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Super-resolution fluorescence imaging with single molecules

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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.

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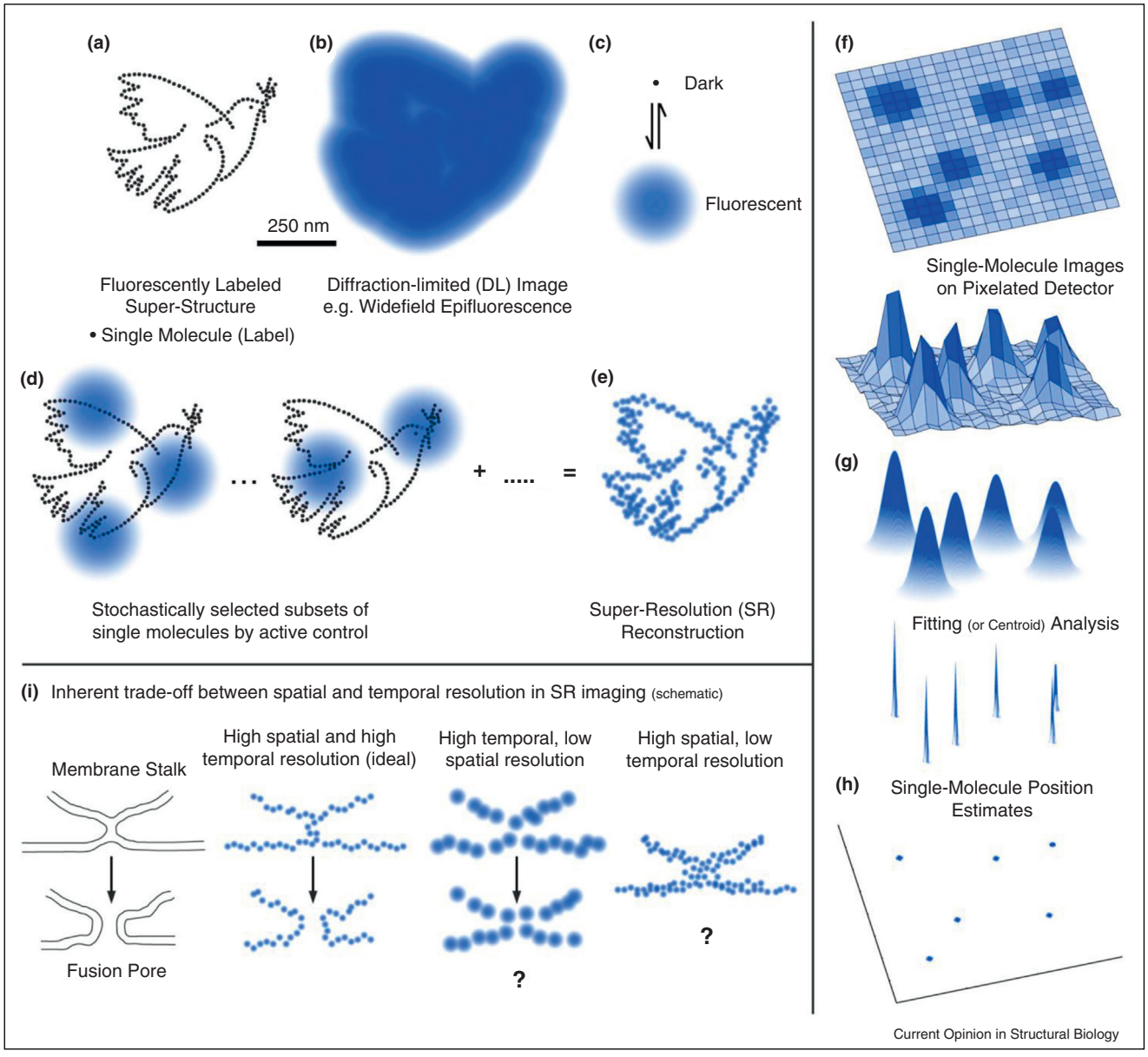
Introduction

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.

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.

