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Multicolor Super-resolution Imaging with Photo-switchable Fluorescent Probes

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Abstract

Recent advances in far-field optical nanoscopy have enabled fluorescence imaging with spatial resolution of 20 - 50 nm. Multicolor super-resolution imaging, however, remains challenging. In this report, we introduce a family of photo-switchable fluorescent probes and demonstrate multicolor stochastic optical reconstruction microscopy (STORM). Each probe consists of a photo-switchable “reporter” fluorophore that can be cycled between fluorescent and dark states, and an “activator” that facilitates photo-activation of the reporter. Combinatorial pairing of reporters and activators allows the creation of probes with many distinct colors. Iterative, color-specific activation of sparse subsets of these probes allows their localization with nanometer accuracy, enabling the construction of a super-resolution STORM image. Using this approach, we demonstrate multi-color imaging of DNA model samples and mammalian cells with 20 - 30 nm resolution. This technique will facilitate direct visualization of molecular interactions at the nanometer scale.

As one of the most versatile imaging modalities in biology, fluorescence microscopy allows noninvasive imaging of cells and tissues with molecular specificity. The availability of fluorescent probes in many colors and the ability to label specific gene products enable visualization of molecular interactions in biological samples. However, the spatial resolution of optical microscopy, classically limited by the diffraction of light to ~ 300 nm, is inconveniently situated 1 - 2 orders of magnitude above the typical molecular length scales in cells. Various “super-resolution” optical imaging techniques have been developed to overcome this limit (1,2). Among these methods, stimulated emission depletion microscopy (STED) and reversible saturable optically linear fluorescent transition (RESOLFT) techniques (2,3), saturated structured illumination microscopy (SSIM) (4), stochastic optical reconstruction microscopy (STORM) (5) and photoactivated localization microscopy (PALM) (6,7) have achieved 20 - 50 nm resolution in the far field and promise to preserve the inherent noninvasive imaging capability of optical microscopy. In certain cases, binding kinetics or translational motions of individual molecules have also been used to paint high-resolution structures in cells (8-10).

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One sentence summary

A family of photo-switchable fluorescent probes enables multicolor, super-resolution fluorescence imaging of cells with 20 - 30 nm spatial resolution.

Nonetheless, multicolor super-resolution imaging remains a major challenge, leaving many biological questions beyond the reach of optical methods. Among the various multicolor imaging techniques, fluorescence resonance energy transfer (FRET) is particularly powerful for probing molecular interactions on the nanometer scale (11-14), although it is difficult to determine the precise locations and organizations of molecules from a FRET image. Fluorescence colocalization offers another approach to detect molecular interactions, but has been traditionally used within the boundaries of diffraction-limited imaging; the power of this approach would enhance significantly when used in combination with super-resolution techniques (15). Here, we report a family of photo-switchable probes with distinct colors and demonstrate multicolor STORM imaging with 20 - 30 nm resolution.

STORM relies on the detection of single fluorescent molecules (16) and the localization of these molecules with nanometer accuracy (17-21). Limited only by the number of photons detected (17), localization accuracies as high as 0.1 - 1 nm can be achieved for bright fluorescent or scattering objects (22-25). By employing photo-switchable probes, the fluorescence emission profile of individual fluorophores can be modulated in time such that only an optically resolvable subset of fluorophores are activated at any moment, allowing their localization with high accuracy. Over the course of multiple activation cycles, the positions of numerous fluorophores are determined and used to construct a high-resolution STORM image (5-7). The development of multicolor STORM thus depends on the construction of bright, photo-switchable probes with distinct colors.

To search for chromatically distinguishable photo-switchable probes, we turned to cyanine dyes with various lengths of poly-methine chains (fig. S1) (26-28). We constructed a series of dye pairs, each consisting of a cyanine dye and a shorter-wavelength chromophore, as inspired by our earlier work on photo-switchable Cy5 (27). These dye pairs were conjugated to double-stranded DNA or antibody and immobilized on microscope slides for single-molecule detection (26). Remarkably, a series of cyanine dyes with distinct absorption and emission spectra displayed robust photo-switching behavior when paired with a Cy3 molecule in close proximity (fig. S1) (26). Figure 1A shows fluorescence time traces obtained from single molecules of Cy5, Cy5.5, and Cy7 as they undergo multiple switching cycles. Upon illumination with a red laser (657nm), each of the three dyes was initially fluorescent and then quickly switched into a non-fluorescent, dark state. A brief exposure to a green laser pulse (532 nm) led to reactivation of the dyes back to the fluorescent state. Without a proximal Cy3, the reactivation of Cy5, Cy5.5, or Cy7 was barely detectable under our excitation conditions. We therefore refer to the Cy3 dye as the “activator” and Cy5, Cy5.5, or Cy7 as the photo-switchable “reporter”. These reporters typically can be switched on and off for hundreds of cycles before permanently photobleaching.

