D Gaussian localisations, absolute photon numbers were obtained from a calibration of the camera read-out (see S4).

TEM imaging. Transmission electron microscopy was performed on a Hitachi-7100, at an accelerating voltage of 75kV and 10000X magnification, with digital image acquisition on an axially-mounted (2K x 2K pixel) Gatan Ultrascan 1000 CCD camera. A 5µL aliquot of the

diluted streptavidin functionalised 585 nm QDs were spotted on pre-cleaned copper grids and buffer removed by blotting with lens tissue prior to staining with 1% uranyl acetate solution for 1 min and drying under air. QD diameters are measured as the average derived from the pixel area of the dense core-shell centre, segmented from the less opaque protein functionalised surface.

S3. Stage drift correction

Correction procedure. Drift of the sample stage over time will cause a decrease of the accuracy of QD distance measurements using both DORIS and centroid localisation by 2D Gaussian-fitting of the PSF. A procedure to correct for mechanical stage drift following data acquisition was developed and routinely applied prior to analysis of the all image stacks. For QDs dispersed randomly in the field of view, single QDs located near the four corners of an image were isolated and their positions determined over time using the 2D Gaussian fitting described above. Each frame in the image stack was then corrected by translating in *x*, *y* by the mean displacement of the four reference QDs from their initial positions (**Fig. S3**). The uncertainty due to stage drift was defined by the standard deviations, σ_x and σ_y , of the displacements in *x* and *y* from the mean coordinates of the QD centre and given by⁶

Typically the standard deviation in the emitter position was reduced from 12.6 nm (**Fig. S3a**) to 6.8 nm after correction (**Fig. S3b**).

S4. Uncertainties in QD localisations and distances

EMCCD calibration. The number of detected photons was retrieved from the camera read-out in analogue-to-digital units (ADUs) by considering the contributions to the output counts N_{out} , and the variance in the ouput, σ_{out} from analogue-to-digital conversion, EM gain and the excess noise factor arising from the electron multiplication process. For a detected photon number N_{in} with variance σ_{in} due to photon statistics, output counts scale as $N_{out} = CGN_{in}$, where *C* is an analogue-to-digital conversion factor, *G* is the EM gain, while the variance in output is

given by $\sigma_{out}^2 = C^2 G^2 F^2 \sigma_{in}^2$ where *F* is the excess noise factor. Given the detected photons are Poisson distributed, $\sigma_{in}^2 = N_{in}$, which gives by substitution⁷

$$\sigma_{\rm out}^2 = CGF^2 N_{\rm out}$$
 S3

For a gain of G = 1, the EMCCD is expected to perform as conventional CCD and for which there is no electron multiplication and the excess noise factor F = 1. In this case, measuring the variance in ADU counts against the mean ADU count for different photon fluxes gives the conversion factor *C* as the gradient. Measurement of the conversion factor then allows the excess noise factor at arbitrary gain *G* to be determined from a repeat of the measurements. We generated variance/gain vs mean counts for G = 1 and the EM gain G = 300, used in the analysis of the coupled dsDNA-QD samples. Coverslip blanks were imaged under uniform illumination at increasing laser intensities and the mean ADU counts and variance in a 100 x 100 pixel ROI measured after appropriate correction to the flat-field effect. The resulting calibration curves along with a linear regression analyses (**Fig. S4a**) give the conversion factor C = 0.408 +/- 0.005ADU/pe per unit gain and the excess noise factor $F^2 = 1.83 +/- 0.06$ at gain G = 300. Conversion from N_{out} in ADU counts to photon count is then given by $N_{in} = N_{out}/CG$, where CG = 122.4pe/ADU at gain 300.

PSF localisation uncertainties. The accuracy in localising the PSF is ultimately limited by the fundamental characteristics of the imaging system and the number of photons collected. The uncertainty in the centroid position of a PSF was first expressed by Thomson *et al.*⁸ but a more rigorous model has been developed by Mortensen *et al.*⁹, which takes into account the excess noise present in the EMCCD camera. In the latter formulation the uncertainty σ_i , in a given coordinate *i* is given by

$$\sigma_i = F_{\sqrt{\frac{16(s_i^2 + a^2/12)}{9N} + \frac{8\pi b^2(s_i^2 + a^2/12)}{a^2N^2}}}$$
S4

Where F is the excess noise factor, N is the total photon count under the PSF, s_i is the width of the PSF in direction *i*, expressed as a standard deviation, *a* is the pixel resolution and *b* the

background photon count. The uncertainties in x and y combine in quadrature to give an uncertainty, Δr_{photon} in PSF position due to photon noise analogous to that for stage drift. The overall uncertainty in position is then given by

$$\Delta r_{system} = \sqrt{\Delta r_{drift}^2 + \Delta r_{photon}^2}$$
 S5

The uncertainty due to the photon collection converges below 1 nm for photon counts around 30000 and rises to about 5 nm at counts of 1500 photons, the typical peak signal strength of the QD at 100 ms integration. As a lower limit on the integrated photon counts under the PSF, uncertainties due to photon noise are typically low and uncertainty in QD localisation is primarily limited by the stage drift correction to around 6 nm (**S4c**).

