Supporting Online Material for
Multicolor Super-Resolution Imaging with Photo-Switchable Fluorescent Probes

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Corrected 12 September 2007:
In the section “Photon number analysis of the photo-switchable dyes,” the theoretical localization accuracy for Cy5, Cy5.5, and Cy7 should have been calculated using the background noise level ($b$), rather than the background signal level as appeared in the previous version of the Supporting Online Material. We have changed the text accordingly and recalculated the localization accuracies for the three dyes. The new values are slightly smaller than our original estimates, and the Supporting Online Material has been updated with the new values. This correction does not affect any of the conclusions of the manuscript.
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**Multicolor Super-resolution Imaging with Photo-switchable Fluorescent Probes**

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Materials and Methods

Single-molecule imaging of photo-switchable activator-reporter pairs

To characterize the switching kinetics of the photo-switchable probes reported in this work, the two fluorescent dye molecules (activator and reporter) were conjugated to the end of a double stranded DNA (dsDNA) construct, and the construct was immobilized on a quartz surface for single-molecule imaging. The DNA constructs were labeled using the same procedure as previously described (5). Briefly, PAGE purified DNA oligonucleotides with biotin and/or amine modification at the ends were obtained from Operon. The oligos (30 base pairs (bp) in length) were labeled with amine reactive dyes (Cy2, Cy3, Cy5, Cy5.5, and Cy7 were obtained from GE Healthcare, and Alexa Fluor 405 and Alexa Fluor 647 were obtained from Invitrogen) post-synthetically following the protocol provided by the manufacturers. The dye-labeled oligos were purified using reverse phase HPLC. Complementary strands of DNA, each labeled with an activator or a reporter dye, were annealed to form biotinylated dsDNA by mixing equimolar amounts of the two complementary strands in 10 mM Tris-Cl (pH 7.5), 50 mM NaCl. This allowed a pair of activator and reporter dyes to be brought into close proximity, as illustrated in fig. S1D, facilitating the immobilization of dye pair to a microscope slide via biotin-strepavidin linkage.

To immobilize the labeled dsDNA on a surface, quartz microscope slides (G. Finkenbeiner) were cleaned using Alconox detergent, sonicated in 1M KOH, ethanol, and 1M KOH sequentially before being rinsed with MilliQ water and flame dried. A biotinylated bovine serum albumin (b-BSA, Sigma) solution (1.0 mg/mL) was first added to the slides, followed by 0.25 mg/mL streptavidin (Invitrogen), and finally the DNA sample at a low concentration (~ 30 pM) in order to obtain a low surface density of DNA molecules such that individual molecules were well separated and optically resolvable from each other. The slides were rinsed prior to the addition of each reagent. Single-molecule imaging was performed in a standard imaging buffer that contains 50 mM Tris, pH 7.5, 10 mM NaCl, 0.5 mg/mL glucose oxidase (Sigma, G2133), 40 μg/mL catalase (Roche Applied Science, 106810), 10% (w/v) glucose and 1% (v/v) β-mercaptoethanol.

Single-molecule imaging was performed on an Olympus IX-71 inverted microscope equipped with prism-type total internal reflection fluorescence (TIRF) configuration. A red 657 nm diode laser (RCL-200-656, Crystalaser) was used to excite fluorescence from the reporter fluorophore and to switch them off to the dark state. A 532 nm diode-pumped solid state laser (GCL-200-L, Crystalaser), the 457 nm line of an Ar ion laser (35-LAL-030-208, Melles Griot), and a 405 nm diode laser (CUBE 405, Coherent) were used to reactivate the reporters by exciting the different activators. The fluorescence signal from the reporter dyes was collected by a 60x, NA 1.2 water immersion objective (Olympus) and then imaged on to an EMCCD camera (Andor Ixon DV897DCS-BV) after passing through a band pass fluorescence emission filter (Chroma, HQ710/80m for Cy5 and Cy5.5 and HQ740LP for Cy7). A 1.6 × tube lens was used to set the final imaging magnification to ~100 ×.

