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Parallel super-resolution imaging

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Abstract

Massive parallelization of scanning-based super-resolution imaging allows fast imaging of large fields of view.

Ever since Ernst Abbe formulated the theory of diffraction in optical imaging, resolution has been thought to be restricted to approximately half the wavelength of light: the diffraction limit. Much work has been done to circumvent this limit; early work involved methods such as structured light illumination, first pioneered by Lukosz and Marchand[1] and subsequently popularized by the late Gustafsson[2]. More recent developments such as stimulated emission depletion (STED) microscopy[3], photoactivated localization microscopy (PALM)[4], stochastic optical reconstruction microscopy (STORM)[5], reversible saturable optical fluorescence transitions (RESOLFT)[6] and their variants have made great progress in bringing optical imaging to the 10-nm scale, but fast imaging of large fields of view using these techniques has been an elusive goal. In this issue, Chmyrov *et al.*[7] present a novel parallelized RESOLFT approach that promises to eliminate the speed bottleneck in super-resolution imaging at these scales.

Techniques based on point spread–function engineering (such as RESOLFT and STED) typically generate an image by raster scanning a single point through a sample. After fluorophores are switched on within a diffraction-limited volume, RESOLFT works by switching off most of the fluorophores, allowing fluorescence from just one small region to

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dominate. Switching off is usually achieved using a 'donut beam', which has a finite intensity close to the center of the beam but a node at the very center where the light intensity drops to 0. The light from the donut beam is used to suppress the fluorescence from surrounding fluorophores, either by stimulated emission in the case of STED or by switching the fluorophore off photochemically in a typical implementation of RESOLFT, thus ensuring that the fluorophores at only the very center of the donut beam will be observed. The key to the RESOLFT principle is saturation: the idea that, with a sufficiently high light intensity, all the fluorophores in a particular region can be made not to fluoresce. Through the maintenance of a sufficiently high intensity in the donut beam, the remaining fluorescent region can be made arbitrarily small, limited only by the power that can be applied to the sample.

Conventional RESOLFT requires raster scanning of both the switch-on and the switch-off beams to generate a volumetric image. In comparison, techniques that can image the whole field of view in parallel, such as PALM and STORM, work by ensuring that only a statistically random subset of fluorophores are active simultaneously, such that the probability of having more than one fluorophore emitting within a diffraction-limited volume is negligible. Given these assumptions, the centroid of each fluorophore can be determined with nanometer precision, and the process can be repeated with many different subsets to create a high-resolution image.

In general, super-resolution imaging techniques are slow with small fields of view: the speed of raster-scanning techniques is limited by their sequential approach, and the speed of full-field techniques such as PALM and STORM is limited by the need for multiple exposures. Chmyrov *et al.*[7] overcome the limitations of raster scanning with a new form of nanoscopy that greatly improves imaging speed by massively parallelizing the photochemical RESOLFT approach, resulting in imaging times of 400 ms for a 50 μm × 50 μm field of view or 2–4 s for a 100 μm × 120 μm field of view. Parallelized switch-off is achieved by projecting two orthogonal incoherent standing-wave patterns of high frequency on the sample. This generates a multitude of zero-intensity spots or 'donuts', each of which confers super-resolution and is scanned to form a super-resolved image (Fig. 1). Importantly, at saturation, the cross-sections of the ~110,000 donuts are effectively circular, and no rotation of the pattern is needed to ensure that resolution is isotropic.

Using this highly parallel super-resolution setup, the researchers were able to image keratin 19–rsEGFP(N205S) expressed in PtK2 cells; part of the cytoskeleton could then be visualized with 80-nm resolution. In another demonstration, the growth of neurites from a neuron expressing the Lifeact-Dronpa-M159T fusion protein was measured over time. Here each super-resolution frame was measured in 2 s, allowing the growth to be monitored with high spatial and temporal resolution.

An important goal in the methodological development of this field is to achieve three-dimensional (3D) super-resolution imaging over a substantial volume and at video rates. Chmyrov *et al.*[7] do not quite achieve this: the frame rates, despite showing major improvement over those seen in previous efforts, are still low, and super-resolution is demonstrated in 2D rather than 3D, although the authors report a commendable *z* resolution

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of around 580 nm. Other methods such as 3D STORM[8] and biplane fluorescence PALM[9] have been shown to deliver sub-100-nm axial resolution, but the stochastic nature of these approaches results in substantially longer imaging times.