For the images not to overlap and thus be identifiable individually, a sparse turn-on is required by some mechanism of 'active control', where the experimenter maintains a balance of the vast majority of molecules off (dark) and only a tiny fraction on (fluorescent). The basic requirement is that single molecules (SMs) disclose their position by emitting a large number of photons in one go (a photon burst) before returning to a non-fluorescent state, so as to enable other molecules in the structure to enter the signal-giving state and contribute their location (Figure 1d). The first reports of SM-based SR imaging achieved this by photo-activation of sparse sets of fluorescent proteins followed by their bleaching in (fluorescence) photoactivated localization microscopy ((F)PALM) [22,23] or by fast switching of Cy5-Cy3 dye constructs in stochastic optical reconstruction microscopy (STORM) [24]. The same idea was applied to dye molecules stochastically binding to and unbinding from a structure, experiencing a dramatic fluorescence enhancement during the binding. The resulting single-molecule blinks sampled the underlying structure in a method termed points accumulation for imaging in nanoscale topography (PAINT) [25]. The light-induced switching and blinking of many fluorescent proteins [26] provides a widely applicable alternative — and facilitates the use of genetically encoded probes [27]. Indeed, long-lived dark states are common to many fluorophores, which allows super-resolution microscopy with just one imaging laser per dye/protein employed, especially when additives are used to control dark redox state fractions [28–30]. A great many variants of the same idea have been put forward, often with their own acronym, but in all cases, the experimenter must actively select reading intensities, pumping wavelengths, photochemical mechanism, or sample additives to control the emitting concentration at a single-molecule level in each imaging frame. Collectively, these microscope schemes may be referred to, in a mechanism-independent fashion, as single-molecule active control microscopy (SMACM).

While a necessary condition, it is important to note that high spatial precision of individual molecule positions does not always translate into high spatial resolution. The average spacing of labels in the super-structure must be no larger than half the desired resolution to satisfy Nyquist–Shannon sampling, and if the assumption of a quasi-static structure is violated — as in time-lapse imaging of more or less dynamic objects — additional sampling considerations apply, highlighting an inherent trade-off between spatial and temporal resolution [31]. Such a trade-off is inherent in all imaging but becomes worth considering especially for the present SR techniques, because the registration of large numbers of sampled SM positions requires time. While for a static structure, the acquisition time is less critical (assuming any sample drift is properly accounted for), for a dynamic sample, choices have to be made to follow the time-course of a structural rearrangement. If SM positions from shorter time intervals are pooled together to infer a super-structure in each case, the number of molecules may be too small to sample sufficiently in space and spatial resolution is sacrificed. Conversely, if longer time windows are combined for SR reconstructions, the structural determination can suffer as an effective time-average is measured over the time during which the structure itself evolved, and this is not compensated for by more molecules recorded (Figure 1i). The goal must be to increase switching speeds to acquire more positions over shorter times, and indeed several groups have pushed SMACM recordings down to the time-scale of order 1–10 s (Figure 2e–g).

