Sub-10-nm distance measurements between fluorophores using photon-accumulation enhanced reconstruction (PACER)

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Abstract

Single-molecule localization microscopy (SMLM) precisely localizes individual fluorescent molecules within the wide field of view. However, the localization precision is fundamentally limited to around 20 nm due to the physical photon limit of individual stochastic single-molecule emissions. Using spectroscopic SMLM (sSMLM) to resolve their distinct fluorescence emission spectra, we can specifically distinguish and identify individual fluorophore, even the ones of the same type. Consequently, the reported photon-accumulation enhanced reconstruction (PACER) method accumulates photons over repeated stochastic emissions from the same fluorophore to significantly improve the localization precision. This work showed the feasibility of PACER by resolving quantum dots that were 6.1 nm apart with 1.7-nm localization precision. Next, a Monte Carlo simulation is used to investigate the success probability of PACER’s classification process for distance measurements under different conditions. Finally, PACER is used to resolve and measure the lengths of DNA origami nanorulers with inter-molecular spacing as small as 6 nm. Notably, the demonstrated sub-2-nm localization precision bridges the detection range between Förster Resonance Energy Transfer (FRET) and conventional SMLM. Fully exploiting the underlying imaging capability can potentially enable high-throughput inter-molecular distance measurements over a large field of view.

Graphical Abstract

This study demonstrates photon-accumulation enhanced reconstruction (PACER), a novel spectroscopic single-molecule localization microscopy (sSMLM) technique which improves the localization precision and enable distance measurements with sub-10-nm resolution. Here, PACER is demonstrated experimentally using quantum dots and Alexa Fluor 647 nanorulers with as low as 6 nm molecular spacing.
1. Introduction

Super-resolution imaging technologies, such as stimulated emission depletion (STED) microscopy and single-molecule localization microscopy (SMLM), surpassed the diffraction limit with demonstrated resolution of 10–20 nm\(^1\)–\(^5\). In particular, SMLM technologies, including photoactivated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), and point accumulation for imaging in nanoscale topography (PAINT), employ stochastic switching or binding approaches to regulate the density of fluorescent blinking within a recorded image frame. Thus, the location of each fluorescent molecule can be precisely estimated from the recorded image frame. Accumulating these localization events from thousands of recorded image frames not only creates a super-resolution image, but also establishes the means for quick quantification of intermolecular spacing over a wide field of view (FOV). Despite being widely successful, the prerequisite stochastic process of single molecule florescence often produces a rather limited number of photons, which practically restricts the localization precision of SMLM techniques to around 20 nm. Thus, limiting the utility of SMLM for resolving and measuring structures with short length scales.

To further improve the localization precision, DNA-PAINT and MINFLUX have been developed\(^6\)–\(^9\). DNA-PAINT exploits transient binding between a docking strand and dye-conjugated imager strands to replace the stochastic switching process to eliminate the influence of photobleaching and allow the use of brighter non-blinking dyes. DNA-PAINT is capable of imaging discrete arrangements of fluorescent molecules with ~5 nm spacing on synthetic DNA nanostructures\(^6\),\(^7\) but long imaging times and more sophisticated labeling strategies are needed for imaging biological samples. On the other hand, MINFLUX relies on prior knowledge of the donut-shaped illumination to triangulate the spatial location of individual emitters with greatly improved precision of ~2 nm, but at a reduced FOV\(^8\),\(^9\).