Switching kinetics analysis of the photo-switchable dyes

To measure switching kinetics, the DNA samples were first illuminated with the red imaging laser (657 nm) to switch the reporter molecules into the dark state, and the rate at which they
switched off ($k_{\text{off}}$) was measured by recording the number of fluorescent molecules as a function of time and fitting it to a single exponential function. For measurements of $k_{\text{on}}$, after switching the reporter fluorophores off and while the red imaging laser remained on, the sample was exposed to the activation laser (405 nm, 457 nm, or 532 nm), which caused the fluorophores to switch back on, reaching equilibrium between activation and deactivation. The number of fluorophores in the fluorescent state at equilibrium was measured, and the activation rate constant ($k_{\text{on}}$) was then calculated from the independently determined value of $k_{\text{off}}$ and the fraction of molecules ($F$) in the fluorescent state at equilibrium, according to the relation $F = k_{\text{on}} / (k_{\text{on}} + k_{\text{off}})$.

**Photon number analysis of the photo-switchable dyes**

We have measured the number of photons detected per switching cycle for Cy5, Cy5.5, and Cy7 when they were paired with Cy3 as the activator on DNA and antibody molecules. In all cases, the average number of photons detected per switching cycle was a constant independent of the excitation laser intensity. The photon number, however, depended on the emission filters and imaging geometry used. Using the prism-type TIRF imaging geometry, 657 nm imaging laser, and two stacked HQ665LP emission filters for Cy5 and Cy5.5 and a HQ740LP emission filter for Cy7, the photon numbers detected were ~ 3000 for Cy5 and Cy5.5 and ~500 for Cy7. These numbers correspond to a theoretical limit of localization accuracy (in terms of standard deviation or s.d.) of 3 nm for Cy5 and Cy5.5 and 9 nm for Cy7, calculated using a previously established formula $s.d. = \sqrt{S^2 + a^2/12}/N + 4\sqrt{\pi S^3 b^2/N}$.

In the formula, $S$ is the standard deviation of the point spread function of our imaging setup, $a$ is the edge size of the area imaged on each CCD pixel, $b$ is the background noise level, and $N$ is the number of photons detected ($S'_1 = 173$ nm for Cy5/Cy5.5 and $= 200$ nm for Cy7, $a = 165$ nm, $b = 6$ for Cy5/Cy5.5 and $= 1$ for Cy7 in our experiment). In this work, we typically use full-width-half-maximum (FWHM) to describe imaging resolution. The FWHM values corresponding to the localization accuracies quoted above are 8 nm for Cy5 and Cy5.5 and 22 nm for Cy7. Using the objective-type TIRF imaging geometry, 657 nm imaging laser, and stacked HQ665LP and HQ710/70BP emission filters for Cy5 and Cy5.5 and stacked HQ740LP and 800WB80 emission filters for Cy7, the photon numbers detected were ~ 6000 for Cy5 and Cy5.5 and ~1000 for Cy7, which were approximately twice as high as the numbers obtained in the prism-type TIRF geometry, as expected ($S_1$). The number of photons detected for Alexa 647, a cyanine dye with a similar structure to that of Cy5 (see fig. S1A), was identical (within 10%) to the number detected from Cy5. The photon numbers detected from the activator-reporter-labeled DNA samples were slightly smaller than the numbers detected from the corresponding antibody samples. The HQ665LP, HQ740LP, and HQ710/70BP filters were obtained from Chroma and the 800WB80 filter was from Omega.

**Three-color STORM imaging of model DNA samples**

Three different DNA constructs, each labeled with an Alexa 405-Cy5 pair, a Cy2-Cy5 pair, or a Cy3-Cy5 pair were mixed in solution and co-immobilized onto a quartz slide as described above. A concentration of 500 pM of each DNA was used to reach a high surface density of immobilized molecules. Due to a moderate Cy5 quenching effect that occurred when a Cy3 molecule was positioned in very close proximity, in this experiment these two dyes were separated by 9 base pairs on a 43 bp dsDNA, instead of being attached to the end of a dsDNA.
This Cy5 quenching effect was less significant when Alexa 405 or Cy2 was positioned in very close proximity. Fluorescent beads (Molecular probes, F8801) were added to the sample slide as fiducial markers for the purpose of drift correction.

