Supplementary Box 2 | **Diffraction limit and super-resolution microscopy**

Membrane domains in cell membranes are assumed to measure below 20 nm in diameter[1], and consequently are not resolvable on a conventional optical microscope. The spatial resolution of any lens-based microscope is limited to about 200-250 nm for visible light. Since such a lens-based microscope employs focussed light, the light interacts with fringes of intricate objects, and it broadens to a diffraction pattern as it propagates. This diffraction pattern contains a central disk containing most of the information on the object (Airy disk), but becomes larger than the object masking the subtle details of it. This diffraction limit, as formulized by Ernst Abbe in 1873: $d = \lambda/2NA$ (where d is the diameter of the focal spot (or Airy disk at full-width-half-maximum of the focused light intensity), λ is the wavelength of the light and NA is the numerical aperture of the objective) prevents the separation of two close-by objects with the standard illumination (**see Figure**).

A remedy to this physical limit is the reversible inhibition of fluorescence, ensuring that the measured signal stems from a region of the sample that is much smaller than 200-250 nm, as realized in super-resolution optical microscopy or nanoscopy (for a review see ref[2]). Stimulated emission depletion nanoscopy (STED) nanoscopy uses a depletion laser whose focal spot exhibits at least one intensity zero such as a donut-shaped intensity distribution to inhibit spontaneous fluorescence emission at the periphery of the airy pattern, leaving the central fluorescence signal untouched (**see Figure**). The resulting effective fluorescence spot is thus reduced to sub-diffraction scales, and, consequently, the STED nanoscope produces images with <<200 nm spatial resolution. On the other hand, photoactivatable localization microscopy (PALM) or stochastic optical reconstruction microscopy (STORM) switches the employed fluorescence labels between dark and bright states in such a way that in a single camera image only a few molecules are bright and detected, allowing a precise determination (or localization) of the spatial positions of molecules (**see Figure**). Switching on and off all fluorescence labels in subsequently recorded image frames allows establishing a final image out of all molecular positions. Since the localization precision is <<200 nm, the final PALM or STORM images feature sub-diffraction spatial resolution. Both methods have their own advantages and disadvantages. While STORM/PALM may deliver higher spatial resolution in day-to-day use, dynamic live-cell imaging is less straightforward and requires extensive post processing. In contrast, STED nanoscopy delivers direct images, and its spatial resolution can be continuously tuned by increasing the STED laser intensity, which offers unique possibilities such as combination with fluorescence correlation spectroscopy (STED-FCS). However, high laser power of the depletion laser entail the careful use of the STED technique in living cells. Further techniques such as structured-illumination microscopy achieve an up to 2-fold improvement in spatial resolution (in contrast to the in principle unlimited increase in STED and PALM/STORM microscopy) by employing spatial patterns in the illuminating laser light only and no reversible photoswitching of fluorescence emission.

References

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2 Eggeling, C., Willig, K. I., Sahl, S. J. & Hell, S. W. Lens-based fluorescence nanoscopy. *Quarterly reviews of biophysics* **48**, 178-243, doi:10.1017/s0033583514000146 (2015).

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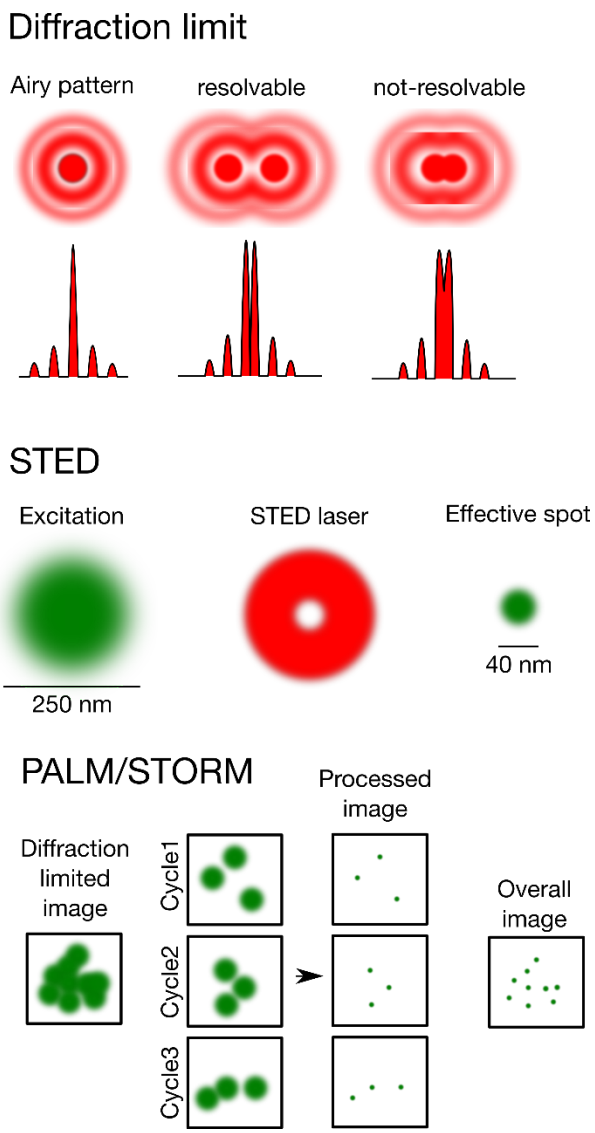

Figure: Principle of diffraction limit, STED and PALM/STORM super-resolution microscopy.