In fact, commonly used blinking dyes produce a large number of photons over the repeated occurrence of stochastic blinking events. However, due to the lack of molecular discrimination, the repeated stochastic blinking from the same molecule are all treated as independent events with limited localization precision around 20 nm. Thus, a method to identify the molecular origin of the stochastic blink events and subsequently accumulate the emitted photon from the same dye molecule can significantly improve the localization precision, without the need to reinvent the labeling and imaging protocols. To illustrate this concept, let’s consider a single fluorescent molecule located at the center of the 1×1 \(\mu\text{m}^2\).
FOV as a representative example (Figure 1a). The diffraction-limited imaging system forms a blurred image (Figure 1b), which can be mathematically represented by a point spread function (PSF) with a typical diffraction-limited resolution of ~200 nm (Figure 1c). The calculated centroid location of the recorded PSF is used to approximate the true location of the fluorescent molecule with a precision greatly exceeding the diffraction-limited resolution$^{10-12}$. By programmatically switching the majority of the fluorescent molecules to the “off” state, SMLM detects a small subset of the fluorescent molecules that are stochastically restored to the “on” state. The centroids of the sparsely distributed PSFs from a subset of molecules can be individually localized. These localized events are then accumulated over thousands of recorded image frames to build-up a super-resolution image. The localization precision in this process is inversely proportional to the square root of the photon number for individual blinking events$^{13}$. Figure 1d shows a simulated SMLM image of 100 repeated stochastic blinking events from the same molecule, with 800 photons per stochastic blinking (see Supplementary Information 1 for simulation details). Despite the fact that the photons originated from the same molecule, each stochastic blinking event is individually localized at photon-number limited precision of 14 nm (Figures. 1e and 1f). Since all the photons originated from the same molecule, in principle, repeated blinking events can be aggregated to accumulate a much larger photon number to improve the localization precision as compared to localization precision being limited to each event (Figures. 1g-i). Figure 1j shows simulation results which demonstrate how accumulating photons from different numbers of repeated blinking events can improve the localization precision. As shown in Figures. 1k and 1l, localization precision is inversely proportional to the square root of the number of blinking events (NB). After accumulating photons from 100 sequential blinking events, the localization precision is improved by 10-fold to ~ 1.4 nm, showing that the centroid location agrees well with the ground truth (Figures. 1g-i).

Thus, accumulating photons to improve localization precision can be accomplished if the true origin of the stochastic blinking can be specifically identified. This can be implemented via spatial clustering when the fluorescent molecules are sparsely distributed. As previously demonstrated, aggregating photons over prolonged detector exposure time can reach a localization precision of 1.5 nm in well-separated single molecule tracking$^{10}$. However, imaging biological systems often requires consideration of the spacing between individual molecules and thus, spatial locations alone become insufficient for identifying the sources of emitted photons to specific individual molecules among a densely packed ensemble. In addition to their spatial locations, the fluorescence spectra of emission events may provide additional dimensionality to specifically identifying individual molecules for potential photon accumulation$^{11, 12}$. Simultaneously recording the spatial and spectral information of each stochastic fluorescent emission event was recently made possible using spectroscopic single-molecule localization microscope (sSMLM) developed by us and other groups$^{14-16}$. In our previous study, we have demonstrated the use of sSMLM to identify fluorescent molecules of different species based on their distinct emission spectra$^{15}$. Interestingly, single-molecule spectroscopy studies suggest individual fluorescent molecules of the same species also exhibits distinct dissimilarity in their emission spectra, this phenomenon is often referred to as the spectral heterogeneity (SH)$^{16, 17}$. Thus, capturing the inherent SH of
individual fluorescent molecules further enables for spectroscopic discrimination of individual molecules of the same species in sSMLM\textsuperscript{18, 19}.

In this work, we report photon-accumulation enhanced reconstruction (PACER), which accounts for photons in repeated emission events from the same molecules through spectroscopic discrimination by sSMLM. Using PACER, we demonstrated a 1.7-nm localization precision using quantum dots (QDs), which exhibit high SH. We further showed that even with fluorophores with low SH (Alexa Fluor 647, AF647), PACER resolved DNA origami nanorulers with inter-molecular spacing as small as 6 nm.

2. Results

2.1 Measuring the distance between Quantum dots using PACER

We first experimentally demonstrate the feasibility of PACER in achieving sub-2-nm localization precision using QDs. Figure 2a shows the schematic of the sSMLM system. It employs a dispersive optical component to simultaneously capture the full fluorescent emission spectrum along with the spatial location of every single-molecule emission event. We chose QDs for their high SH due to structural variation and crystalline defects\textsuperscript{20}. The high SH enabled us to easily distinguish individual QDs using their distinct fluorescence spectra. Before imaging, we sparsely dispersed QDs (Lumidot, #694614, Sigma-Aldrich Co.) onto cover slides. Using sSMLM, the captured emission spectra are associated with the locations of individual blinking events. Figure 2b shows one frame of the spatial images and Figure 2c shows the simultaneously acquired spectral image of the QDs. Among all the recorded localization events, some “outliers” caught our attention. Specifically, the red dashed box highlights a region, within which we observed more frequent stochastic blinking events compared to the surroundings. The corresponding spectral image also appears to be much wider than the typical single QD emission spectrum. The repeated occurrence of single molecule blinking events within this highlighted region are spatially clustered in close vicinity. In conventional SMLM image reconstruction, these blinking events are treated independently, leading to a cluster of localizations without knowledge of their exact origins as shown in Figure 2d.

