FLUORESCENCE NANOSCOPY: PRINCIPLES AND APPLICATIONS IN LIFE SCIENCES

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For a long time, the resolution of any lens-based optical microscope has been limited to $d = \lambda/(2n\sin\alpha) > 200$ nm in the focal plane and about $\lambda \approx 400$-700 nm along the optic axis, with $n\sin\alpha$ denoting the numerical aperture of the lens and λ the wavelength of light. While this diffraction barrier has prompted the invention of electron microscopy, the 3D-imaging of the interior of (living) cells requires the use of focused visible light. The resolution issue began to change with the advent of 4Pi microscopy[1,2] which improved the axial resolution by a factor of 3-7 along the optic axis in fixed[3] and living cells[4,5]. However, 4Pi microscopy and the related I^5M concept [6,7] could not improve the resolution in the focal plane and were still diffraction-limited.

Here, I will report on the principles and applications of lens-based fluorescence microscopy concepts that are no longer constrained in resolution by the wavelength of light. Emerged in the wake of the discovery that elementary transitions between the states of a fluorophore can be used to eliminate the limiting role of diffraction[8-11], these concepts share a common basis: They employ a transition between a bright and a dark fluorophore state to switch the fluorescence of 'inseparable' features either on or off, such that these features emit fluorescence sequentially in time[12,13].

The first concrete and viable concept was Stimulated Emission Depletion (STED) microscopy[8], where the features located at the outer part of a scanning focal spot of excitation light are transiently switched off by de-excitation through stimulated emission. As a result, only features located within a narrower spot of diameter $d \approx \lambda/\left(2n\sin\alpha\sqrt{1+I/I_s}\right)$ are able to fluoresce[11,14,15]. I_s is a characteristic of the fluorophore whereas $I > I_s$ is the intensity of the beam inducing the de-excitation. Confining fluorescence to a narrower spot that is scanned through the sample increases the spatial resolution. For $I/I_s \rightarrow \infty$ it follows that $d \rightarrow 0$, meaning that, unlike in lens-based optical microscopes known until then, the resolution is no longer fundamentally limited by the wavelength.

In an initial application[16], STED microscopy was employed to investigate the fate of synaptic vesicle proteins after fusion to the presynaptic membrane. Its nanoscale resolution discerned individual vesicles in the synapse and showed that synaptotagmin I, a protein resident in the vesicle membrane, remains clustered in isolated patches on the presynaptic membrane, regardless of whether the nerve terminals were mildly active or intensely stimulated. These findings suggested that at least some vesicle constituents remain together during the vesicle protein recycling. This and other early applications[17-19] demonstrated the power of the emerging field of lens-based fluorescence nanoscopy to solve biological questions.

A video-rate recording variant of STED microscopy was used to map and describe the mobility of vesicles inside the axons of cultured living neurons, particularly within the highly confined space of synaptic boutons[20]. These results evidenced the possibility to image intracellular physiological processes with nanoscale resolution in real-time.

Live-cell STED microscopy has also been used to noninvasively image activity-dependent morphological plasticity of dendritic spines [21]. The results indicate that the resolution provided by STED has the potential to supersede the use of electron microscopy in many cases. Time lapse STED imaging of dendritic spines of YFP-positive living hippocampal neurons in organotypic slices outperformed confocal microscopy in the quantification of morphological parameters, such as the neck width and the curvature of the heads of spines, which are considered to play an important role for the function and plasticity of synaptic connections.

In another application, the dynamics of individual lipid molecules in the membrane of a living mammalian cell was detected and characterized[22]. The up to 70 times smaller focal spot area created by STED (as compared to confocal microscopy) directly revealed fundamental differences between the diffusion of phospho- and sphingolipids under physiological conditions. Single sphingolipids, but not phospholipids, are transiently (< 10 ms) and locally (< 20 nm) trapped in the plasma membrane, mediated by cholesterol. Thus, STED directly revealed inhomogeneities of lipid diffusion in the plasma membrane of a living cell on a single molecule basis, setting an upper bound for the size of cholesterol mediated lipid nanodomains ('rafts') in the cell membrane[22].

The concept of STED microscopy can be expanded by employing other transitions that shuffle the fluorophore between a dark and a bright state, such as (i) shelving the fluorophore in a dark (triplet) state[10] and (ii) switching between a 'fluorescence activated' and a 'fluorescence deactivated' (conformational) state[11,14,23,24]. Examples for the latter include photochromic organic compounds and switchable fluorescent proteins. Optical switching between long-lived bright and dark states of switchable proteins has been shown to overcome the diffraction barrier at ultra-low light levels[25]. More recent nanoscopy schemes switch the molecules individually and stochastically to a state that emits $m >> 1$ detectable photons in a row before returning to a dark state. Detection of these photons on a camera and calculation of the position of these molecules with high precision renders an image consisting of molecule positions. Contrary to earlier (fluorescent protein) switching concepts[11,24,25], these single fluorophore switching concepts[26-30] require only a single switching cycle[12,13] per fluorophore to generate an image, thus greatly expanding the fluorescence nanoscopy toolbox.

In a nutshell, lens-based fluorescence nanoscopy is an unexpected and fascinating development in the physical sciences with high relevance to the life sciences. Owing to their simplicity, commercial availability, and proven usefulness I expect far-field fluorescence nanoscopes to enter most life science laboratories in the near future.

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