The rate constant of switching from the fluorescent to the dark state, designated as k_{off} , scaled linearly with the red laser power (Fig. 1B). Consistent with this observation, the average number of photons detected per switching cycle was a constant, independent of the laser power (data not shown). The number of photons detected was ~ 3000 for Cy5 and Cy5.5 and ~ 500 for Cy7 under prism-type total internal reflection fluorescence (TIRF) imaging geometry, and these numbers doubled under objective-type TIRF imaging geometry (26). The activation rate, k_{on} , from the dark to the fluorescent state, was linear with respect to the green laser power (Fig. 1B). The linear dependence of both rate constants extended over the entire range of laser powers used, suggesting that faster switching rates are possible at higher laser intensities. As demonstrated previously, their distinct emission spectra (fig. S1) allow Cy5, Cy5.5, and Cy7 to be distinguished at the single-molecule level (29), suggesting one approach to accomplish multicolor STORM imaging.

The above results indicate that chromatically distinguishable photo-switchable reporters can be activated by the same activator dye. As a further exploration, we tested whether other dyes with distinct spectral properties can be used as activators. To this end, Cy3, Cy2, and Alexa Fluor 405 (fig. S1) (26) were paired with Cy5. Switching traces for individual pairs are shown in Fig. 1C. Again, Cy5 was quickly switched into the dark state upon illumination with a red laser. The activation of Cy5, however, required different colored lasers corresponding to the absorption wavelengths of the activators. The Alexa 405-Cy5 pair was efficiently activated by a violet laser (405 nm), but was much less sensitive to blue (457 nm) and green (532 nm) activation light. Similarly, the Cy2-Cy5 pair and Cy3-Cy5 pair were most sensitive to the blue and green lasers, respectively. To determine the activation specificity quantitatively, we measured the activation rate constants for these dye pairs at the three wavelengths, 405 nm, 457 nm, and 532 nm. For each wavelength, the pair with the appropriate activator was activated rapidly, with a rate at least 10 times higher than those measured for the other two pairs (Fig. 1D), indicating high activation specificity.

This selective activation provides a second parameter for multicolor detection. Not only can different activator-reporter pairs be distinguished by their emission color, as determined by the reporter dye, but they can also be differentiated by the color of light which activates them, as determined by the activator dye. A combinatorial pairing of the three reporters and three activators reported here could generate up to nine distinguishable fluorescent probes, but requires only four light sources for excitation and three spectrally distinct channels for detection. Photoswitching appears not to be limited by the spectral separation between the dyes, as illustrated by efficient activation of Cy7 by Alexa 405 (fig. S2), despite the fact that the two dyes lie at opposite extremes of the visible spectrum with absorption maxima ~ 350 nm apart (fig. S1) (26). We expect this combinatorial scheme to significantly expand multi-color imaging capabilities at both conventional and sub-diffraction limit resolutions.

Here, we use the selective activation scheme as an initial demonstration of multicolor STORM imaging. Three different DNA constructs labeled with Alexa 405-Cy5, Cy2-Cy5, or Cy3-Cy5 were mixed and immobilized on a microscope slide at a high surface density such that individual DNA molecules could not be resolved in a conventional fluorescence image (fig. S3) (26). To generate a STORM image, the sample was first exposed to a red laser (633 nm) to switch off nearly all Cy5 dyes in the field of view. The sample was then periodically excited with a sequence of violet (405 nm), blue (457 nm), and green (532 nm) laser pulses, each of which activated a sparse, optically resolvable subset of fluorophores. In between activation pulses, the sample was imaged with the red laser. The image of each activated fluorescent spot was analyzed to determine its centroid position (referred to as a localization), and a color was assigned according to the preceding activation pulse. As the same imaging laser and detection channel were used for all three dye pairs, there was no need for correction of chromatic aberration. After thousands of activation cycles, a STORM image was constructed by plotting all of the colored localizations (Figs. 2A-C). In contrast to the conventional image (fig. S3), the STORM image showed clearly separated clusters of localizations, each cluster corresponding to an individual DNA molecule and resulting from repetitive localizations of a single Cy5 molecule over multiple switching cycles. The majority of the localizations within each cluster displayed the same color, identifying the type of activator dye present on the DNA and illustrating multicolor imaging capability through selective activation. We identified two origins of crosstalk between colors: false activation by laser pulses of incorrect colors (for example, activation of the Cy3-Cy5 pair by blue pulses) and non-specific activation by the red imaging laser (26). Both effects were quantitatively small (fig. S4) (26). The localizations within each cluster approximately follow a Gaussian distribution with a full-width-half-maximum (FWHM) of 26 ± 1 nm, 25 ± 1 nm, and 24 ± 1 nm for the three color channels (Fig. 2D), suggesting an imaging resolution of ~ 25 nm for 3-color STORM imaging. This resolution

is lower than the theoretical limit predicted from the number of photons detected (26), likely due to the residual stage drift that was not completely accounted for by the drift correction.