Evidently, uncertainties in the localisation of the PSF centre necessarily propagate in distance calculations made between PSFs. The distance between two PSFs located at (x_1, y_1) and (x_2, y_2) is given by $= \sqrt{\Delta x^2 + \Delta y^2}$, where $\Delta x = x_1 - x_2$ and $\Delta y = y_1 - y_2$. The uncertainties $\sigma_{x_1}, \sigma_{y_1}$ and $\sigma_{x_2}, \sigma_{y_2}$ in the coordinates of the two PSFs combine to give an uncertainty in the calculated distance given by¹⁰

$$\sigma_d^2 = \frac{(\Delta x)^2}{(\Delta x)^2 + (\Delta y)^2} \times \left(\sigma_{x_1}^2 + \sigma_{x_2}^2\right) + \frac{(\Delta y)^2}{(\Delta x)^2 + (\Delta y)^2} \times \left(\sigma_{y_1}^2 + \sigma_{y_2}^2\right)$$
 So

S5. Effects of fluorescence modulation on DORIS

Image sets for two closely-spaced fluorophores undergoing random and independent temporal fluctuations in their emission were simulated to investigate the effect fluctuation-amplitude on object resolution. Image sets were combined in sum to form a dimer set that was analyzed by DORIS to obtain mean intensity-amplitudes from the set of difference-images for a range of modulation-depths in the single molecule fluorescence (**Fig. S5**). For two molecules separated by only 50 nm the difference-amplitude is observed to increase with modulation depth, and saturates at the limiting value for binary on-off blinking (~0.74 at 50nm in Fig. S1d) as the fluctuation-amplitude approaches several multiples of the mean intensity. Here, the fluctuation

amplitude is defined by the standard deviation of the normal distribution from which the intensities of each fluorophore are independently sampled over time. At increasing amplitudes the fraction of fluctuations leading to the complete suppression of fluorescence is substantial, with DORIS-amplitudes derived from binary-like on- and off-states contributing increasingly to the mean. For molecules undergoing intensity fluctuations with a standard deviation of only 300 cts. about a peak-mean of N = 1000 photons (Fig. S5 inset), resolution of the fluorophores by image subtraction remains clear (in selected *i.j* stamped difference-images) despite incomplete suppression of fluorescence from either emitter. It was found that in the shot-noise limit where the SNR = \sqrt{N} , intensity-amplitudes in the difference-image and their standard-deviation decrease as the inverse of the shot noise or $1/\sqrt{N}$, reaching close to the noise-free amplitudes (Fig. S1d) at a signals around 1000 counts for QD separations ≥ 20 nm. Importantly here, the presence of two fluorophores in the simulation is not evident in the combined fluorescence intensity trajectory of the dimer (intensity sum). While direct distance measurement by DORIS may be subject to large uncertainties in the amplitude-to-separation conversion at low fluctuation-amplitudes, DORIS nonetheless allows identification of emitting centres in multimeric structures, where flickering in the fluorophore fluorescence may prohibit unambiguous resolution by standard localisation methods or identification of multiple fluorophores from analysis of the intensity trajectory.

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Figure S1. Simulations of 2D diffraction-limited point-spread-functions for two single fluorophores, in sum and difference and separated by a) 5nm, b) 100 nm and c) the Rayleigh diffraction-limit $d = 0.61\lambda/NA = 223$ nm. (scale bar 235 nm). Below 100 nm, the image of the combined PSF is barely discernible from that of a single object, but subtraction of the PSFs reveals a distance dependent amplitude in the difference-image. 2D PSFs are simulated with $\lambda = 530$ nm and NA = 1.45 at a pixel resolution of 47 nm and standard deviation given by $\sigma = \lambda/4NA\sqrt{2ln2}$. d) DORIS amplitude-to-separation conversion curves. Amplitudes are derived from the difference between intensity maxima and minima in the difference-image between two simulated PSFs for a distance between fluorophores of 5-80 nm in steps of 5 nm.



Figure S2. Schematic of the dsDNA-coupled QD dimer used in the experimental determination of fluorophore separations by difference imaging. 5' biotinylated single strand sequence (blue) is pre-annealed to the complementary strand (grey) prior to conjugation with streptavidin (SAV) functionalised QDs (585 nm).



Figure S3. Correction to temporal stage drift via single QD localisations. Single QD localisations via PSF-fitting with a 2D Gaussian shows the localisations of a typical QD undergoing movement due to stage drift over a period 30 seconds recorded with an acquisition time of 100 ms. a) before correction to stage drift (red points) and b) after correction (blue points) by the mean displacement of four widely spaced QDs from their initial position, in each successive image of the QDs in a movie-stack.



Figure S4. EMCCD calibration and photon count localisation uncertainties. a) EMCCD signal variance vs mean count curves for gains G=1 (gray points/blue line) and G=300 (blue point/red line). The slope obtained by fitting mean ADU counts against the variance in count rate in a 100×100 ROI of images taken under increasing light intensities. Gradients are 0.408 ± 0.005 (=*C*) at *G*=1 and 0.746 ± 0.026 (=*CF*²) at *G*=300. b) Uncertainty in QD localisation due to photon statistics (orange dots), stage drift (blue line) and total uncertainty (red dots).



Figure S5. Difference-imaging in flickering fluorophores. Plot of the mean intensity-amplitude in the difference-image as a function of the fluctuation-amplitude of two closely spaced fluorophores. Simulations were performed for two molecules separated by 50 nm, undergoing random fluctuations that are normally distributed about a mean count of 1000 photons/frame with standard deviations of 100 cts and 500 to 4000 in steps of 500 cts (magenta points). The DORIS amplitude approaches the limit for binary on-off blinking (0.74 at 50 nm, Fig. S1b) as the modulation amplitude extends beyond the mean count rate. Inset (bottom right) are representative intensity trajectories for two fluorophores (blue and red) fluctuating with a standard deviation of 300 cts, as well as the combined trajectory (green) of the dimer. Also shown are two difference-images (top left) showing resolution of the two fluorophores in opposing differential-patterns of type [1-1] and [-11] in binary notation. Scale bar 200 nm.