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Extending the performance capabilities of isoSTED

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If you try to focus light with a lens, you will soon realize that you cannot create a point. Instead, the intensity of light that you focus will always be wider and longer because of the diffraction of light. The created intensity pattern is called a point spread function (PSF). In the 19th century, the physicist Ernst Abbe was able to show that the size of the PSF depends on the wavelength of the used light and the light collection angle of the lens (1). According to Abbe, when a single lens is used, the PSF will be shaped like an ellipsoid, with a width of approximately half the wavelength of the input light in the focal plane and a length two to threefold that size along the optical axis. The PSF of a microscope is of fundamental importance because its shape ultimately defines the resolution limit of a conventional microscope. Structures smaller than the PSF will appear to be the same size as the PSF and have the same elongated distortion along the optical axis. However, the anisotropy of the PSF can be reduced if two opposing lenses are used. This arrangement, referred to as 4Pi, doubles the light collection angle and increases the resolution along the optical axis by three- to sevenfold (2–4). However, although 4Pi microscopes can reach a nearly isotropic resolution, they remain diffraction-limited.

Superresolution microscopes or nanoscopes also experience the diffraction limit of light. Still, they are theoretically diffraction-unlimited because they take advantage of molecular state transitions, typically between a fluorescent (ON) and nonfluorescent (OFF) state (5). Stimulated emission depletion (STED), reversible fluorescent saturable optical transition (RESOLFT), and photoactivation localization/stochastical optical reconstruction microscopes all use the ON and OFF states of fluorophores either in a controlled or random way in time or space to overcome the diffraction limit.

A STED microscope operates similarly to a confocal microscope. However, in STED, two concentric and synchronized laser beams are scanned across the specimen. They control the transition of the fluorophores between their excited (ON state) and their ground state (OFF state) to create a diffraction-unlimited image. The first beam (ON switching beam) excites fluorophores from their OFF to their ON state. Then, the second donut-shaped beam (OFF-switching beam) pushes excited fluorophores back to their OFF state before they can emit fluorescence. Only signal from fluorophores in the center of the donut that were not pushed back to their OFF state can be detected. So, although both beams are diffraction-limited, using them to control the ON and OFF state of the fluorophores breaks the diffraction limit. RESOLFT microscopy functions similarly but switches the fluorophores between different conformational states, which requires a less powerful OFF-switching beam. However, it takes longer to acquire an RESOLFT image than an STED image because the pixel dwell times required for switching between conformational states with the currently available fluorophores are, on average, at least one order of magnitude larger than for switching between ground and excited states of fluorophores. Despite the advances provided by superresolution microscopes, even these technologies can benefit from the earlier developed 4Pi arrangement when tasked with reaching diffraction-unlimited three-dimensional (3D) resolution. Here, the incredibly sharp and light-efficient patterns/modulations created in 4Pi microscopes, and the doubled light detection have been shown to significantly improve the images collected using STED (6), RESOLFT (7), and photoactivation localization/stochastical optical reconstruction (8,9). All in all, applying the 4Pi scheme to superresolution approaches is a mighty optical scheme for 3D fluorescence nanoscopy.

In this issue of the *Biophysical Journal*, Siegmund et al. (10) present two ways to extend the capabilities of the combination of 4Pi microscopy and STED microscopy, an approach also termed isoSTED (fluorescence

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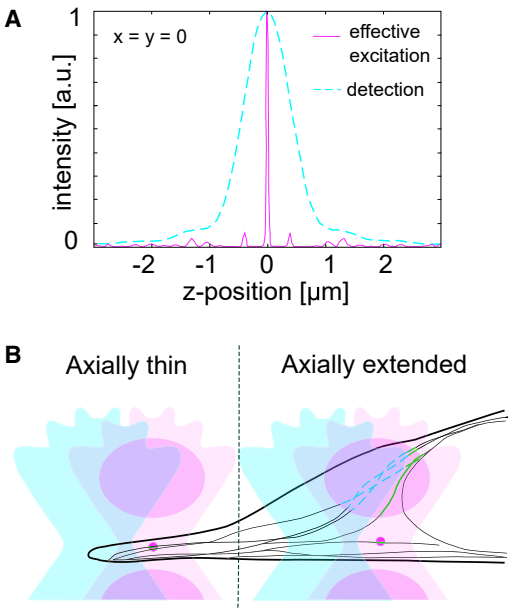

FIGURE 1 How the effective excitation profile contributes to OOFCs in thick specimens. (*A*) Intensity profile of the effective excitation (*solid line*) along the optical (*z* axis) shows the magnitude of PSF side lobes (OOFCs) in comparison to the central excitation point; all are collected by the diffraction-limited detection (*dashed line*). (*B*) In a thin region of the cell, only the main focus (*small circle*) contributes to the detected signal, whereas, in a thick region of the cell, OOFCs (*large oval*) contribute to the signal and can be detected by a laterally shifted detector for subsequent removal (dashed microtubules). Modified from (6). To see this figure in color, go online.

microscopy setup with isotropic 3D focal spot) (6), broadening its usefulness to biomedical research. First, the authors use water-immersion lenses for the first time in isoSTED to image structures embedded in aqueous media. Second, they present a new detection method to quantify and remove out-of-focus contributions (OOFCs) that would otherwise increase the background and therefore decrease the practical resolution of the microscope.

