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Super-resolution fluorescence imaging with single molecules

Steffen J Sahl and WE Moerner

The ability to detect, image and localize single molecules optically with high spatial precision by their fluorescence enables an emergent class of super-resolution microscopy methods which have overcome the longstanding diffraction barrier for far-field light-focusing optics. Achieving spatial resolutions of 20–40 nm or better in both fixed and living cells, these methods are currently being established as powerful tools for minimally-invasive spatiotemporal analysis of structural details in cellular processes which benefit from enhanced resolution. Briefly covering the basic principles, this short review then summarizes key recent developments and application examples of two-dimensional and three-dimensional (3D) multi-color techniques and faster time-lapse schemes. The prospects for quantitative imaging — in terms of improved ability to correct for dipole-emission-induced systematic localization errors and to provide accurate counts of molecular copy numbers within nanoscale cellular domains — are discussed.

Address
Department of Chemistry, Stanford University, Stanford, CA, USA

Corresponding author: Moerner, WE (wmoerner@stanford.edu)

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Introduction

Optical imaging's most serious drawback — the limited spatial resolution [1] — has been radically overcome for the important case of fluorescence with the advent of a number of methods termed super-resolution (SR) microscopies. Realizing that the molecules which constitute a labeled structure are *themselves* nanoscale sources of light [2–5], the key to rescinding the limiting role of diffraction in most techniques has been to switch the fluorescence of molecules residing closely packed within a diffraction-limited region of the sample on and off, actively controlling the emitting concentration at a very low level, and to localize stochastically available single molecules in a time-sequential manner [5,6]. Thus, with recordings of the positions of single molecules (1–2 nm size) as the light emitters to high spatial precision (10–40 nm), an increase in resolving power by an order of magnitude and more has been demonstrated over the much coarser diffraction-limited (DL) level of resolution (200–300 nm laterally, 500–700 nm axially) accessible by focusing light through even the best modern microscope lenses. A separate set of SR fluorescence methods including stimulated emission depletion (STED) [7–9], reversible saturable/switchable, optically linear fluorescence transition (RESOLFT) [10–12], and (non-linear) structured illumination (SIM) [13–15] microscopies achieve subdiffraction resolution by directly reducing the effective microscope point spread function (PSF) via toggling molecules between fluorescent and non-fluorescent states with carefully prepared beam shapes, often in a laser-scanning setup. This second set of methods is discussed elsewhere.

Beyond diffraction: nanometer-scale resolution by precise localization and active on/off control of single-molecule emitters

The challenge is illustrated in Figure 1. For conventional imaging, for example in a wide-field epi-fluorescence or total internal reflection fluorescence (TIRF) system, all molecules in a certain spatial arrangement (a super-structure, Figure 1a) are excited and fluoresce simultaneously. As a result, their diffraction-limited images overlap seriously on the camera detector. Information about the underlying super-structure is irretrievably lost (Figure 1b). If, however, individual sparse subsets of single molecules that are spatially separated further than the DL can be made to emit while all others remain dark, their positions may be extracted in a time-sequential manner by finding the center of a mathematical description (fit) of the single-molecule image shapes, and a super-resolution reconstruction may be assembled from the list of estimated positions (Figure 1c–e). More than two decades after the first detection of single molecules in condensed phases [16] and single-molecule imaging [17–19], sufficient sensitivity to allow imaging of single-molecule labels (i.e. attaining sufficient signal-to-noise ratio) remains one essential requirement. The ability to determine the position of each single molecule from pixelated recordings [20,21], a process sometimes termed super-localization, is a second essential requirement. Even at relatively modest signal to noise, digitizing and fitting of the single-molecule image (Figure 1f–g) allows the center (x,y) to be determined much more precisely (Figure 1h) than the width of the shape, which is the DL PSF. In situations where a single object is emitting, crucially, this knowledge then allows one to interpret the center of the PSF as a measurement of the location of this emitter. It is worth noting that the above two points taken together do not lead to super-*resolution* images without a clever modification to standard single-molecule imaging.

