Biophysical Letter

Dual-Color 3D Superresolution Microscopy by Combined Spectral-Demixing and Biplane Imaging

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ABSTRACT Multicolor three-dimensional (3D) superresolution techniques allow important insight into the relative organization of cellular structures. While a number of innovative solutions have emerged, multicolor 3D techniques still face significant technical challenges. In this Letter we provide a straightforward approach to single-molecule localization microscopy imaging in three dimensions and two colors. We combine biplane imaging and spectral-demixing, which eliminates a number of problems, including color cross-talk, chromatic aberration effects, and problems with color registration. We present 3D dual-color images of nanoscopic structures in hippocampal neurons with a 3D compound resolution routinely achieved only in a single color.

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Single-molecule localization microscopy (SMLM) techniques (1,2) rely on stochastic activation and precise localization of individual fluorophores. Such techniques allow the resolution of important cellular structures in unprecedented detail. Major challenges in technical development now lie in multicolor and three-dimensional (3D) imaging.

Regarding multicolor imaging, the two most widely used strategies include either excitation of spectrally well-separated fluorophores with multiple laser lines and detection through a dichroic beamsplitter or different emission filters (3), or the use of an activator/reporter dye system (4). In the former approach, the compound multicolor resolution will also depend on the precision with which the two images can be registered, and accuracies of $<$10 nm involve very elaborate and sophisticated calibration experiments, even more so in three dimensions (5). In the latter approach, the same reporter dye can be switched by spectrally distinct activator dyes in close proximity. Although being completely free from chromatic aberrations and requirement for image registration, the activator/reporter method can be prone to color cross-talk, owing to spontaneous or nonspecific activation. Methods to estimate and correct for this exist (6), but rely on additional experiments and the extent of cross-talk will vary with the employed activator/reporter conjugated reagent.

Several optical methods have been developed to access the axial dimension for subdiffraction 3D resolution. The z position of a single molecule can be obtained from the shape of its point-spread function (PSF) by employing two focal planes as in biplane imaging (7) or after shaping the PSF by using an astigmatic (8), double-helical (9), or self-bending PSF (10). Other methods employ interferometric detection, such as iPALM (11) and 4PI-SMS (12). Some of these methods provide isotropic or close-to-isotropic 3D resolution, but require complex optical setups as a tradeoff.

In this Letter we provide a simple and robust approach to two-color SMLM imaging in three dimensions by combining an established dual-color scheme based on spectral-demixing (13–16) with biplane imaging. Existing setups can be readily extended to two-color imaging capability with only minor changes. In spectral-demixing, a single laser is used to excite two spectrally close fluorophores. Here we used the far-red dyes AlexaFluor647 (AF647) and CF680, which are both highly performing in terms of brightness and duty-cycle in the same conventional blinking buffer (Fig. S1 in the Supporting Material). Their partially overlapping emission is spectrally separated by a dichroic beamsplitter and imaged onto two separate parts of the camera chip. Both channels exhibit cross-talk in either direction, and an emitting fluorophore produces a localization on each side of the camera chip. The assignment of this localization pair to either dye is based on the ratiometric fluorescence intensity. The same dichroic can also be used to introduce an axial separation between the focal planes of the two channels to implement biplane 3D imaging (Figs. 1 *A* and S2).

The principle is illustrated in Fig. 1 *C*. Reconstruction of the two-color 3D image is based on localizations only from the short-wavelength channel and hence all optical surfaces

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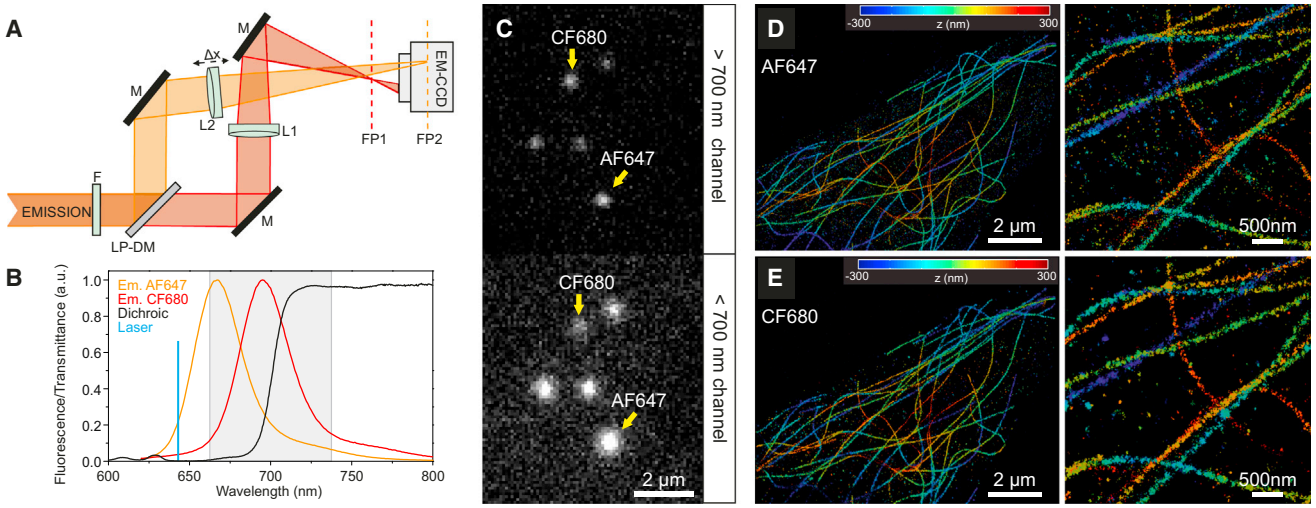