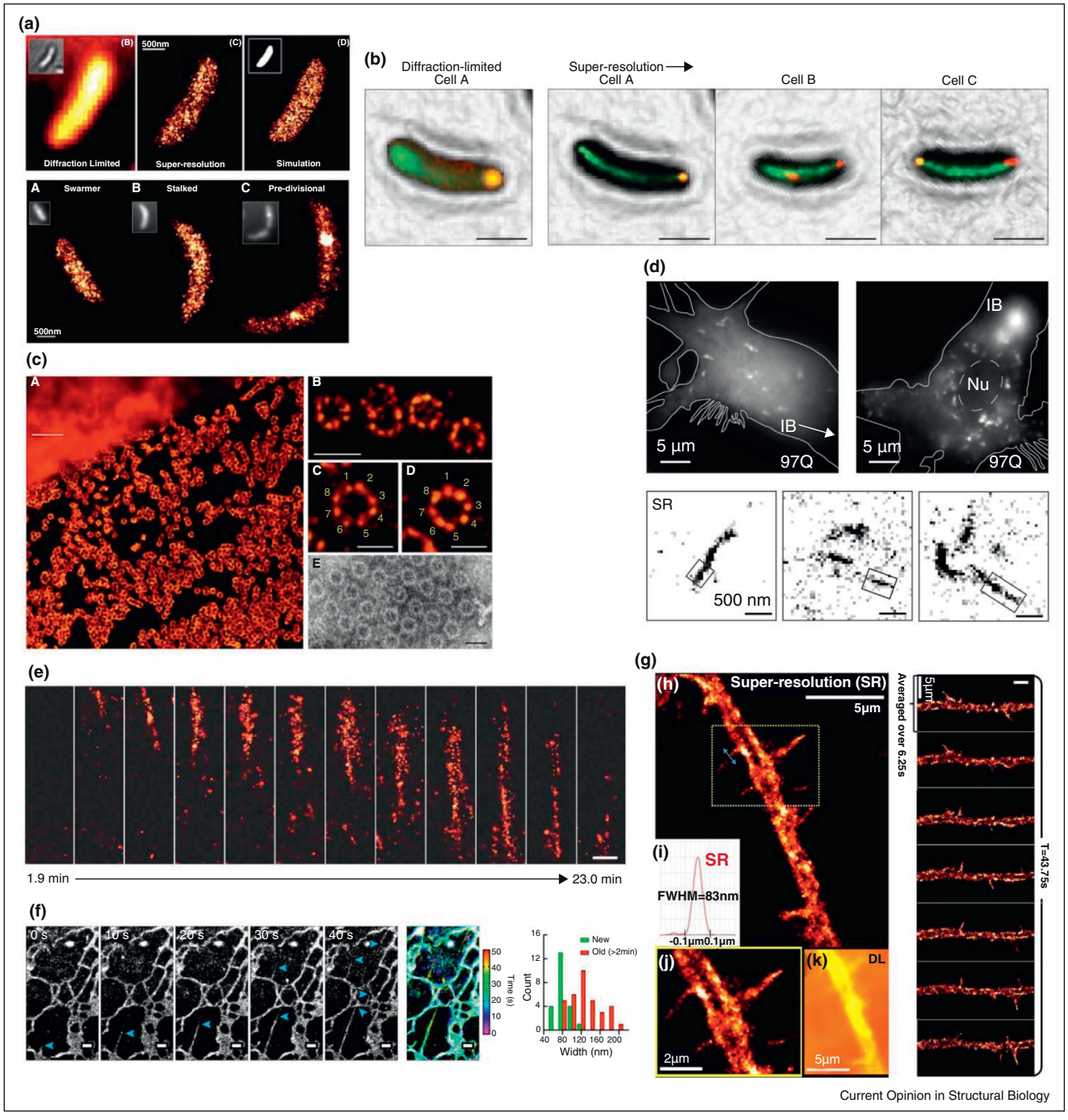
Selected examples of applications

New mechanisms of active control (to keep the majority of molecules dark in a given imaging frame) have been explored, and new biological systems are now being studied at an astonishing pace (see Table 1). Great interest exists in the organization of the cytoskeleton, which is rather dense in many mammalian cell types. Microtubules have served as test structures in SR demonstrations for some time due to their piecewise linearity and well-defined subdiffraction width, but the modeling and dynamic re-modeling of intricate three-dimensional microtubular or actin networks [32–34] will be explored more as methods become faster (see next section on dynamics).

Bacterial cell and developmental biology can especially benefit from SR analysis because of the small length scale. The distribution and organization of DNA in bacterial cells is an area of particular interest. By fusing EYFP to the nucleoid-binding protein HU, one can use the intensity-dependent photoinduced blinking and photobleaching of EYFP to extract information about the locations of relatively nonspecific protein binding sites on the DNA. This idea was demonstrated for HU-EYFP in fixed *Caulobacter crescentus* cells (Figure 2a), and the resulting single-molecule distributions were characterized by spatial-point statistics to be relatively uniform in the quiescent parts of the cell cycle, but strongly clumped in pre-divisional cells [35]. In *E. coli* in contrast, the H-NS distribution was strongly clumped for most of the cell cycle [36]. Other bacterial examples have included the study of the clustering of chemotaxis proteins in *E. coli*, generating a stochastic nucleation model [37], and the discovery of a ~40 nm thick spindle of the protein ParA, which guides chromosome segregation in *C. crescentus* [38] (Figure 2b).

Direct labeling of DNA with a DNA-associating dye, Picogreen, was recently demonstrated in living mammalian (U2OS) cells under favorable blinking conditions for the dye [39]. Direct STORM imaging visualized the eightfold symmetry of gp210 proteins around the nuclear

