MICROSCOPY

Taking nanoscopy to the limit

MINFLUX nanoscopy can obtain 1-nm localization precision with orders of magnitude fewer emitted photons.

In super-resolution microscopy, a method that delivers 1-nm localization precision independent of the wavelength with fewer than 500 detected photons and fast temporal resolution might sound too good to be true. This is in part because single-molecule localization microscopy (SMLM) methods rely on high photon counts to achieve resolutions around 10–30 nm. High photon counts improve the precision with which the fluorophore-generated centroid of the diffraction pattern can be determined, but the need for high photon counts is also associated with long image acquisitions and dependence upon bright and photostable fluorophores.

Super-resolution microscopy approaches like stimulated emission depletion (STED), also rely on large numbers of photons for high resolution. However, these photons come from the illumination lasers rather than the sample itself, as the precise position of the fluorophore is determined by the position of the excitation and depletion beams. These methods, while powerful, do not specifically resolve single emitters.

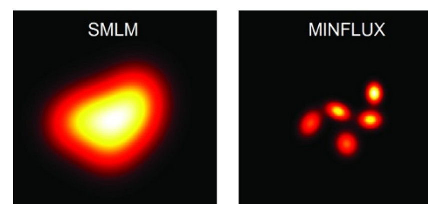
A new approach from Stefan Hell, postdoctoral fellow Francisco Balzarotti and graduate students Yvan Eiles and Klaus Gwosch at the Max Planck Institute for Biophysical Chemistry, Göttingen both marries and surpasses SMLM and coordinate-targeted imaging. The method is called MINFLUX in reference to the minimum photon flux needed, and it combines the stochastic photoswitching necessary for SMLM with a donut-shaped laser, typically used with STED, for determining the position of the emitter.

From there, MINFLUX diverges from existing approaches. In MINFLUX the emitter position is determined not by where fluorescence is present, but by where it is absent. To conceptualize this, Hell notes in a thought experiment that if the position of an emitter was known a priori, then its position could be tracked using a donut-shaped excitation beam in which the emitter was always located in the donut center (where the fluorophore would not be excited). Note that this is in contrast to the donut-shaped depletion beam used in STED. In this way, the emitter's position could be precisely determined and tracked without a single emitted photon. Indeed, any emission would merely prove that the fluorophore was not in the center of the donut and could be used to recenter it.

In practice, an emitter's position cannot be known in advance, and photoactivation of an individual fluorophore is followed by conventional imaging to find the fluorophore for subsequent determination of the precise location with the donut-shaped excitation beam. Also, a single measurement is not sufficient for determination of localization. The researchers found that rapidly moving the donut to image at four positions directly over and adjacent to the emitter was sufficient to accurately localize its position with 500–1,000 photons.

The advantages of MINFLUX were demonstrated on DNA origami structures, where fluorophore spacing was tightly controlled. Here, in an unprecedented demonstration, the researchers showed they could image individual fluorophores as close as 6 nm apart with 1-nm precision. The same emitters could not be resolved with SMLM. The team then showed the advantages of MINFLUX for single-particle tracking in living bacteria; they labeled the 30S ribosome with a photoswitchable fluorescent protein and carried out high-resolution single-particle tracking with 5- to 10-fold higher photon efficiency than that of conventional methods. The increased photon efficiency also allowed them to track with a 100-fold higher time resolution than that of conventional methods.

Hell believes that his unique thinking about the essence of super-resolution microscopy is the basis of this innovative technique. In his view, both SMLM and STED rely on an on–off state transition for feature separation, with the fundamental difference being the way the position of the fluorophores is established. "Whereas SMLM relies on emitted photons, in STED the position is determined using the photons from the laser. Since the precision always scales with the number of photons, it makes sense to do as much localization as possible with the excitation laser." He had thoughts along these lines as early as 2008 when writing his super-resolution Perspective in *Nature Methods*, and first filed a patent on this idea in 2011. He recalls that MINFLUX was already quite advanced by 2014, saying, "While I stood there winning the Nobel Prize, I knew there was something coming that was going to be important."



MINFLUX (right) enables visualization of individual emitters as close as 6 nm apart, while SMLM (left) does not. Adapted from Balzarotti *et al.* (2016) with permission from AAAS.

MINFLUX is still in its early days. Unlike SMLM, MINFLUX requires specialized optics for generating the excitation beam, and commercial instruments will likely be needed to see broad adoption of this method. In addition, while current implementations of MINFLUX are poised to impact quantitative super-resolution imaging and single-particle tracking, future versions should expand the method to 3D and multi-color applications.

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RESEARCH PAPERS
Balzarotti, F. *et al.* Nanometer resolution imaging and tracking of fluorescent molecules with minimal photon fluxes. *Science* http://dx.doi.org/10.1126/science.aak9913 (2016).

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