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Nanometer Distance Measurements Between Multicolor Quantum Dots

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Abstract

Quantum dot dimers made of short double-stranded DNA molecules labeled with different color quantum dots at each end were imaged using multicolor stage-scanning confocal microscopy. This approach eliminates chromatic aberration and color registration issues usually encountered in other multicolor imaging techniques. We demonstrate nanometer accuracy in individual distance measurement by suppression of quantum dot blinking, and thoroughly characterize the contribution of different effects to the variability observed between measurements. Our analysis opens the way to accurate structural studies of biomolecules and biomolecular complexes using multicolor quantum labeling.

Recent advances in fluorescence microscopy such as STED¹, PALM², STORM³ and others (reviewed in ref. ⁴) have pushed the resolution of far-field imaging down to the ten nanometers range. These techniques have recently been extended to multicolor imaging, providing powerful tools to investigate the supramolecular architecture of cells^{5–7}. On the other hand, true nanometer-resolution distance measurements between individual nano-objects does not require high resolution imaging techniques and has therefore been available for quite some time (reviewed in ref. ⁸). These methods rely on the accurate center localization of an individual fluorescence source's image, which does not necessitate high-resolution imaging. A number of approaches to obtain accurate localization have been demonstrated in the past (FIONA⁹, SHRIMP¹⁰, SHREC¹¹, etc, reviewed in ref. ¹²). Whereas these approaches can easily achieve nanometer distance measurement between individual probes, this performance is limited to single color signals. Achieving such a distance resolution with multicolor signals is much more challenging for several reasons: (i) in wide-field imaging approaches, chromatic aberrations in optical elements may result in slightly different image scale and focus plane for different colors; (ii) image registration may be highly non-linear or not reproducible from one acquisition to the next rendering calibration difficult; (iii) finally, for confocal imaging techniques, using different excitation laser lines may result in additional alignment issues compromising the proper registration of images¹³. In wide-field multicolor imaging techniques, the best distance resolution reported so far is thus on the order of 10 nm or more ^{11, 14–17}. Using confocal microscopy with a common excitation wavelength for all emitted colors, we have previously shown that distance resolution better than 10 nm could be achieved with quantum dots or TransFluoSpheres^{18, 19}. However, some limitations due to quantum dot (QD) blinking remained and no independent validation of these measurements was provided. Here we show how the elimination of QD blinking renders true nm-resolution in distance measurement between multicolor probes readily accessible. In particular, we validate our measurements with

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Supporting Information Available. Supplementary Material and Methods as well as Supplementary Figures are available free of charge at <http://pubs.acs.org>.

samples having known distances between QDs. These new advances make this approach particularly attractive e.g. for structural studies of macromolecular complexes or gene and protein binding sites on DNA or chromatin.

QD blinking has been the topic of intensive studies since its discovery in 1996²⁰. Its precise origin is still debated, but theoretical arguments as well as recent experimental developments support the original interpretation by Efros & Rosen²¹ that surface defects may be involved. In this model, charge carriers can be trapped, resulting in Auger recombination of each new exciton thereby leading to a non-emitting QD (reviewed in ref. ²²). Although blinking can be a useful feature, for instance to verify that one is observing a single QD rather than a cluster of several QDs, it has a number of drawbacks for imaging applications: long off-times result in lower intensity images, and “patchy” images in confocal imaging, which both adversely affect the accuracy of QD localization. Blinking can be almost suppressed by improving the passivation of defect sites at the surface of QDs, for instance by adding small molecules containing thiol groups in the surrounding medium²³ or directly to the QD’s surface²⁴. Another solution is to grow a thicker shell of higher bandgap material around the QD core at the expense of the nanoparticle size^{25, 26}. As we used commercially available functionalized QDs in this work, we chose a passivation method, embedding our samples in a thin layer of dithiothreitol-(DTT-) containing polymer as described in ref. ²⁷ and Supporting Information. With this treatment, QDs imaged for several minutes exhibited only very rare and short off periods (Fig. S1). The resulting point-spread-function (PSF) could be well fitted with a 2-dimensional Gaussian, whose center position could be determined with nanometer accuracy (see Supporting Information for details).

To obtain samples consisting of different color QDs separated by known distances, we borrowed a DNA-based approach that has been successfully used in the past to build gold nanoparticles dimers and trimers^{28–30}, as well as gold-QD dimers³¹. In brief, we used commercially available streptavidin-functionalized QDs (SAV-QD585 and SAV-QD655) attached to short complementary single-stranded DNA (ssDNA) molecules functionalized with biotin (see Supporting Information). Separate gel electrophoresis and extraction of the species comprised of a single QD bound to a ssDNA molecule was performed for each QD color (Fig. 1). This method resulted in dimers comprised of exactly one dsDNA connecting two QDs, a critical ingredient for precisely estimating the distance between individual probes.