From the spectral image, we extracted spectral centroids (SCs)\textsuperscript{21, 22} to represent the spectroscopic signature of each QD. We calculate SC as

$$\lambda_{SC} = \frac{\sum_{\lambda} \lambda I(\lambda)}{\sum_{\lambda} I(\lambda)},$$

where $\lambda$ is the emission wavelength and $I(\lambda)$ is the spectral intensity at $\lambda$. As shown in Figure 2e, the SC distribution of the blinking events from the highlighted region reveals three distinct distributions. Such a SC distribution suggests that the highlighted region may contain three QDs and these QDs demonstrated distinct spectroscopic signatures, which are 580.3 ± 3.3 nm (QD1), 592.5 ± 1.6 nm (QD2), and 619.4 ± 2.8 nm (QD3), as the result of SH. For reference, individual frames showing each spatial and spectral image of QD1, QD2 and QD3 with the corresponding emission are shown in Figure S1. Frames where multiple QDs emitted at the same time were excluded based on the width of the emission spectrum.
Using the SCs of the QDs, we can identify and classify the origin of each detected blinking event to one of the three QDs. The first 100 repeated blinking events originating from each of the three QDs after classification were further selected to demonstrate the principle of PACER. Their corresponding spectra and SCs were shown in Figure 2f.

The observed SH establishes the foundation for PACER to discriminate individual molecules from the ensemble populations based on their spectroscopic signature. In PACER, the emitted photons originated from repeated blinking of the same QD and can all be combined to collectively improve the localization precision. Figure 2g illustrates the improved localization precision with respect to increasing number of NB, where we overlay three rendered QDs with pseudo-colors (see Supplementary Information 2 for methods of image rendering). When NB=1 the localization precision is limited to 17.0 nm, corresponding to an average of 250 photons in the spatial image. Such a localization precision is insufficient to spatially distinguish the three QDs in the reconstructed image (Figure 2g, NB=1). After applying PACER, we can positively classify all stochastic blinking events to their respective true origins based on their unique heterogeneous fluorescence emission spectra. After classifying the origins of the blinking events, we accumulate photons from repeated blinking events that are from the same QD from multiple recorded frames to increase the total photon number and, thus, to improve the localization precision (see Supplementary Information 3 for details of photon-accumulation and Figure S2 for rendered images with increasing NB to 5, 10, 50, and 100). Accumulating photons from 10 blinking events (Figure 2g, NB=10) leads to a more than 3-fold improvement in localization precision to ~4.9 nm, where three QDs can be resolved in the reconstructed sSMLM image. When NB=100, an approximated 10-fold improvement in localization precision to ~1.7 nm shows that QD2 and QD3 are 6.1 nanometers apart. Considering QDs have the mean diameter of 5.2 nm, it is possible that QD2 and QD3 are almost in contact. Figure 2h shows the line profiles of the reconstructed image across the QD2 and QD3 with NB of 1, 10, 100, further illustrating the improvement in localization precision as NB increases.

2.2 Simulating the success probability of molecular discrimination for PACER

We aimed to understand how PACER can be used to confidently classify different blinking events to their respective origins based on SH. To answer this question, we performed a Monte Carlo simulation of the classification process under different experimental conditions. We consider a model system which comprises of two fluorescent emitters of the same species located in close proximity to each other (M1 and M2 in Figure 3a). M1 and M2 represent the predefined true locations of the two emitters with a predefined inter-emitter spacing (d). We simulate experimental localizations of the two emitters based on a given localization precision in the spatial image (referred to as spatial precision). The total number of simulated experimental localizations is \( N_{\text{tot}} \). The probability distributions of the two simulated experimental localizations are represented by the two dashed circles (Figure 3a), with the radius corresponding to the spatial precision. In the spectral image (Figure 3b), we predefine the emission maximum and spectral heterogeneity (h) based on the selected emitter species\(^\text{17}\). We then simulate SCs of the M1 and M2 based on a given precision in the spectral image (referred to as spectral precision). The probability distributions of the two simulated SCs are collectively determined by the spectral heterogeneity (blue dash line) and
the imaging parameters (the dashed arrow), including the number of photons, system noises, and the system spectral dispersion\textsuperscript{22} (see Supplementary Information 4 for details of the Monte Carlo simulation).