Figure 2



Examples of single-molecule-based super-resolution imaging in biological applications. **(a)** The nucleoid-associated protein HU imaged at different stages in the cell cycle of asymmetrically dividing *C. crescentus* bacterial cells, by light-induced blinking of enhanced yellow fluorescent protein fusions. **(b)** A spindle-like apparatus of ParA is part of the asymmetric division machinery in *C. crescentus* bacterial cells. **(c)** Nuclear pore complexes (gp210 proteins) with eight-fold symmetrical subunits in isolated *Xenopus laevis* oocyte nuclear envelopes. **(d)** Cytosolic nanoscale fibrillar aggregates of mutant huntingtin exon 1 proteins inside intact neuronal model cells (PC12m). IB: inclusion body. Nu: nucleus **(e)** tdEos-tagged focal adhesion molecules (paxillin) imaged in 2D at 55-s time resolution in a live CHO cell. (**f**) Endoplasmic reticulum (ER) dynamics in live BS-C-1 cells. (left) A time-series of 10-sec STORM snapshots. Blue arrowheads: Tips of extending tubules. (middle) A composite image containing all these snapshots with each localization colored by its time of appearance according to the shown color map. (right) Distribution of the widths of ER tubules. Green bars: Newly extended tubules. Red bars: Old tubules that had already existed for at least 2 min. (**g**) Voltage-gated ion channels recognized by fluorescently-labeled saxitoxin ligands, imaged at 6 s/reconstruction. Spines are constantly extending and retracting from the extended neuritic process in the neuronal model cell PC12. Scale bars as noted and 1000 nm (b, c left), 500 nm (e, f), 250 nm (c top right), 150 nm (c bottom right (C–E)). Examples reproduced with permission from [35] (a), [38] (b), [40] (c), [44•] (d), [31] (e), [51] (f), [46] (g).

Table 1

Selected structural biological measurements by single-molecule-based super-resolution approaches

Biological super-structure(s)	Significance/comments	Ref
Integrin-based focal adhesions	Integrins and actin are vertically separated by a ~40-nm focal adhesion core region consisting of multiple protein-specific strata including integrin; talin, a further component, has polarized orientation, indicating a role in organizing the focal adhesion strata	[90]
MreB, CreS, PopZ, FtsZ in *C. crescentus*	Cytoskeletal and polar as well as midplane-located protein superstructures in live bacteria	[27,88,89,91]
Aggregates of mutant huntingtin exon 1 protein	Fibrillar aggregates, possibly transient protofibrils, with a sharp cut-off length of ~1.5 μm observed to co-exist with mature inclusion bodies	[43,44•]
Cycling nitro-reductase	Visualization in live *B. subtilis*	[45]
Sodium channels in live neuronal model cells by fluorescent saxitoxin	Novel labeling by *de novo* synthesis of fluorescent forms of saxitoxin, fast (<10 s) dynamics of neuritic spine movements	[46]
Nucleosome-binding protein HU in live *C. crescentus*	Spatial point statistics analysis (2D) of chromosomal DNA	[35]
ParA/ParB in live *C. crescentus*	Division spindle components for chromosome segregation	[38]
Chemotaxis network in *E. coli*	New model triggered for localization patterns	[37]
Actin and spectrin	Highly periodic protein arrangements in axons	[33,34]
Synaptic proteins Bassoon and Homer1	Organization of key proteins at the synapse in brain tissue	[92]
Spine and excitatory synapse density in the hippocampus (bassoon and glutamate receptor 1/2)	Afadin, a Ras/Rap effector, is a key intracellular signaling molecule for cadherin recruitment and is necessary for spine and synapse formation *in vivo*	[93]
Podosome formation and dissociation	Visualized using an mCherry-tagged truncated talin construct (live cell)	[81]
DNA	Direct dye labeling	[39]
Various organelles	Live cell, high temporal sampling using membrane probes (1–2 s/SR reconstruction)	[51]
Proteins central to centrosome formation	The carboxy terminus of pericentrin-like protein (PLP) is revealed to be at the centriole wall, radiating outwards into a matrix domain where it is organized into clusters having quasi-nine-fold symmetry	[94]

pore complex and resolved the central channel with ~15 nm resolution [40] (Figure 2c).

The aggregation of misfolded proteins in neurodegenerative disorders can be studied at high resolution in experimental systems relevant to Alzheimer's [41], Parkinson's [42] or Huntington's diseases, both *in vitro* and inside cellular models [43,44•] (Figure 2d).

An alternative strategy to 'turn-on' fluorophores for SR imaging can be provided by enzymatic generation of fluorophores, where the catalytic reaction converts a dark fluorogen into a good emitter. The concentration can be kept low in any imaging frame by a combination of limiting the concentration of fluorogenic substrate and high reading intensity to photobleach emitters before diffusion can occur. This concept was recently demonstrated in *Bacillus subtilis* using a neutral, cell-penetrating fluorogenic nitro-DCDHF dye which is converted to a hydroxyl-amino derivative by native nitroreductase [45].