QD-dimer samples were spin-cast on cleaned glass coverslips and imaged with a dual-color stage-scanning confocal microscope comprised of a nm-resolution closed-loop scanner, a high numerical aperture oil immersion objective lens and single-photon counting avalanche photodiodes (SPAD) similar to the setup described in ref. ^{18, 19}. The main advantage of this approach is possibility to form a high resolution image comprised of pixels of adjustable size and to record the fluorescence intensity of the sample emitted at well-defined locations. Since the excitation point source is identical for all color QDs and the fluorescence is collected simultaneously on all channels without introduction of color-specific aberration, there is in principle no issue of image registration.

Stage-scanning confocal images of blinking QDs (not embedded in DTT-PVA) showed the usual patchy fluorescence pattern visible in Fig. S1. As mentioned previously, blinking has two noticeable effects on the image PSF of a QD: (i) some pixels are dark, (ii) the intensity of the bright pixels does not vary as smoothly as expected from the theoretical PSF profile. Whereas it is easy to discard dark pixels during the PSF fitting step by setting a background threshold, non-dark pixels with intensity lower than expected due to blinking affect the fit quality and result in larger localization errors than expected. This error is best estimated by bootstrap analysis (ref ^{19, 32} and Supplementary Information). Indeed, standard error analysis methods designed for non-blinking emitters result in overly optimistic estimates due to their

expectation of a Poisson-distributed signal^{19, 33, 34}. As an example, the predicted localization accuracy for Fig. S1B is 0.3 nm using the standard shot-noise limited formula, but jumps to 4.2 nm using bootstrap analysis. This uncertainty is reduced for non-blinking QDs, as shown in Fig. S2A, where bootstrap and shot-noise analyses both result in a similar localization accuracy of ~ 0.5 nm.

The uncertainty on the distance measured between two probes localized with a finite accuracy in both spatial directions can be estimated exactly. A formula was recently published to compute the distance probability density function (PDF) between two probes localized with an identical uncertainty σ in both directions, from which the uncertainty on the distance can easily be extracted³⁵. Since bootstrap analysis results in a distribution of localization probability which is usually not a symmetric Gaussian, this formula does not apply in general. However, it is possible to compute the corresponding distance PDF numerically using a similar approach (ref.¹⁹ and X. Michalet, in preparation). In practice, for (average) localization uncertainties σ small compared to the measured distance, the distance PDF turns out to be very close to a Gaussian, characterized by a standard deviation σ close to the average localization uncertainty. The histogram of standard deviation of the distance is represented for the 60 bp dimer in the inset of Fig. 2. The typical uncertainty of 60 bp dimer distance was $\sim 1.3 \pm 0.5$ nm. Distances measured on many QD-dimers were represented as histograms, as shown in Fig. 2. Distance histograms for 60 bp (resp. 90 bp) QD-dimers were well fitted by Gaussians with mean and standard deviation 40.4 ± 3.4 nm (resp. 49.7 ± 3.3 nm).

This standard deviation is larger than the uncertainty on individual dimer distance measurements. Several factors can explain this difference: (i) QD size and shape distribution, (ii) SPAD alignment, (iii) dsDNA elasticity and (iv) deposition protocol.

QD size and shape distribution

QD size comprises the core-shell dimension, polymer coating and functionalization layer (avidin), resulting in rather large particle compared to the original core-shell material. QD size can be measured using different techniques such as FCS, DLS or TEM. However, in the case of non-spherical particles such as the red emitting QDs, it is especially important to know the exact shape of the particle, as attachment of the DNA along one symmetry axis of the particle rather than along the orthogonal one will result in different measured distances. The best technique to obtain this information is TEM of counterstained nanoparticles³⁶, which renders the extent of polymer and functionalization layers readily observable. Measurements performed as described in Supporting Information revealed that the SAV-QD585 were fairly spherical, with an average diameter of 18.1 ± 1.3 nm, whereas the SAV-QD655 were clearly oblong, with a long axis of 25.2 ± 2.5 nm and a short axis of 16.5 ± 2.1 nm (Fig. 3). Since there is no reason to suspect a preferred attachment point of the DNA molecule to the QD, we assumed a uniform attachment probability. We modeled the SAV-QD655 as ellipsoids of revolution (Fig. 4A), first with fixed major and minor axes (Fig. 4B & 4C) and then with major and minor axes taken from two Gaussian distributions with parameters corresponding to the experimentally measured one (Fig. 4D & 4E). Further assuming that the DNA orientation is perpendicular to the QDs, we generated a large number of DNA binding sites and measured the resulting center-to-center distance. In the fixed shape case (Fig. 4B & 4C), the resulting distance distribution was strongly peaked close to the shortest distance (corresponding to a most probable attachment along the larger diameter). By introducing an uncertainty in the exact dimension of the QDs (second model, Fig. 4D & 4E), the distance distribution was smoothed out, resulting in a quasi-Gaussian distribution of distances with a standard deviation of ~ 1.4 nm.