In the simulation, the criterion for successful identification of M1 and M2 is defined as identification in either the spatial image or the spectral image. The total number of successfully identified localizations is defined as $N_{id}$ (see Supplementary Information 4 for detailed description of the criterion). We varied $d$ and $h$ to evaluate the success probability $P_{sc}$ which is defined as $N_{id} / N_{tot}$. We first simulate the case using the conventional SMLM method at an averaged 6000 photons per recorded stochastic blinking event and a background of 10 photons per pixel. All the photons are allocated to the spatial image (Figure 3c, blue dash line). The success probability decays rapidly from 100\% to 2\% as $d$ decreases from 50 nm to 2 nm, rendering it difficult to successfully identify M1 and M2 in the spatial image when $d$ is smaller than 5 nm. This can be attributed to the photon number limited precision in the conventional SMLM method. In comparison, sSMLM captures spectroscopic signatures of individual emitters and thus, provides an additional clue to better identify M1 and M2, other than the spatial image. In the simulation, emitted photons are distributed between the spatial image and spectral image at a 1:1 ratio, resulting in a signal level of 3000 photons and a background level of 5 photons per pixel in each image. We consider a dye molecule with high spectral heterogeneity ($h = 10$ nm) for semiconductor nanoparticles. As shown in Figure 3c, sSMLM further utilizes the recorded spectral image to capture the inherent spectral heterogeneity of M1 and M2 and thus, significantly increases the success probability from 2\% to 42\% in comparison to SMLM which uses the spatial image alone.

Figure 3d further investigates how $d$ and $h$ influence $P_{sc}$. As expected, reduced inter-emitter spacing and the spectral heterogeneity are found to unfavorably lower the success probability. While high success probability > 90\% can be obtained when $d$ is larger than 20 nm, significantly reduced success probability < 20\% are found in the cases when $d$ is smaller than 7 nm and $h$ is smaller than 2 nm. Thus, simultaneous occurrence of lower $d$ and $h$ represents a challenging condition in faithfully distinguishing two fluorescent molecules. We also investigated the influence of photon numbers on the success probability with respect to $d$ and $h$. As the photon number decreases, both spatial and spectral precisions are reduced\textsuperscript{13,22}. Thus, it becomes increasingly difficult to specifically identify and discriminate each of the dye molecule. As the results, it unfavorably reduces the success probability as shown in the Figure S6. For example, we observed significantly reduced success probability < 20\% when $d$ is smaller than 12 nm and $h$ is smaller than 7 nm at 1000 photons. Notably, in the case with low photon numbers, using the dye molecules with high spectral heterogeneity can help to increase the success probability. Since QDs often exhibit large spectral heterogeneity, whereas many of commonly used molecular labels have smaller $h$\textsuperscript{17}. To further explore the benefit of PACER using dyes with smaller $h$ we measured the lengths of a variety of DNA nanorulers functionalized with AF647.
2.3 Measuring the length of DNA nanorulers

Upon successful demonstration of improved localization precision using QDs sample, we further experimentally validated PACER using the model system with known inter-molecular spacing. Figure 4 shows the results of PACER resolving up to 6-nm spacing in DNA nanorulers (Gatta-Storm Nanoruler, Gattaquant GMBH) labeled with AF647 fluorescent molecules. The nanoruler comprises DNA origami backbone with precise placement of two fluorescence molecules at controllable inter-molecular spacing\(^{23, 24}\) (Figure 4a). Unlike DNA-PAINT models, the dyes on the DNA nanoruler are permanently bound to the structure enabling the spectra of the dyes to be used for molecular discrimination. We acquired 2,000 frames with an integration time of 10 ms per frame. The recorded spectra of all the individual stochastic blinking from the nanoruler sample labeled with two AF647 molecules are plotted in Figure 4b and the corresponding histogram of the SCs is shown in Figure 4c. Nanoruler samples with contrast in the measured SC histogram exceeding the threshold value of 20% are accepted. Two distinct peaks with mean SCs separated by approximately 10 nm, which underlie the effect of SH of AF647, can be clearly observed in Figure 4c. Each individual molecule exhibits consistent emission spectra during stochastic switching, with the measured spectral precision less than 2 nm, as estimated by the Standard Deviation (S.D.) of the SC distribution. The averaged fluorescent spectra of the two AF647 molecules are shown in Figure 4d after classification based on Figure 4c.