A large class of cellular labels can be contemplated based on fluorescent ligands which bind to cell-surface receptors. As an example, Ondrus and Lee *et al.* prepared a fluorescently-labeled saxitoxin molecule which is known to block voltage-gated sodium (NaV) channels. When PC12 cells are incubated with medium-nM concentrations of the labeled ligand, molecules free in solution diffuse so fast that they only produce an increased background on the imaging detector. However, when the molecules bind to the NaV channels in the cell membrane, the emission from each single molecule appears as a single spot which can be localized to high precision and quickly photobleached. This type of target-specific PAINT imaging provides samples of the NaV channel locations on the living cell surface [46] (Figure 2g).

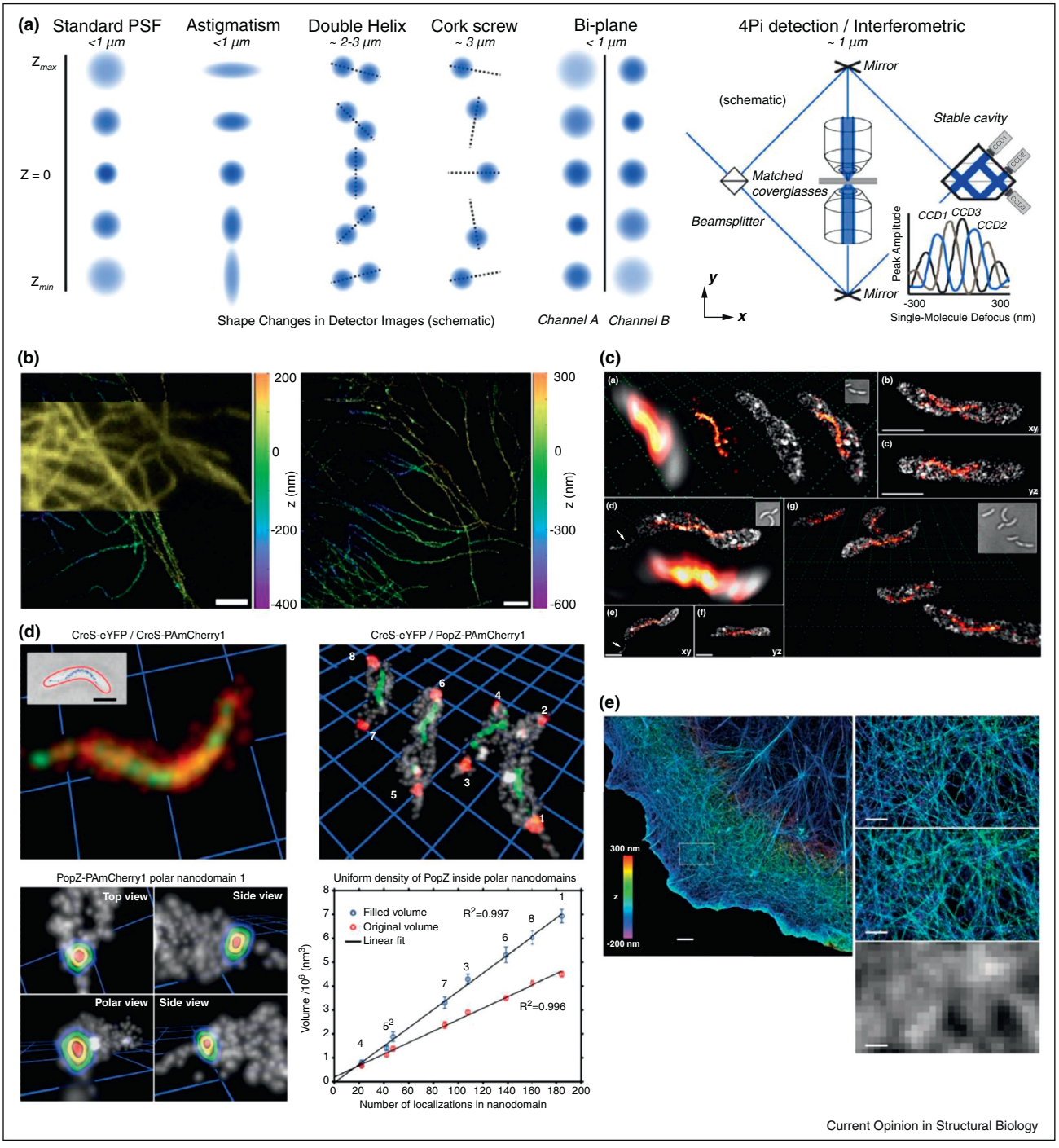
Dynamics: time-lapse imaging in organelles, protein complexes and beyond