Applying STORM to cell imaging, we first performed single-color immunofluorescence imaging of microtubules, filamentous cytoskeleton structures important for many cellular functions. BS-C-1 cells were immunostained with primary antibodies and then with activator-reporter-labeled secondary antibodies (26). Cy3 was used as the activator and Alexa Fluor 647, a cyanine dye with very similar structural, spectral and photo-switching properties to Cy5, was used as the reporter (fig. S1). The STORM image shows a drastic improvement in the resolution of the microtubule network as compared to the conventional fluorescence image (Figs. 3A-F). In the regions where microtubules were densely packed and undefined in the conventional image, individual microtubule filaments were clearly resolved by STORM (Figs. 3C-F). As an example, two filaments 80 nm apart appeared well separated in the STORM image (Fig. 3G). Whole-cell STORM images, including $\sim 10^6$ single-molecule localizations, were acquired in 2 - 30 minutes, an improvement of 1 - 2 orders of magnitude over the previously reported imaging speed of PALM (6). Super-resolution microtubule structures, in fact, began to emerge after only 10 - 20 seconds of STORM imaging.

To determine the imaging resolution more quantitatively, we identified point-like objects in the cell, appearing as small clusters of localizations away from any discernable microtubule filaments. These clusters likely represent individual antibodies nonspecifically attached to the cell. The width (FWHM) of the localization clusters was 24 ± 1 nm (fig. S5) (26), giving a measure of the image resolution inside the cell. Next, we measured the apparent width of a microtubule filament in the STORM image by examining its cross-sectional profile. Figure 3H shows a cross-sectional distribution of localizations for a microtubule segment. A simple Gaussian fit to the profile yielded a FWHM of 51 nm but deviates significantly from the actual distribution, which appeared flat-topped presumably due to the finite width of microtubule. A significantly better fit was obtained by simulating the actual microtubule cross-section as a rectangular function of width d , but convolving it with a Gaussian function with FWHM r to model our image resolution. From the fit, we obtained an intrinsic imaging resolution of $r = 22$ nm, consistent with the localization accuracy determined from the point-like objects. The microtubule width derived from the fit, $d = 56$ nm, agrees with the 60 nm width of antibody-stained microtubules previously determined using electron microscopy (30). However, this apparent width is significantly larger than the diameter of a bare microtubule without antibody coating, pointing out a commonly appreciated aspect of super-resolution imaging - the effective resolution is determined by a combination of the intrinsic imaging resolution and the size of the labels. Improvement in the effective resolution may be achieved by using direct immunofluorescence staining with dye-labeled primary antibodies or Fab fragments.

To demonstrate multi-color STORM imaging in cells, we simultaneously imaged microtubules and clathrin-coated pits (CCPs), cellular structures used for receptor-mediated endocytosis. Microtubules and clathrin were immuno-stained with primary antibodies and then activator-reporter-labeled secondary antibodies. The activator-reporter pairs used were Cy2-Alexa 647 for microtubules and Cy3-Alexa 647 for clathrin. The 457 nm and 532 nm lasers were used to selectively activate the two pairs. Figure 4 shows the two-color STORM image presented at different scales. Crosstalk between the two color channels due to false and non-specific activations were subtracted from the image after statistical analysis (26). The intrinsic imaging resolution, as determined from the FWHM of point-like clusters of localizations was 30 ± 1 nm for each of the two color channels, slightly larger than the resolution determined for the single-color STORM images (fig. S6) (26). The green channel (457 nm activation) revealed filamentous structures as expected for microtubules, with a width of 53 nm, quantitatively similar to that obtained from the single-color STORM image. The red channel revealed predominantly spherical structures, representing clathrin-coated pits and vesicles (Figs. 4 and

S7A) (26). In contrast to the STORM images, the conventional TIRF images of CCPs appeared as diffraction limited spots without any discernable shape (fig. S7A). Side-by-side CCPs that were clearly resolved by STORM appeared as a single spot in the conventional image (fig. S7A). Interestingly, many of the spherical CCPs appeared to have a donut shape with a higher density of localizations towards the periphery, which is consistent with the two-dimensional projection of a three-dimensional cage structure. Finally, the STORM image allowed us to measure the size distribution of CCPs (fig. S7B), which agrees quantitatively with the size distribution determined using electron microscopy (31). In summary, our two-color STORM images of cells clearly revealed ultra-structural information not discernable in conventional fluorescence images. This technique may be readily expanded to include more colors using the photo-switchable activator-reporter pairs reported here, significantly enhancing our ability to visualize molecular interactions in cells and tissues. Further development of cell delivery methods with molecular specificity may allow live cell imaging with these probes (14,32).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Fig. 1.