The reason for using water-immersion objectives in microscopy when imaging samples embedded in aqueous media, including living cells, is straightforward: you want to create a homogenous immersion system to avoid light reflection or refraction and achieve the maximal resolution. In the ideal case, one needs to match the refractive index and numerical aperture of the objective, the immersion media, the cover glass, and the mounting medium. However, because cover glasses with the refractive index of water are not available, only the refractive index and numerical aperture of the objective, immersion media, and mounting media can be matched. Most water objectives can compensate for the major effects of this refractive index mismatch with a correction collar; however, the system is still very sensitive to the tilt of the cover glass. Strong deformations of the PSF (aberrations) can easily be created if the cover glass is not perpendicular to the optical axis. Therefore, either your sample and its holder have to ensure such a positioning during imaging, or your imaging system's stage needs to be capable of correcting for tilt, typically with a goniometer (7). However, here Siegmund et al. successfully integrate water-immersion objectives into an isoSTED system with a sample preparation protocol that does not require them to implement an otherwise necessary tilt compensation.

The usefulness of 4Pi microscopy combined with water-immersion objectives for live-cell imaging has been shown using confocal and RESOLFT microscopy (7,11). However, Siegmund et al.'s implementation of ON and OFF switching between the ground and excited state during isoSTED enables them to acquire isotropic superresolved images with water-immersion objective much faster than ever before. Siegmund et al. reach voxel dwell times of 20 μs and capture a volume of $\sim$14.6 μm $\times$ 8 μm $\times$ 0.6 μm in under a minute, which is suitable for imaging the dynamics of living cells. While in the current implementation, the duration of time-lapse studies is short due to photobleaching, the authors can capture the movement of microtubules with 60-nm isotropic resolution while keeping the integrity of mitochondria intact over tens of minutes. Future integration of temperature control would further boost the system's adaption in biomedical imaging.

The authors also developed a new way to deal with OOFCs. OOFCs can arise if the OFF switching of the fluorophores is insufficient. Siegmund et al. indicate that OOCFs are likely to originate in isoSTED because the effective excitation PSF has still side lobes outside of the focal point, which excite and turn on fluorescence at points along the optical axis (Fig. 1 *A*). These fluorescence sources increase the background signal and make thicker sample regions blurry. Their removal is essential, because similar to standard confocal microscopy, the signal-to-background ratio can severely impact an image's quality. In the worst case, it can even be the primary factor that practically limits the resolution of a microscope and not the actual specific resolution of the microscope itself. In isoSTED, the impact of background can be even more severe because of its small effective excitation volume.

Here, instead of utilizing multiple ON- and OFF-switching beams to eliminate OOFCs like in 4Pi-RESOLFT microscopy (7), Siegmund et al. implement a new technique that exploits the simultaneous detection of emitted fluorescence onto several sightly offset detectors surrounding

the central detector. Whereas the main detector in the center captures the signal like in a typical confocal microscope, the offset detectors surrounding it primarily detect various OOFCs (Fig. 1 *B*). The authors show that if the signal of the offset detectors is appropriately scaled and subtracted from the main detector, the image quality of the superresolved image significantly improves. To realize the detection system, the authors used four channels of a 1-to-7 fan-out fiber. They suggest that implementing their newly developed detection might also boost the image quality of single lens-based 3D STED systems. Notably, the approach does not require any prior knowledge of the sample or the system's PSF.

The reported study is a promising next step toward developing isoSTED to image fast enough to capture the dynamics within living cells. Siegmund et al.'s new detection method for correcting OOFCs gives isoSTED a significant advance toward imaging truly isotropically at the single-molecule level. This system has the potential to enhance the performance of other systems as well, especially STED-FCS (fluorescence correlation spectroscopy) microscopy. Moreover, they propose several schemes for further development that will increase acquisition speed and decreasing photobleaching, such as brighter dyes and implementing a scanning system with low latency and a large field of view where the pixel dwell time can be locally adapted to the specimen (12,13). If they can implement these enhancements, then their approach even more useful for biological imaging.

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