We imaged nanorulers with inter-molecular spacing values of 23 nm (Figure 4e), 11 nm (Figure 4k), and 6 nm (Figure 4o). We recorded 5 acquisitions from randomly selected FOVs for each sample and pooled the detected nanorulers for analysis. After classifying each localized event to one of the two AF647 molecules and correcting the stage drift based on the position of a fiducially marker, the scatterplot of all the localized blinking events is color-coded in red and green to represent the origins of the two AF647 molecules (Figure 4f). Two molecules can be readily separated with colors indicating distinct spectral signatures after classification as shown in Figure 4g. After PACER, the localization precision improved from 18.2 nm to 2.6 nm, as denoted by the red and green solid lines in Figure 4h. The measured distance between the images of the two molecules is 22.7 nm (Figure 4i). Figure 4j shows the histogram of the measured inter-molecular spacing from all the 57 nanoruler samples, which shows a mean value of 23.2 nm with a S.D. of 0.8 nm. It agrees well with the expected value of 23 ± 1 nm according to manufacturer’s specification.

Figures 4k-m illustrate the case of nanorulers with an inter-molecular spacing of 11 nm. The color-coded scatter plot of the localization events and the reconstructed PACER image after drift correction of one representative sample are shown in Figures. 4k and 4l, respectively. Figure 4m is the histogram of the measured inter-molecular spacing from 35 nanoruler samples, showing the mean spacing of 10.8 nm with a S.D. of 0.9 nm. Again, it agrees well with the expected value of 11 ± 1 nm according to manufacturer’s specification. Finally, we performed PACER on the nanoruler sample with inter-molecular spacing of 6 nm (Figures. 4n-p). The corresponding results are shown in Figures. 4n and 4o. The histogram of the measured inter-molecular spaces from 15 nanoruler samples shown in Figure 4p indicates the mean spacing of 6.1 nm with S.D. of 0.8 nm, which agrees well with the expected value of 6 ± 1 nm according to manufacturer’s specification. Thus, even in a rather challenging
case, using AF647 with rather weak SH, PACER can still successfully resolve the fluorescent molecules at the intermolecular spacing of 6-nm distance, which represent 5-fold improvement in the localization precision under the identical experimental conditions.

3. Conclusion

In this study, we demonstrated a single-digit nanometer resolution that can be achieved by combining sSMLM and PACER. We first validated its feasibility of distinguishing molecules/particles of the same type by capturing the intrinsic SH. By using the spectroscopic signature as a unique identifier, photons from individual molecules can now be accumulated in achieving greatly improved localization precision. We have experimentally validated sub-2-nm localization precision using QDs, and synthetic DNA origami nanorulers with the smallest intermolecular spacing of 6 nm. The experimental results indicate that spectroscopic signature of individual molecules would greatly benefit molecular identification and resolution improvement using PACER.

It is worthwhile to note that photo bleaching of the fluorescence dyes imposes a practical limit on the number of repeated blinking from the same dye molecule to 25–100 times\textsuperscript{25}. It corresponds to about 5- to 10-fold improvement in the localization precision using PACER. As the number of available blinking from one molecule limits the precision improvement of PACER, it would be advantageous to use labeling methods to increase the number of blinking from the dye molecules. Furthermore, PACER is not applicable to the methods like PAINT, which rely on transient binding of dye molecules to the target labeling sites. As the dye molecules being continuously replaced during image acquisition process, spectroscopic signatures can no longer been used as the reference to discriminate the specific labeling site.