SPAD alignment

As discussed in more details in Supporting Information, although there is in principle no image registration issue with our approach, we discovered that SPAD misalignment can contribute a *constant* offset of a few nm between positions measured in different channels. The contribution of a constant offset to the distribution of measured distances can be estimated by numerical simulations. However, since measurements were taken on different days with different alignments which were not systematically characterized, we were in practice dealing with a data set comprised of distances measured with a distribution of offsets. Careful analysis of the effect of this random offset showed that it was well approximated by a linear broadening of the distance distribution as a function of offset (see Supporting Information and Fig. S3–S6). For instance, starting from a distribution of distances with a standard deviation of 1.4 nm, addition of a random offset in the range of 0 to 8 nm (as observed in our experiments) resulted in an observed distribution of distances with a standard deviation of ~ 3.4 nm. In other words, the distance distributions reported in Fig. 2 were well accounted for by the previous two effects with the reported experimental parameters.

dsDNA elasticity

Long dsDNA strands are well described by a worm-like chain model with a persistence length of ~ 150 bp (~ 50 nm) in standard buffer³⁷. It is usually inferred from this property that dsDNA fragments shorter than the persistence length are fairly straight, although recent experiments have challenged this simple interpretation^{38–40}. It is therefore possible that some flexibility of the dsDNA strand used in this work may contribute to the dispersion of measured distances. For instance, recent X-ray scattering experiments between gold nanocrystals attached to DNA have led to the suggestion that cooperative stretching elasticity results in the standard deviation of the end-to-end distance to scale linearly with the DNA strand length, with a proportionality factor of $0.21 \text{ \AA}$ per base pair⁴⁰. In our case, this would add a standard deviation of 1.3 nm to the 60 bp dimer distance distribution and 1.9 nm for the 90 bp dimers. Assuming that all effects (QD shape, SPAD offset and DNA cooperative elasticity) are independent from one another, the total variance is the sum of all variances due to these various effects. Due to the large contribution of the SPAD offset and QD shape distribution, DNA cooperative elasticity would only add a few Angstroms to the observed standard deviation of the distance. In order to precisely quantify the contribution of this effect, much larger statistics would be necessary, as well as a smaller (ideally zero) SPAD offset.

Deposition protocol

QD-dimers spin-coated on a glass surface may in principle adopt a conformation that differ from their conformation in solution. In our analysis of QD shape effect, we have implicitly assumed that each QD-dimer contacts the surface via a first QD, rotates as a rigid object and comes to rest when the second QD touches the surface. However, several other scenarios could happen instead of this simple 3-dimension to 2-dimension projection⁴¹. For instance, it is well known that long tethered dsDNA can be stretched by flow⁴² or surface tension⁴³. Although there is buffer flow during spin-coating, the only time when the QD-dimers are tethered (to the surface) and therefore susceptible to stretching, they are in nm-proximity to the surface, where the flow velocity is exactly zero. Similarly, it is likely that surface tension effects have no influence on the QD-dimers conformation, since they would be anchored to the surface via the two QDs by the time a meniscus would pass over them. For these reasons and the fact that we do not need to account for any unexpected broadening of the distance PDF, we can safely exclude any major influence of the deposition protocol on our results.

So far, we have not discussed the experimentally measured distances, focusing first on their standard deviations and the different factors responsible for the observed dispersion. Indeed, absolute distance measurement requires a perfect knowledge of probe size and shape, mode of attachment (and length of the linker itself) and an understanding of how the previous effects affect individual distance measurements. For instance, calculations of the effect of QD shape presented in Fig. 4 were performed using TEM measurements reported previously and a QD-to-QD distance equal to the dsDNA length calculated using the canonical B-DNA scale of 0.334 nm/bp⁴⁴. However, TEM measurements are affected by an uncertainty that can easily reach 1 nm due to the low contrast and rough contour of the organic coating (white areas in Fig. 3A & 3C). Moreover, DNA is attached to both QDs via biotin linkers with 6 carbons, potentially adding up to 1 nm to the total inter-QD distance. These considerations would accordingly increase the center-to-center distances calculated in Fig. 4D & 4E. Another potential contributor to the distance uncertainty comes from the SPAD offset. SPAD offsets slightly increase the average measured distance. However, this effect remains smaller than 0.5 nm for offsets smaller than 8 nm (Fig. S4C) and is completely negligible in the case of random SPAD offset of amplitude smaller than 8 nm (Fig. S5C). For instance, assuming that all reported TEM QD dimensions are underestimated by 1 nm (and proportionally rescaled standard deviations), that the C6 linkers contribute 1 nm to the QD separation, the maximum probability for the 60 pb and 90 bp dimer distances is found at 40.1 nm and 50.2 nm respectively, very close to the measured values of 40.4 nm and 49.7 nm. The remaining small differences are probably due to the lesser known SPAD offset effect. The expected distance dependence as a function of base pair separation is represented on Fig. 5 for values ranging from 0 to 100 bp, and is well approximated by a linear dependence: $d = 20.3 + 0.334 \times \text{base pairs}$.