STORM imaging was performed on an Olympus IX71 inverted microscope in the prism-type TIRF configuration. A 633 nm HeNe laser (25-LHP-928-249, Melles Griot) was used as the imaging laser and the violet (405 nm), blue (457 nm), and green (532 nm) lasers mentioned above were used as the activation light sources. The sample was first exposed to the red imaging light to switch off nearly all Cy5 dyes in the field of view. Then the sample was periodically activated with a sequence of violet, blue, and green laser pulses each of which switched on a sparse, optically resolvable subset of fluorophores which were then imaged with the red laser. Fluorescence from these probes was detected with the CCD camera after passing through a long pass emission filter (Chroma, HQ645LP). During the STORM data acquisition, the camera recorded the fluorescence signal at a constant frame rate of 19 Hz. In each switching cycle, one of the activation lasers was turned on for 1 frame, followed by 9 frames of illumination with the red imaging laser. Under our typical imaging conditions, an average fluorophore remains in the fluorescent state for three frames after activation, and ~ 3000 photons per molecule were detected during each switching cycle.

**STORM imaging of microtubules and clathrin-coated pits in cells**

Green monkey kidney BS-C-1 cells were plated in LabTek II 8 well chambered coverglass (Nunc) at a density of 30K per well. After 16 to 24 hr, cells were rinsed with phosphate buffered saline (PBS) buffer, fixed with 3% formaldehyde, and 0.1% glutaraldehyde at room temperature in PBS for 10 min, and quenched with 0.1% sodium borohydride in PBS for 7 min to reduce the unreacted aldehyde groups and fluorescent products formed during fixation. The sodium borohydride solution was prepared immediately before use to avoid hydrolysis. The fixed sample was permeabilized in blocking buffer (3% BSA, 0.5% Triton X-100 in PBS) for 10 min, stained with one or both of the primary antibodies against tubulin and clathrin (2.5 μg/mL mouse anti-β tubulin, ATN01 from Cytoskeleton and 2 μg/mL rabbit anti-clathrin heavy chain, ab21679 from Abcam) for 30 min in blocking buffer. The sample was then rinsed with washing buffer (0.2% BSA, 0.1% Triton X-100 in PBS) three times. Corresponding secondary antibodies labeled with photo-switchable probes (2.5 μg/mL) were added to the sample in blocking buffer and then thoroughly rinsed after 30 min. Cell imaging was performed in a standard imaging buffer that contains 50 mM Tris, pH 7.5, 10 mM NaCl, 0.5 mg/mL glucose oxidase, 40 μg/mL catalase, 10% (w/v) glucose and 1% (v/v) β-mercaptoethanol. We found that β-mercaptoethanol was critical for the observed photo-switching behavior of Cy5, Cy5.5, and Cy7, but even a low concentration of β-mercaptoethanol (as low as 0.02% v/v) supported photo-switching. β-mercaptoethanol at low concentrations (0.1% and 0.02%) was compatible with live cell imaging. Photo-switching was also observed when β-mercaptoethanol was replaced with cysteine (100 mM), which was also compatible with live cell imaging. Glucose oxidase was used as an oxygen scavenger system to increase the photostability of the cyanine dyes, and cell morphology was normal at the reported glucose oxidase concentration for at least 30 min. In this work, all STORM imaging experiments were performed on fixed cells.

Goat anti-mouse antibody (Invitrogen) and goat anti-rabbit antibody (Abcam) were each labeled with a mixture of amine-reactive activators and reporters. Cy2 and Cy3 were used as the activators. Alexa 647 (Invitrogen), which has similar structural and optical properties to Cy5 (fig.
S1) (S2), was used as the reporter. The concentrations of the reactive dyes were controlled such that each antibody had, on average, two activator molecules and 0.3 – 0.4 reporter molecules. The photo-switching behavior was relatively insensitive to the number of activators per antibody. The labeling ratio of two activators per antibody was chosen to ensure that the majority of antibodies had activators and thus to optimize the staining efficiency. However, when more than one reporter molecule was attached to the same antibody, we found that the close proximity of the reporter molecules significantly lowered the off rate. To assess this effect more quantitatively, dsDNA molecules labeled with two Cy5 dyes of known separations were prepared. The off rate of the construct having two Cy5 dyes separated by 2 nm was ~ 5 times slower than that for a construct with a single Cy5. For constructs where the two Cy5 dyes were separated by 7 nm or 14 nm, the off rates were roughly comparable to that of the single-Cy5 construct. This self-interaction effect was slightly less pronounced for Alexa 647 as compared with Cy5. Practically, when labeling antibody, we chose a relatively low dye/protein ratio for the reporter (0.3 – 0.4) such that the majority of reporter-labeled antibody molecules have only one reporter.