This technique offers significantly improved localization precision over the conventional SMLM technique but remains compatible with the existing fluorescence labels and imaging protocols. It is worth noting that the demonstrated sub-2-nm localization precision bridges the detection range between Förster Resonance Energy Transfer (FRET) and conventional SMLM. The broad accessibility and the molecular-scale resolution can potentially enable an increased understanding of interactions of biological and synthetic molecules at short length scales.

4. Methods

4.1 Optical setup

The optical setup contains an inverted optical microscope (Eclipse Ti-U with perfect-focus system, Nikon), equipped with 645-nm and 445-nm solid-state lasers with 500-mW maximum output, a high numerical aperture objective lens (100x, NA1.49, Nikon CFI apochromat TIRF) for TIRF illumination, and a home-built transmission spectrometer. The illumination power was controlled by a set of linear polarizers. The imaging filter set was consisted of a laser clean-up filter (FF01–642/10–25, Semrock), a dichroic mirror (FF649-D101–25X36, Semrock), and a long-pass filter (BLP01–647R-25, Semrock) at the emission port to reject the reflected laser beam. The fluorescence image was then coupled into a transmission spectrometer featuring a blazed dispersive grating (150 grooves mm\textsuperscript{-1}). The
image further divided into a non-dispersed zero-order spatial image and a spectrally
dispersed first-order spectral image and can be simultaneously collected by a high-sensitivity
electron multiplying charge-coupled device (EMCCD, ProEM, Princeton Instruments).

4.2 sSMLM imaging procedure

The samples were placed on the microscope stage and imaged under a TIRF objective
(Nikon CFI apochromat 100X, 1.49 NA), with an additional magnification of 1.5X by a tube
lens. We used a 445-nm laser to excite fluorescence from QDs and a 645-nm laser to excite
fluorescence from Alexa Fluor 647 on DNA nanorulers. The illumination intensity was set
to be 10 kWcm$^{-2}$ and 3 kWcm$^{-2}$ to create stochastic radiations from QDs and Alexa Fluor
647 for sSMLM imaging, respectively. The EMCCD camera acquired images from the
monochromator with integrating time of 10 ms at a frame rate of 85 Hz. Unless specifically
noted, 5,000 frames were recorded for imaging reconstruction using PACER. To avoid
overlapping events and minimize localization errors, we filtered out some PSFs using the
sigma value of fitted PSFs and the SCs during the post-data processing. We also excluded
the molecules simultaneously recorded in the same row of the spectral image. Details of the
post-data processing in the spectral image are available in an ImageJ plugin module –
RainbowStorm$^{26}$.

4.3 Preparation of quantum dots samples

The QD nanoparticles used in the experiment is core-shell CdSe/ZnS Lumidots$^{TM}$ (product
#: 694614) with mean particle size of 5.2 nm and emission peak at 610 nm. QDs were
immobilized on a coverslip (#1.5, VWR) by spin-coating of their suspension (~100 ng mL$^{-1}$)
and covered with silicon oil to protect them from photo-oxidation.

4.4 DNA nanoruler

The nanorulers was customized from Gattaquant DNA Nanotechnologies with mark-to-mark
distances of 23 nm, 11 nm, and 6 nm, respectively. Each mark only contains one Alexa Fluor
647 molecule. All samples were delivered in solution and then immobilized on BSA-biotin-
neutravidin surface in LabTek (VWR) chambers.

4.5 Immobilization of DNA nanoruler

The LabTek chamber (VWR) was washed three times with 500 μL PBS and then incubated
with 200 μl of BSA-biotin solution (1 mg ml$^{-1}$ in PBS) for 5 min. After removing the BSA-
biotin solution, the chamber was washed 3 times with 500 μl of PBS. Then the chamber was
incubated with 200 μl of neutravidin solution (1 mg ml$^{-1}$ in PBS) for 5 min and washed
three times with 500 μl of 1x PBS supplemented with 10 mM magnesium chloride. 1 μl of
the DNA origami solution were diluted with 200 μl of 10 mM magnesium chloride. The
chamber was incubated with the diluted DNA origami solution for 5 min to achieve the
desired surface density of DNA origami structures (~ 0.1 μm$^{-2}$) and then washed three times
with 500 μl of PBS. The chamber was filled with the imaging buffer prior to imaging.
4.6 Imaging buffer