Photo-switchable probes constructed from activator-reporter pairs. (A) Spectrally distinct reporters exhibit photo-switching behavior. The lower panel shows the fluorescence time traces of three photo-switchable reporters, Cy5 (dark yellow line), Cy5.5 (red line), and Cy7 (brown line) when paired with a Cy3 dye as the activator on a DNA construct. Traces were shifted relative to each other for clarity. The upper panel shows the green laser pulses (532 nm) used to activate the reporters. The red laser (657 nm) was continuously on, serving to excite fluorescence from the reporters and to switch them off to the dark state. (B) Switching rate constants k_{on} and k_{off} of the Cy3-Cy5, Cy3-Cy5.5, and Cy3-Cy7 pairs as a function of green and red laser power. Error bars indicate SEM from ~3 data sets. We note that the laser power to intensity calibration may vary between different samples due to moderate differences in the laser spot size at the sample. (C) The same reporter can be activated by spectrally distinct activators. The lower panel shows the fluorescence time traces of Cy5 paired with three different activators, Alexa 405 (magenta line), Cy2 (blue line) and Cy3 (green line). The upper panel shows the violet (405 nm, magenta line), blue (457 nm, blue line), and green (532 nm, green line) activation pulses. (D) Normalized activation rate constants per unit laser power of the three pairs at three activation wavelengths, 405 nm, 457 nm, and 532 nm. The values obtained for Alexa 405-Cy5, Cy2-Cy5 and Cy3-Cy5 were used for normalization at 405 nm, 457 nm and 532 nm, respectively. The absolute activation rates were rapid for each pair at its corresponding optimal wavelength, with values ~ 10 s⁻¹ or greater at only a few hundred μW of laser power. The activation rate of the Alexa 405-Cy5 pair by the 532 nm laser was too small to be measured reliably.

Fig. 2.

Three-color STORM imaging of a model DNA sample. (A) Three-color STORM image of three different DNA constructs labeled with Alexa 405-Cy5, Cy2-Cy5, or Cy3-Cy5 mixed at a high surface density on a microscope slide. The image was plotted by rendering each localization as a Gaussian peak, the width of which was scaled with the theoretical localization accuracy given by the number of photons detected (26). Each colored spot in this image represents a cluster of localizations from a single DNA molecule. A conventional fluorescence image of the same area is shown in fig. S3 for comparison. (B, C) Higher magnification views of the boxed regions in (A) show several examples of closely spaced DNA molecules. Here, each localization was plotted as a cross, colored according to following code: if the molecule was activated by a 405 nm, 457 nm, or 532 nm laser pulse, the color of the cross was assigned as blue, green, or red, respectively. (D) The localization distributions of the blue, green, or red clusters. The 2D histograms of localizations were generated by aligning multiple (50 - 60) clusters by their center of mass. The histograms were fit to a Gaussian profile to determine their FWHM.

Fig. 3. STORM imaging of microtubules in a mammalian cell. (A) Conventional immunofluorescence image of microtubules in a large area of a BS-C-1 cell. (B) STORM image of the same area. (C, E) Conventional and (D, F) STORM images corresponding to the boxed regions in (A). (G) Cross-sectional profiles of two nearby microtubule filaments in the cell. The inset shows the STORM image, and the histogram shows the cross-sectional distribution of localizations within the small regions specified by the white box. (H) Cross-sectional profile of a microtubule segment determined from the STORM image. A relatively long segment (~ 7 μm) was chosen to obtain good statistics. The histogram shows the cross-sectional distribution of localizations. The green line is a single Gaussian fit with FWHM = 51 nm. The red line shows the fit obtained by convolving a rectangular function of width = d with a Gaussian function of FWHM = r . The fit yields $d = 56$ nm and $r = 22$ nm, corresponding to the microtubule width and the imaging resolution, respectively.

Fig. 4.

Two-color STORM imaging of microtubules and CCPs in a mammalian cell. (A) STORM image of a large area of a BS-C-1 cell. The secondary antibodies used for microtubule staining were labeled with Cy2 and Alexa 647, while those for clathrin were labeled with Cy3 and Alexa 647. The 457 nm and 532 nm laser pulses were used to selectively activate the two pairs. Each localization was colored according to the following code: green for 457nm activation and red for 532 nm activation. (B) STORM image corresponding to the boxed region in (A) shown at a higher magnification. (C) Further magnified view of the boxed region in (B).