In conclusion, our work demonstrates that it is possible and rather straightforward to measure distances between multicolor QDs, using far-field optics, with *true nanometer resolution* using stage-scanning confocal microscopy, a feat not easily achieved with any other imaging approach. In particular, our method allows measuring distances larger than 10 nm, not accessible by fluorescence resonance energy transfer (FRET)^{45, 46}. Here, we used quantum dots with a significant size and shape dispersion, forcing us to measure many dimer distances in order to obtain an accurate average value. With better defined probes, it should be possible to obtain accurate distance information from a single measurement. For instance, a 10 % size dispersion for perfectly spherical QDs of average diameter 13 nm as recently reported⁴⁷ would result in sub-nanometer accuracy at the single distance measurement level. By ensuring before the measurements that the SPAD alignment is contributing a negligible offset, true nanometer resolution distance measurement at the single-dimer level would be achievable.

This approach could for instance be used to better constrain structural models of biomolecular complexes present in limited copy numbers in the cell or high-resolution mapping of binding sites of DNA-binding proteins on combed DNA⁴⁸, among many other possible applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Agarose gel electrophoresis of DNA-QD dimers. A: Titration of SAV-QD585 with 90 bp 5'-biotinylated ssDNA. Lane 1: ssDNA. Lane 2: SAV-QD585. Lane 3: SAV-QD585 mixed with a 25-fold molar excess of biot-DNA, in which multiple DNA's bind to the SAV-QD585. Lane 4: DNA mixed with an 8-fold molar excess of SAV-QD585, in which a single-DNA/single-QD conjugate is formed (arrow). B: Lane 5: single-DNA-SAV-QD585 conjugate (extracted from the band indicated in Lane 4 as described). Lane 6: SAV-QD585. Free SAV-QD585 are not visible, indicating that the DNA-QD linkage remains intact after purification. C: Lane 7: single-DNA-SAV-QD655 conjugate. Lane 8: Free SAV-QD655.

Fig. 2.

Distance histograms. A: 60 bp dimers. The Gaussian fit yields $d = 40.4 \pm 3.4$ nm. Inset: distribution of uncertainty on the distance, as obtained from bootstrap analysis. B: 90 bp dimers. The Gaussian fit yields $d = 49.7 \pm 3.3$ nm. C: Example of a single QD-dimer distance measurement. Top: histograms of localization of 1,000 bootstrap replicas of the SAV-QD655 image (red, left) and the SAV-QD585 image (orange, right). Each QD was localized with 0.5 nm accuracy and the measured distance was 36.4 ± 0.7 nm. Bottom: Images of each QD. The fitted center is indicated by a white cross and the fitted PSF is indicated as a red curve along the corresponding orthogonal intensity profiles.

Fig. 3. TEM images of QDs. QD size and shape measurements. A: TEM images of SAV-QD585, B: corresponding size histogram. C: TEM images of SAV-QD655, D: Scatter plot of (length, width) measurements for the SAV-QD655. A typical rod-like aspect ratio of 1.5 is observed.

Fig. 4.

Estimation of the effect of QD geometry of the measured distribution of distance. A: The geometrical model used here represents the SAV-QD585 (green) as spheres of diameter 18.1 ± 1.3 nm and SAV-QD655 (red) as ellipsoids of revolution with a long axis of 25.2 ± 2.5 nm and a short axis of 16.5 ± 2.1 nm. DNA (blue) is orthogonal to the ellipsoid and the sphere. The computed distance is the distance between the two centers (orange rod). B, C: Distribution of computed distances obtained when assuming that perfect ellipsoids with long (resp. short) axis of 25.2 nm (resp. 16.5 nm) are connected by 20.04 nm (B) or 30.06 nm (C) long rod-like DNA attached randomly on the QDs. The curves were fit with a stretched exponential. D, E: Distribution of computed distances obtained when the ellipsoid parameters were taken from

the observed distribution. The fitted curves are asymmetric Gaussian distributions. In (B–E), the values reported on the graphs correspond to the positions of the peaks.

Fig. 5.

Measured distance as a function of DNA length (in bp). Square data points correspond to the optical measurements of this work. Filled circles correspond to simulated data including the effect of QD shape distribution, a 1 nm linker contribution, and 0.334 nm per bp (but no SPAD offset since it has minimal effects on the average distance). The dashed line corresponds to $d = 20.3 + 0.334 \times \text{base pairs}$.