STORM imaging was performed on the Olympus IX71 microscope with an objective-type TIRF configuration. A custom polychroic beamsplitter (z458/514/647rpc, Chroma) reflected the excitation laser light onto the sample through an objective (100x oil, NA 1.4, UPlanSApo, Olympus), and fluorescence emission from the sample was collected by the same objective. Emitted light was filtered with two stacked dual-band emission filters (51007m, Chroma, and 595-700DBEM, Omega optical) before being imaged on the EMCCD camera. The use of a dual-band emitter enables fluorescence from Cy3 to be collected in addition to the fluorescence of the reporter dyes. Cy3 fluorescence collected during frames in which the green activation laser was on was used for drift correction purposes and to generate the conventional fluorescence image. For single-color STORM imaging with Alexa 647 as the reporter and Cy3 as the activator, the red laser (657 nm) was used for imaging and green (532 nm) laser pulses were for activation. For two-color STORM imaging with Cy2 and Cy3 as the activators, alternating blue and green (457 and 532 nm) laser pulses were used for activation. Images were acquired at a frame rate of 19 Hz. In each switching cycle, one of the activation lasers was turned on for 1 frame, followed by 9 frames of illumination with the red imaging laser. Because the two stacked dual-band emission filters (51007m and 595-700DBEM) significantly cut fluorescence signal from Alexa 647, only ~3000 photons, instead of ~ 6000, were detected on average from one antibody during each switching cycle. Typical laser powers used for STORM imaging were 40 mW for the red laser and 2 μW for each of the activation lasers.

STORM image analysis

A typical STORM image was generated from a sequence of 2000 – 100000 image frames recorded at 19 Hz. The movie consists of a repetitive sequence of activation frames (in which the activation laser is on) and imaging frames (in which the imaging laser is on). For each imaging frame, fluorescent spots were identified and fit to a Gaussian or elliptical Gaussian function to determine their centroid positions, intensities, widths and ellipticities (5, S3). Based on these parameters, peaks too dim, too wide or too elliptical to yield satisfactory localization accuracy were rejected from further analysis (5, S3). Peaks appearing in consecutive imaging frames with a displacement smaller than one camera pixel were considered to originate from the same fluorescent molecule, and centroid positions of these peaks were connected across frames and organized into a data structure which we refer to as a “string”. Each string represents a
single switching cycle for one fluorescent reporter molecule: the starting point of the string is the frame in which the molecule is switched on and its endpoint is the frame in which the molecule switches off. The final localization of the molecule was determined as the weighted average of the centroid positions across the entire string, weighted by the number of photons detected in each frame. The total number of photons detected for each switching cycle was used as an additional filter to further reject localizations with low accuracy. Strings starting in an imaging frame immediately after an activation frame were recognized as a controlled activation event and color-coded according to the activation laser color. Other strings were identified as non-specific activations, most likely induced by the red imaging laser as the amount of non-specific activation was observed to increase with the red laser intensity (data not shown). Nonspecific activation by the red imaging laser would also occur in the first imaging frame and be counted as a controlled activation event, giving one source of error for color crosstalk.