A standard imaging buffer was freshly made and added to the DNA nanoruler samples prior to imaging. It contained TN buffer (50 mM Tris and 10 mM NaCl), an oxygen scavenging system (0.5 mg ml$^{-1}$ glucose oxidase (Sigma-Aldrich)), 40 μg ml ml$^{-1}$ catalase (Sigma-Aldrich) and 10% (w/v) glucose (Sigma-Aldrich), and 143 mM βME (Sigma-Aldrich).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Schematic showing how image resolution is improved by molecular discrimination. (a) Due to the wave nature of light, when light comes from a point emitter focused by an optical imaging system, the interference can result in a blurred distribution of light called a point spread function (PSF). (b-c) The size of the PSF sets up the fundamental resolution limit of an optical imaging system, namely Abbe diffraction limit. (d-f) In single-molecule localization microscopy (SMLM), the probable location of a single fluorescent molecule can be estimated from the centroid of the fluorescence diffraction pattern produced on a camera. The localization precision is determined by the photon count collected in each captured frame. The scatter plot and rendered image of 100 blinking events indicate a localization precision of ~14 nm. (g-i) Through molecular discrimination, blinking events can be combined, resulting in an improved localization precision of sub-2 nm. (j) Improved resolution using photon accumulation with respect to the number of blinking events (NB). White crosses denoted positions of all localizations in each test and red crosses denoted their centroids (upper panel). Reconstructed images only using centroids (middle panel). Localization precision is calculated along the x-axis and its intensity was then normalized for comparison, which are 14.10 nm, 6.31 nm, 4.46 nm, 3.20 nm, 2.01 nm, and 1.41 nm for
NB=1, 5, 10, 20, 50, and 100, respectively (lower panel). (k) Simulated localization precision with respect to NB. Error bars are from 100 independent simulations. (l) The black line is the curve calculated by $\sigma_0 / \sqrt{NB}$, where $\sigma_0$ is the localization precision at NB=1.
Figure 2.
Experimental demonstration of PACER in achieving a 1.7-nm localization precision. (a) The schematic of sSMLM. (b) One frame of the spatial images and (c) one frame of the simultaneously acquired spectral images of the QD sample. (d) The scatter plot of localization events in the red dashed box in (b). (e) Histogram of the SC distribution. (f) Fluorescence spectra of three QDs after classifying by SCs using spectral intensity threshold of 300, 460, and 480 photons, respectively, and spectral windows of 575-585 nm, 585-600 nm, and 615-625 nm, respectively, as filtering criteria. The corresponding SC of each fluorescence spectrum is noted as an open circle in the plot. (g) Rendered sSMLM images after combining multiple emission events with NB of 1, 10, and 100, respectively. (h) Line profiles across two QDs in (g) with NB of 1, 10, 100.
Figure 3.
Model system consists of two fluorescent emitters of the sample type located in the close vicinity of each other in (a) the spatial domain and (b) the spectral domain for the success probability estimation. (c) Success probability with respect to the inter-molecular spacing (d) at the spectral heterogeneity (h) of 10 nm of PACER and the conventional SMLM with the same signal level of 6000 photons and a background level of 10 photons per pixel. A splitting ratio of the sSMLM system for PACER is set to be 1 to 1. (d) Semi-log plot of success probability with respect to \( d \) and \( h \).
Figure 4.
Experimental validation of PACER using DNA nanoruler samples. (a) Schematic illustration of a DNA nanoruler labeled with a pair of AF647 featuring a predefined mark-to-mark distance. (b) Representative emission spectra of two molecules on one nanoruler. (c) Histogram of SCs indicates the existence of two molecules with distinct spectral signatures on one nanoruler. (d) The average spectra of the two molecules separated by SC at the wavelength of 669 nm. (e) Schematic of a DNA nanoruler featuring mark-to-mark distance of 23 nm. (f) The scatter plot and (g) the rendered sSMLM image of localization events with colors indicating distinct spectral signatures after stage drift correction. (h) Comparison of molecule location in SMLM image (the dashed black line) and sSMLM images without (the dashed colored lines) and with (the solid colored lines) PACER. (i) Calculated location of molecules through PACER. (j) Histogram of mark-to-mark distance. The representative results of a DNA nanoruler featuring mark-to-mark distance of (k-m) 11 nm and (n-p) 6 nm.