At present, the authors note that it is the camera and the state transition kinetics of the fluorophores that limit the frame rate. Today camera technology is advancing rapidly and is likely to become a nonissue within a few years. On the other hand, the development of more efficient switchable fluorophores represents the key bottleneck. Fortunately, many excellent laboratories are actively on the hunt for better fluorophores. Indeed, with the intense interest in switchable fluorophores for not just RESOLFT but also PALM, STORM, saturated structured illumination microscopy[10] and their derivatives, microscopists can look forward to a future with a wide palette of different fluorophores optimized for nanoscopy. When that happens, wide-field RESOLFT imaging and its variants may well become a commonplace substitute for wide-field fluorescence imaging whenever high-resolution images are required.

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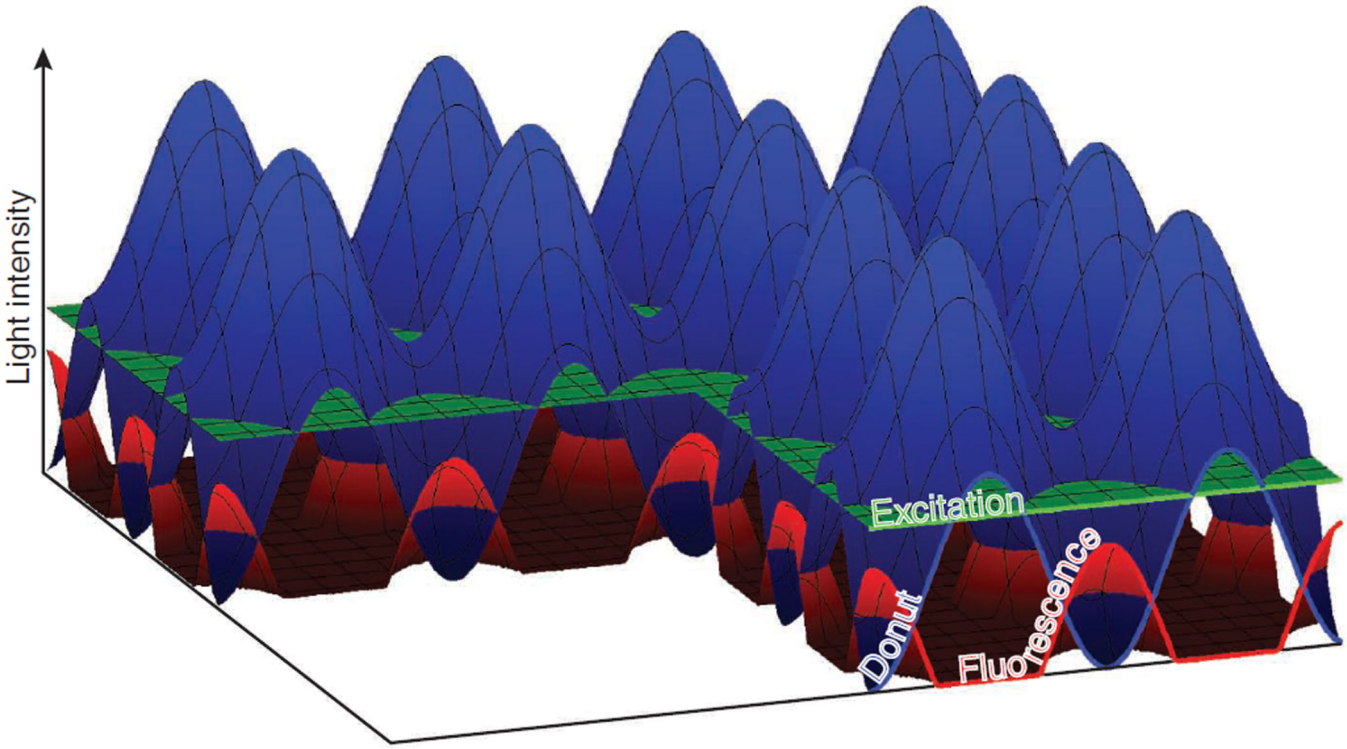

Figure 1.

Parallelized RESOLFT imaging uses ~110,000 donuts to scan a sample. The different colored plots represent the intensities of excitation and emission light during imaging. The donut beam (blue) switches off fluorophores in the sample, whereas a uniform excitation beam (green) stimulates fluorescence (red) from switched-on fluorophore probes at the centers of the donuts.