Besides the number of photons detected in one imaging cycle, another factor that limits the localization accuracy was sample drift during the course of the experiment. We corrected such drift by two methods. The first method involved adding fiducial markers (fluorescent beads) to track the drift of the sample and subtracting the movement of the markers during image analysis (5, 6). In the second method, we imaged the activator fluorophores during the activation frame and calculated the correlation function between the first activation frame and all subsequent activation frames. By tracking the centroid position of the correlation function, the drift of the image can be determined and corrected for in the STORM image. The correlation functions obtained from the fiducial marker images may also be used for drift correction. In some cases, we also found that further drift correction was possible by analyzing the correlation function of the STORM image itself as a function of time.

For image presentation, each localization was assigned as one point in the STORM image. These points were either represented by a small marker (e.g. a cross) or rendered as a normalized 2D Gaussian peak, the width of which was determined by its theoretical localization accuracy calculated from the number of photons detected for that localization event (5, 6). For multicolor STORM images, each localization was also false-colored according to the color of the activation laser pulse. The following color coding scheme was typically used: activations by the violet (405 nm) laser were shown in blue, those by the blue (457 nm) laser were shown in green, and those by the green (532 nm) laser were shown in red.

Crosstalk between different color channels resulted mainly from two effects: nonspecific activation and false activation. As described earlier, nonspecific activations, mostly likely induced by the red imaging laser, can be most easily identified if the string did not start immediately after an activation frame. However, such a nonspecific activation may also occur during the frame immediately after an activation laser pulse and thus be incorrectly assigned a color, although this mis-assignment will occur with a relatively low probability. Three methods can be used to reduce nonspecific activation-induced crosstalk: (1) increasing the activation laser intensity, providing that the density of activated probes remains low enough for single-molecule localization; (2) using a faster frame rate which effectively improves identification of those molecules activated by the activation laser pulse; and (3) decreasing the imaging laser intensity to reduce the non-specific activation rate, but at the cost of reducing imaging speed and/or accuracy. The second source of color crosstalk, false activations, stems from probes which were switched on by the wrong activation laser. Combining these two sources, the overall crosstalk ratios under our typical cell imaging conditions were measured to be 15 – 25% for the leakage of
Cy2 signal into the Cy3 channel and 25 – 35% for the leakage of Cy3 signal into the Cy2 channel. For the three-color STORM imaging of the DNA sample, crosstalk effects were observed to be somewhat smaller because nonspecific activation was observed to be less pronounced (fig. S4), in part due to stronger activation laser powers used.

The crosstalk ratios between different color channels under each imaging condition can be quantitatively determined using samples singly labeled with only one of the photo-switchable probes. Due to the clear separation between clathrin-coated pits and microtubules, the two color cell image itself can also be used to estimate crosstalk quantitatively. Using the crosstalk ratios, we can effectively subtract crosstalk from a multicolor STORM image. In the case of a two-color STORM image, at any given location we have:

\[
\begin{align*}
D_1 &= d_1 + C_{2\rightarrow1}d_2 \\
D_2 &= C_{1\rightarrow2}d_1 + d_2
\end{align*}
\]

where \(D_1\) and \(D_2\) are the observed local densities of spots in color channels 1 and 2, respectively, and \(d_1\) and \(d_2\) are the corresponding true local densities. \(C_{1\rightarrow2}\) and \(C_{2\rightarrow1}\) are the crosstalk ratios between the two channels. The values of \(d_1\) and \(d_2\) can be solved from observed local densities \(D_1\) and \(D_2\) and crosstalk ratios \(C_{1\rightarrow2}\) and \(C_{2\rightarrow1}\). Thus the probability of a localization at a given position in channel 1 being assigned the wrong color is simply \(P_1 = 1 - d_1 / D_1\). This point can thus be removed according to this probability. Similar treatment can be applied to every points in channels 1 and 2. To correct color crosstalk in our two-color STORM images, we chose a radius of 35 nm to calculate the local densities. Due to the finite area required to reliably calculate local densities, a slight erosion effect will arise from the crosstalk subtraction where two different colored structures overlap in space. According to our simulations, if the imaging resolution is 20 – 30 nm, such an operation will reduce the spatial resolution by ~ 20% when the crosstalk ratio is 20% for both channels. A similar statistical approach can also be used to assign colors to nonspecific activations (e.g. the probability of a non-specific activation belong to color channel 1 is \(d_1 / (d_1 + d_2)\), where \(d_1\) and \(d_2\) were obtained from controlled activations as described above), effectively increasing the overall localization point densities in the images, which may help improve resolution in cases where the resolution is point-density limited. Crosstalk subtraction and nonspecific activation color assignment were applied in Fig. 4.
**Fig. S1.** Chemical structures and spectroscopic properties of the activators and reporters used in this work. (A) Structures of the photo-switchable reporters Cy5, Alexa 647, Cy5.5, and Cy7. “R” stands for the place where DNA or antibody was attached. (B) Normalized absorption and emission spectra of Cy5, Alexa 647, Cy5.5, and Cy7 in aqueous solution. The absorption spectra were normalized by the maximum absorption value, and the emission spectra were normalized by the integrated peak area. (C) Normalized absorption spectra of activator dyes, Alexa 405, Cy2, and Cy3 in aqueous solution. (D) Schematic of double-stranded DNA and antibody molecules labeled with a Cy3-Cy5 pair.
**Fig. S2.** Photo-switching behavior of the Alexa 405-Cy7 pair. The lower panel shows a fluorescence time trace of Cy7. The upper panel shows the 405 nm laser pulses used to activate the dye pair. A red laser (657 nm) was continuously on, serving to excite fluorescence from the Cy7 and to switch it off to the dark state.