FIGURE 1 A dual-color 3D single-molecule localization microscopy approach. (*A*) Schematic of the optical setup (*F*, emission filter; *LP-DM*, long-pass dichroic mirror; *M*, mirrors; *L1*, lens; *L2*, translatable lens; *FP1* and *FP2*, focal planes; and *EM-CCD*, electron-multiplying charge-coupled device). (*B*) Emission spectra of AF647 (*orange*) and CF680 (*red*), transmission of the dichroic (*black*), transmission of the emission filter (*gray box*), and the 643-nm laser (*blue*). (*C*) Sample frame showing the split-channel view. Single AF647 and CF680 molecules are distinguishable by their ratiometric intensity while their *z* position is determined from the widths of their PSFs in the different channels. (*D* and *E*) Two-color 3D superresolution measurement of microtubules using antibodies labeled with AF647 (*D*) and CF680 (*E*).

are identical for the two spectrally very close colors. As a result, the approach is close to free of chromatic aberrations and the two colors are inherently registered, laterally as well as along the optical axis. In conventional biplane imaging the fluorescence intensity is separated 50:50 between the two focal planes by means of a nonpolarizing beamsplitter. This is the optimum in terms of z resolution. However, a 50:50 separation between long- and short-wavelength channels is impossible for both fluorophores simultaneously in spectral-demixing. We instead used a ~50:50 separation for the less bright CF680 and ~75:25 for AF647 between short- and long-wavelength channels. As a result of this separation, we obtained similar fluorescence intensities for CF680 and AF647 in the long-wavelength channel. As a first test, we imaged microtubules in fixed cells. We used a primary anti-tubulin antibody and a 1:1 mixture of AF647- and CF680-labeled secondaries. Fig. 1, *D* and *E*, shows the 3D images for both channels acquired in parallel. By using stringent cutoffs for the dye-assignment, the cross-talk in either channel was kept <1% (Fig. S3 B). From a number of short microtubule segments over the field of view and at different z positions, we determined a lateral and axial offset of <9 nm between the two colors and an average lateral and axial full width at half-maximum for the microtubules of 51/57 nm and 79/86 nm for AF647 and CF680, respectively (Fig. S3). These values are close to what is typically reported for single-color experiments.

We then applied our technique to resolve nanoscopic structures in hippocampal neurons. First, we imaged the axonal scaffolding proteins β4-spectrin and AnkyrinG (AnkG), which localize to the axonal initial segment. Only recently has a periodic cytoskeletal ring-structure along the longitudinal axis of the axon with a spacing of ~190 nm been discovered (17). Using our technique, we obtained high-resolution 3D images of β4-spectrin and AnkG, and observed the expected ~190 nm periodicity for both proteins even though the AnkG density or labeling was too sparse to detect solid rings (Fig. 2, *A* and *B*). AnkG interacts with the N-terminus of β4-spectrin via its spectrin-binding domain (18). Indeed, AnkG staining correlated highly with immunostaining against the N-terminus of β4-spectrin (Figs. 2, *C* and *D*, and S4). A transversal x,z cross section confirmed the circular distribution of both molecules around the axon (Fig. 2 *E*). We consistently observed that AnkG is more peripheral than β4-spectrin. Next, we imaged presynaptic Homer1 and postsynaptic Bassoon in neuronal synapses, which we detected as separated lanes (Fig. 2, *Fi* and *G*) or elliptical disks (Fig. 2, *Fii* and *H*), depending on the viewing angle in the 3D image. Finally, we imaged β2-spectrin together with β-tubulin. We observed a periodical ring-structure of β2-spectrin surrounding β-tubulin staining in the center of the axon (Fig. 2, *I* and *J*).

We have here introduced a simple method for dual-color 3D imaging that addresses several important problems. These include complexity, robustness, cost of the experimental setup, buffer compatibility of fluorophores, color cross-talk, chromatic aberration, and color registration.

We note that in principle this approach can be used with other combinations of spectrally close fluorophores. Two-dimensional spectral-demixing FPALM has already been shown by Gunewardene et al. (14), and the combination