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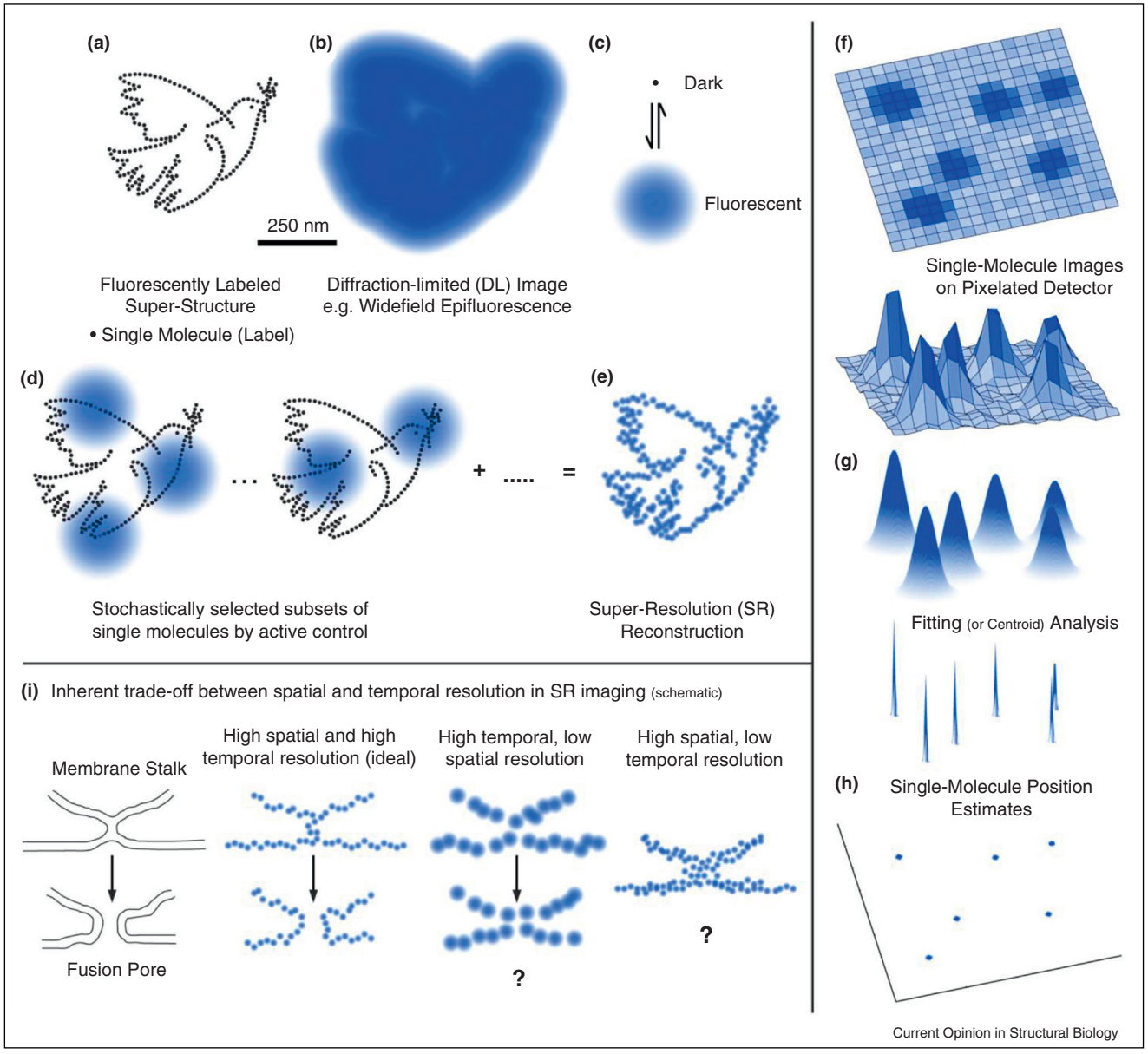
Figure 1



Principles of super-resolution single-molecule active control microscopy. **(a)** A hypothetical arrangement of fluorescent molecules, that is a labeled 'super-structure' (here: outline of 'La Paloma de la Paz' (The Dove of Peace) by P. Picasso, 1961). **(b)** In conventional fluorescence microscopy, all molecules emit simultaneously, so their diffraction-limited images overlap on the detector (camera) and information about the underlying structure is irretrievably lost. (**c**) Addition of on-off control, toggling any one single-molecule emitter between a dark and a fluorescent state. **(d)** If individual sparse subsets of single molecules that are spatially separated further than the diffraction limit are made to emit, their positions may be extracted in a time-sequential manner by finding the center position of a mathematical fit of the single-molecule images. **(e)** From the list of localized molecules, a super-resolution reconstruction is assembled in a post-processing step. Note that if the majority of molecules is detected at least once, the resolution is then governed by the fidelity of the localization estimate of individual localizations. This precision is shown by the blue circles which, for reasonable signal-to-background of single-molecule detection, are dramatically smaller than the extent of the diffraction-limited image given by the microscope PSF. Scale bar: 250 nm. **(f)** The pixelated images of single-molecule emissions in a 2D imaging experiment are typically (**g**) fit by Gaussian functions with variable center coordinates (*x*,*y*) to extract (**h**) single-molecule position estimates. (**i**) Illustration of the inherent trade-off between spatial and temporal resolution when imaging a dynamic process: Cartoon view based on a general membrane fusion scenario, for example SNARE-mediated [87], evolving from a membrane stalk between a vesicle (top membrane) and the plasma membrane (bottom membrane) to a resulting fusion pore. If temporal resolution is prioritized, two or more reconstructions can be obtained, however a lower and possibly insufficient number of position samplings in the reconstructions — possibly also at worse localization precision — leave details unresolved. By contrast, collecting many positions while the structure is changing leads to time-averaging over the acquisition, and a similar loss of information.

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