**Fig. S3.** Conventional fluorescence image of a mixture of three different DNA constructs, each labeled with Cy3-Cy5, Cy2-Cy5, or Alexa 405-Cy5 and mixed at a high surface density on a microscope slide. The fluorescence image was taken from all of the Cy5 molecules in this region before the sample was subjected to any photo-switching. A thermal color scheme is used here to illustrate the intensity, with black indicating low intensity, red higher, and yellow highest. A three-color STORM image of the same region is shown in Figure 2A. The overall intensity profile may appear to be slightly different for the two images due to the different numbers of switching cycles exhibited by individual molecules.
Fig. S4. Crosstalk analysis for the three-color STORM image of the DNA sample. The STORM image shows clearly separated clusters of localizations, each cluster corresponding to an individual DNA molecule (Figs. 2A-C). Each of the localizations was colored according the activation laser used: localizations activated by the 405 nm laser were assigned the blue color, those activated by the 457 nm laser were assigned the green color and those activated by the 532 nm laser were assigned the red color. The majority of the localizations within each cluster displayed the same color, identifying the type of activator dye (Alexa 405, Cy2, or Cy3) present on the DNA molecule. The numbers of localizations of each color were counted for individual clusters and the fractions of localizations assigned to each color channel are plotted here for the Alexa 405, Cy2; and Cy3 clusters. The crosstalk ratios can be calculated from the ratios of incorrectly to correctly colored localizations.
**Fig. S5.** Localization accuracy for the single-color STORM image of the cell. The localization accuracy was determined from point-like objects in the cell, appeared as small clusters of localizations away from any discernable microtubule filaments. Shown here is the spatial distribution of localizations within these point-like clusters. The 2D histogram of localizations was generated by aligning 170 clusters by their center of mass, each cluster containing more than 8 localizations. Fitting the 2D histogram with a Gaussian function gives a FWHM of 24 nm.

**Fig. S6.** Localization accuracy for the two-color STORM image of the cell. The localization accuracy was also determined from point-like objects in the cell, appeared as small clusters of localizations away from any discernable microtubule or CCP structures. Shown here is the spatial distribution of localizations within these point-like clusters. The 2D histograms of localizations were generated by aligning 187 clusters by their center of mass, each cluster containing more than 8 localizations. Fitting the 2D histogram with a Gaussian function gives a FWHM of 30 nm.
**Fig. S7.** STORM images of clathrin-coated pits (CCPs). (A) Comparison of conventional fluorescence images (upper panels) and the STORM images (lower panels). Nearly all CCPs appear to adopt a spherical structure. The rightmost panel shows two close-by CCPs that were resolved in the STORM image, but appeared as a single nearly diffraction-limited spot in the conventional fluorescence image. (B) Size distribution of 300 CCPs determined from the STORM images as shown in (A).

**Supporting references**

