

Primer: fluorescence imaging under the diffraction limit

A brief description of the theory and methods behind super-resolution fluorescence imaging.

Fluorescence imaging 101

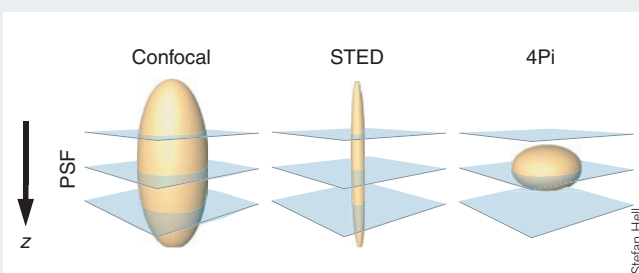
When fluorescent molecules—fluorophores—absorb a photon, they almost always emit a photon of longer-wavelength light about one nanosecond later. Modern detection devices such as charge-coupled devices (CCDs) or photo-multiplying tubes (PMTs) can detect these photons and transform these detection events into quantifiable electrical signals. Provided fluorophores can be attached to biological molecules of interest, these target molecules can be localized and quantified to generate an image of their distribution in a sample. The trick in generating a fluorescent image is to collect these photons in a way that you can determine where they came from.

In fluorescence microscopy this is done by directing a beam of excitation light of the proper wavelength at a biological sample containing molecules of interest that have been individually labeled with fluorophores. Longer-wavelength light emitted by the excited fluorophores is collected by the microscope, separated from unwanted light of shorter or longer wavelengths using a filter and directed to a detector for measurement.

For wide-field imaging, the whole viewing field is illuminated by the microscope, and the entire image is captured at once by a CCD that records the quantity and location of detected photons. In contrast, scanning-based imaging techniques such as confocal microscopy build up the image pixel by pixel, collecting the fluorescent light generated by a focused light beam as it is scanned over the sample and measuring the number of photons collected at each designated pixel position using a PMT or similar detector.

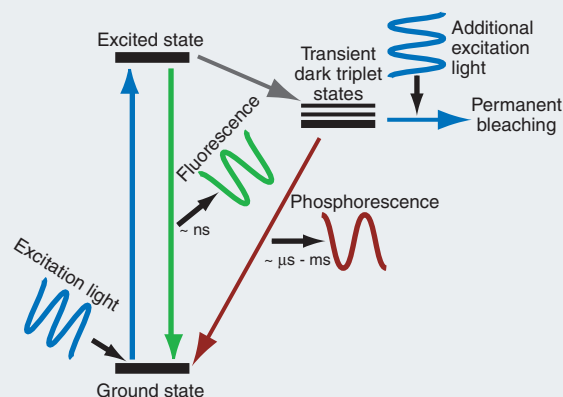
What is the diffraction barrier?

If light always traveled in a straight line, and your detector had the necessary sensitivity and resolution, it would be possible to discriminate all the individual fluorophores in a wide-field fluorescence image. Unfortunately however, interactions between light and the physical medium it travels through do not allow the light from a single fluorophore to be focused to a discrete point in an image. Instead, it forms a spot called a point-spread function (PSF) ~200 nm in diameter (along the x and y directions) and >450 nm long (in the z direction). Emitted light arriving simultaneously from any fluorophores within this volume merges together.



Illustrations of common PSFs.

The required distance between fluorophores that would allow them to be resolved is $d = \lambda / (2n \sin\alpha)$, where λ is the wavelength of the emitted light, n is the refractive index of the medium into which the sample is placed, and α is the angular aperture of the microscope. If the microscope could detect light emitted in all directions (x , y and z), this would simplify to $d \approx \lambda / (2n)$. This theoretical resolution limit can also be expressed in terms of the maximum spatial frequency (the number of emission maxima per meter) that can be observed. This value, called the Abbe limit of detection, is $k = (2n \sin\alpha) / \lambda$. In practice, however, because they cannot detect light from all directions at once, conventional microscopes do not reach this theoretical resolution limit in x , y and z directions.

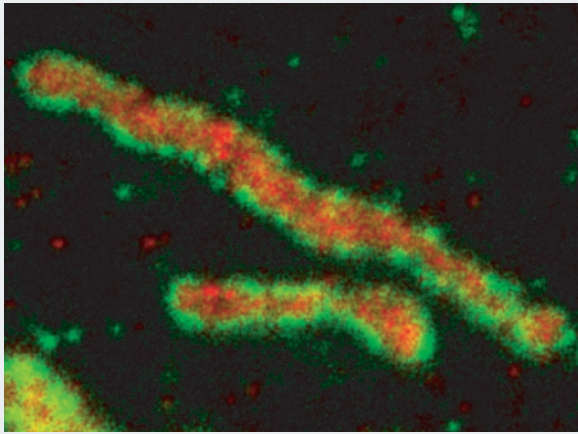


Schematic showing the states and transitions of a fluorophore.