One exciting aspect of the schemes discussed here is that a far-field fluorescence microscope is inherently compatible with time-lapse investigations of biological processes. Much initial work focused on the tracking of individual molecules in cellular membranes or the cytosol at higher and higher spatial and temporal levels [47–49] or to unravel the dynamics of single copies of molecular machines by attaching larger labels and ultimately single molecules. But super-resolution microscopy allows much denser structures to be interrogated over time [50]. Photoswitchable membrane-bound small-molecule probes have enabled live-cell imaging at exquisite spatial and temporal resolution (30–60 nm and 1–2 s) [51] (Figure 2f).

The third dimension: super-resolution in 3D

Life happens in three dimensions, and organelles, cells and tissues are intricately organized in three dimensions

Figure 3



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Three-dimensional (3D) super-resolution fluorescence imaging. **(a)** To extract the location of individual single-molecule emitters with high precision in all three spatial dimensions, the symmetry of the standard microscope PSF (i) must be broken and the PSF re-designed to encode information in the *z* (axial) direction by a well-defined shape change. Widely adopted schemes include: (ii) an astigmatic PSF, where the slowly changing ellipticity of the single-molecule image can be calibrated to provide a *z* estimate. (iii) the double-helix (DH) PSF features two well-defined spots revolving around a common center as a function of *z*. (iv) cork screw PSF (one revolving spot), (v) bi-plane methods assess the relative detected brightness of images formed in two shifted image planes. (vi) 4Pi axial localization methods rely on interferometric detection and two matched objective lenses to collect fluorescence from both sides of the sample. **(b)** Microtubules in mammalian cells extending over a large axial range can be readily imaged with the DH-PSF. **(c)** Visualization of the cytoskeletal protein filament of CreS in pre-divisional *C. crescentus* bacterial cells, jointly with the cell surface by the PAINT approach. **(d)** Quantitative co-localization of a further protein, PopZ (shown in red). The number density of PopZ in the polar nano-domains was demonstrated to be constant between cells. Both (c) and (d) recorded by DH-PSF microscopy. **(e)** Cell-wide arrangement of F-actin by dual-objective astigmatic imaging. Scale bars/grids: 2000 nm (b, e left), 1000 nm (c, d), 500 nm (e right). Examples reproduced with permission from [32] (b), [88] (c), [89] (d), [33] (e).

(3D). The constituent proteins, lipids and other macromolecules interact on various timescales. SR imaging in 3D is challenged by the standard fluorescence microscope's PSF, which contains little information about the axial (z) position of a single emitter. The standard PSF (Figure 3a, left) is highly symmetric axially about the focal plane and changes very little over hundreds of nanometers near it. Because an objective can only produce a spherical cap of a wavefront of light, the PSF inherently is always larger axially than laterally [52]. This may be addressed by using interferometric approaches with two opposing objective lenses [53], which yield very high axial localization precision [54–56]. The instrumental requirements are considerable and the need for high numerical aperture (NA) objective lenses on both sides of the sample may be impractical to implement for some applications. In comparison, single-lens epi-fluorescence wide-field microscopy is widely utilized in biology, and several approaches have been described for 3D high-precision imaging, including astigmatism [57,58], multi-plane [59,60], and the double-helix PSF (DH-PSF) [61] (all in Figure 3a). In the DH-PSF microscope, the PSF response features two prominent spots that revolve around their common midpoint throughout the depth of field, thus appearing as a double helix axially. Unlike other PSF schemes that only moderately evolve with z position, the DH-PSF microscope exhibits highly uniform nanoscale localization precision along an axial range of ~2–3 μm, which is predicted by information-theoretical calculations [62] and has been demonstrated experimentally [63]. In contrast, methods based on astigmatism [57] and biplane [60] improve the axial localization precision over smaller z ranges [62]. An intriguing alternative of a rotating PSF that can facilitate nanoscale localization over a large axial range is the corkscrew PSF (CS-PSF) [64]. 3D SR schemes have been applied to the structural analysis of cytoskeleton proteins in both mammalian and bacterial systems (examples in Figure 3b–e).

Toward counting numbers of molecules in protein super-structures

With imaging of biological structures entering a new era based on single-molecule imaging, it should be possible to arrive at a much more quantitative picture of the numbers of molecules involved in forming molecular assemblies. However, several critical issues must be addressed in dealing with stochastic single-molecule active control and detection. For example, when trying to assess the *number* (rather than primarily the positions) of proteins in a DL region with photoactivatable fluorescent proteins, if photoactivation is irreversible, the number of fluorescent bursts should in principle be a measure of the number of proteins. In practice however, two sources of error play a role. Intrinsic photoblinking leads to an overcounting error [65], while the inevitable simultaneous activation (in some cases) of multiple molecules within one DL region can result in molecular undercounting. A specific photoactivation scheme and careful kinetic modeling are needed to balance overcounting and undercounting, as shown by Lee, Shin *et al.* [66••]. Their work also clearly points to the need to choose and characterize the photoactivatable fluorescent protein to be employed carefully, in their case identifying Dendra2 as superior to mEos2 for this counting application (due to faster bleaching and much less blinking).

True position estimates — unbiased by non-isotropic single-molecule fluorescence emission

As the field pushes to higher spatial precision by virtue of higher number of photons collected, more information-efficient estimators and optimal detection schemes [67], it becomes apparent that one further fundamental aspect has to be considered. For even partially immobile transition dipoles, anisotropic single-molecule emission patterns arise that depend strongly on 3D orientation and z position. Image fitting that fails to account for this may incur a sizable systematic error (mislocalization) of up to ~50–200 nm that can degrade resolution [68]. Backlund, Lew *et al.* recently showed that the DH-PSF uniquely contains parameters in its two lobes which make it possible to extract estimates of both 3D position and orientation of a single molecule. Building a library of the apparent shift as a function of position and orientation, this shift can be subtracted and the error thus corrected, making the (x, y, z) determination not only precise but also *accurate* [69••]. Furthermore, in actual experiments, the degree of mislocalization depends strongly on the rotational mobility of the single-molecule labels; only for molecules rotating within a cone half angle $\alpha > 60^{\circ}$ can mislocalization errors be bounded to $\leq$10 nm. Both low and high rotational/orientational mobility can cause resolution degradation or distortion in super-resolution reconstructions, depending on geometrical details of the structure interrogated [70•].

Conclusions

The resolution gap of fluorescence-based imaging compared to electron microscopy is rapidly closing. Super-resolution microcopy based on the sequential localization of single molecules will be developed further to become a unique 3D tool for dynamics, maintaining the key advantage of high molecular specificity and minimal invasiveness. The labor-intensive sample preparation of electron microscopy in particular can be avoided, circumventing drawbacks like the low labeling efficiency of gold immunofluorescence, and the frequent need to impose symmetry conditions in the analysis. Further improvements will chiefly come from dedicated development efforts in single-molecule probes, notably in two ways. Firstly, increases in probe

photostability (increased detected photons) will directly translate to higher precisions and thus resolution potential [71]. Secondly, it is worth noting that the achievable resolution approaches the size of the fluorescent labels themselves, making labeling density a crucial consideration — and a tunable parameter — to avoid crowding and obscuring of the underlying spatial arrangements interrogated [72]. Novel small, compact labels such as aptamers [73] and nanobodies [74] will have to displace indirect immuno-staining, as primary and secondary antibodies each occupy non-negligible volumes in the labeled structure. Current research has focused on finding parameters and harnessing new technologies for faster imaging [75,76•]. The number of suitable and spectrally distinct labels needs to be further expanded [77,78], and dyes and fluorescent proteins carefully characterized. The described methods rely on detection of single molecules at sufficient signal-to-background. In thick cells or tissue slices optical sectioning must be provided by selective activation and/or excitation of distinct planes in the sample [58,79].

Current research seeks to identify ways to extract the maximum information from SM images, and to find algorithms which can handle multiple active, partially overlapping fluorophores per frame, such as DAO-STORM [80], Bayesian analysis [81], compressed sensing [82], and others. Further analysis tools need to be developed to disentangle structure from the inherently pointillist description in a SMACM experiment and detect, for example, true clustering [83]. SR methods combined with ingenuity of labeling approaches will impact research areas as diverse as genetic engineering and systems biology [84] or biofilm assembly [85,86].

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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