Playing by the rules, but using excitation

By cleverly exploiting the equations above it is possible to actually reach the theoretical resolution limit. Illumination of the sample with spatially structured light (focused or periodically patterned), for instance, provides additional image information about the fluorophores' distribution in space. The use of structured light effectively combines the Abbe limit of detection mentioned above with a similarly constrained Abbe limit of illumination. For example, structured illumination microscopy (SIM) uses full-field illumination with patterned lines of light that are translated and rotated to generate a series of images with higher spatial frequency information. A theoretical two-fold improvement in x and y resolution can be obtained after using these images to compute the final image.

Alternatively, 4Pi and I³M use opposing lenses to increase the angular aperture of the microscope, and improve the illumination and detection performance. This allows detection of emitted light from nearly all directions and provides up to a seven-fold increase in the resolution in the z dimension.

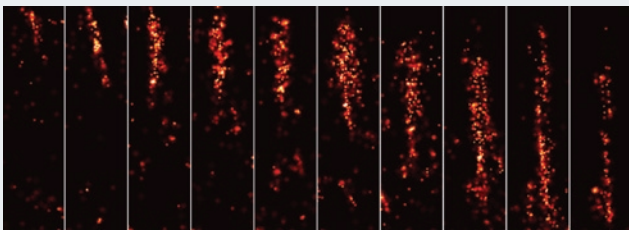


Stefan Hell

Two-color image of a single mitochondrion obtained using a variation of STED microscopy.

Breaking the rules

4Pi, I⁵M and SIM depend only on a standard linear relationship between excitation and emission of a fluorophore, but to really break the diffraction limit it is necessary to move beyond the constraints imposed by the linear equations above and exploit a nonlinear relationship between excitation and emission of a fluorophore. This is possible in fluorescence microscopy because fluorophores exhibit several behaviors that lead to a nonlinear dependence between excitation and emission. For example, intense illumination can trigger photophysical transitions to transient dark states that emit light in the microsecond to millisecond timeframe, or to a permanent dark state (bleaching). Alternatively, light can be used to induce photochemical reactions in photoactivatable or photoswitchable fluorophores to switch these labels between on or off states, or change their color. As long as such a nonlinear dependence can be limited to a subset of the fluorophores in time or space and the transitions are detectable, unwanted signals can be excluded in a defined manner, thus increasing the density of information obtained. Nonlinear effects can be exploited at the ensemble (bulk) or single-molecule level.



Eric Betzig

Time-lapse imaging of individual focal adhesions in a living cell by PALM.

Working with the crowd

Super-resolution at the population level is achieved by driving the fluorophores to one fluorescent state, within regions that are $<\lambda/(2n)$ in size, while separating these regions from one another by a distance $>\lambda/2n$. In the space between these regions the fluorophores have an alternative and easily distinguishable state.

The first useful implementation of this nonlinear dependence was called stimulated emission depletion (STED) microscopy and achieved this by (i) creating a laser scanning focal spot that excites fluorescence emission and (ii) surrounding it with a doughnut of longer wavelength laser light to deplete the fluorophores surrounding the focal spot. Increasing the intensity of light in the doughnut will shrink the size of the spot from which emission is possible to $<\lambda/(2n)$ and increase the width of the depletion region which is $>\lambda/2n$. The spot is then scanned over the sample to generate a subdiffraction image.

An alternative to stimulated emission is to exploit the long-lived triplet state of fluorophores as a dark state. Termed ground state depletion (GSD) microscopy, this approach works with a larger number of fluorophores, but the bleaching sensitivity of the triplet state can be a problem.

Although STED and GSD typically rely on scanning a focal spot, a super-resolution technique based on SIM—called saturated structured illumination microscopy (SSIM) or saturated pattern excitation microscopy (SPEM)—uses a full-field illumination pattern of alternating lines of saturating illumination typically $>\lambda/(2n)$ in width separated by non-emitting lines $<\lambda/(2n)$ in width.

The power of the individual

Whereas the ensemble methods above require that the two distinguishable fluorescent states can be cycled many times without bleaching, this ceases to be a requirement if individual molecules can be temporally distinguished and localized in separate imaging frames. Photoactivatable fluorophores allow this kind of temporal discrimination because the intensity of full-field photoactivation light can be attenuated to induce a physically sparse subpopulation of activated molecules where each is separated from the others by $>\lambda/(2n)$. These are selectively visualized using excitation light specific for the activated molecules, and the centers of the resulting diffraction-limited fluorescent spots are localized with nanoscale precision—typically by fitting a Gaussian curve to the spot. This process is repeated many times until enough fluorophores are localized to generate an image.

The step between visualization of a group of activated molecules and activation of a new group can differ between different methods. In photoactivation localization microscopy (PALM), the activated fluorophores are illuminated during image acquisition until all of them are bleached and then a new subpopulation is photoactivated to begin the next cycle. Conversely, in stochastic optical reconstruction microscopy (STORM) a second photoactivation laser is used to switch the photoactivated molecules back to their starting state after the desired number of photons has been collected. Several variations on these techniques have been published, and recently a GSD approach was described that combines the use of universal photophysical transitions with individual fluorophore localization.

Daniel Evanko

ACKNOWLEDGMENTS

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