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A starter kit for point-localization super-resolution imaging

Suliana Manley, Julia Gunzenhäuser and Nicolas Olivier

Super-resolution fluorescence imaging can be achieved through the localization of single molecules. By using suitable dyes, optical configurations, and software, it is possible to study a wide variety of biological systems. Here, we summarize the different approaches to labeling proteins. We review proven imaging modalities, and the features of freely available software. Finally, we give an overview of some biological applications. We conclude by synthesizing these different technical aspects into recommendations for standards that the field might apply to ensure quality of images and comparability of algorithms and dyes.

Address
Laboratory of Experimental Biophysics, École Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland

Corresponding author: Manley, Suliana (suliana.manley@epfl.ch)

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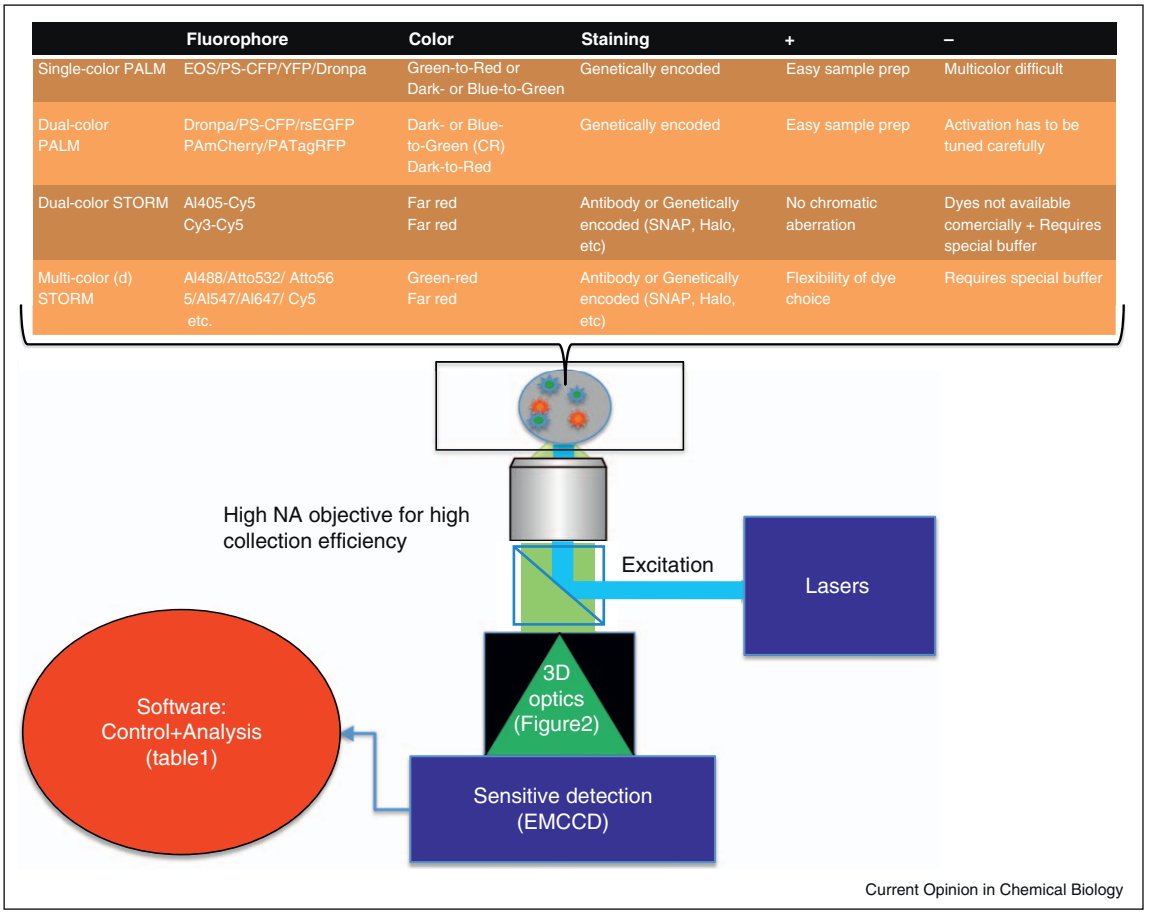
Introduction

Super-resolution fluorescence imaging (SR) is enabling the visualization of the organization and dynamics of biological systems at unprecedented scales. This review focuses on SR derived from the point localization of individual molecules, an inherently single-molecule measurement. Advances in SR are rapidly occurring in the areas of algorithm development for rapid and maximal localization, the implementation of flexible labeling schemes and the diversification of usable fluorescent dyes, and meaningful biological applications. In this short review, we aim to create a 'starter kit' (Figure 1) with the basic information needed to navigate the dyes and software packages for image reconstruction, as well as a concise overview of imaging modalities and select biological applications. For more information, several in-depth reviews have recently been published [1,2], and detailed protocols provide a practical guide [3•,4,5].

The light from a point source, whether it is a star or a molecule, is invariably blurred by diffraction when it is imaged through a finite aperture. The form of this image, first derived by astronomer Sir George Biddell Airy [6], can be used to define the diffraction-limited resolving power of an imaging system. In the case of fluorescence microscopy of a structure densely labeled with dye molecules, diffraction limits the resolution to of order half the wavelength of visible light, or several hundred nanometers. However, with low background noise and efficient photon collection, molecular locations can be determined with high precision [7]. This is because the center of a molecular image or point spread function (PSF) can be localized, typically by fitting to a Gaussian function, even down to the nanometer scale [8]. Thus, by combining point localization with the stochastic switching of thousands of single molecules, resolving molecular distributions at the nanoscale is possible. Developed nearly in parallel by several groups, this method was alternately named photoactivated localization microscopy (PALM) [9••], fluorescence photoactivated localization microscopy (FPALM) [10], and stochastic optical reconstruction microscopy (STORM) [11]. Point-localization SR relies on the control of molecular fluorescence such that less than one molecule per diffraction-limited area emits at a time. Additionally, to achieve SR the density of localized molecules must satisfy the Nyquist criteria: in this context, the intermolecular spacing should not exceed twice the desired resolution [12].

Although a variety of properties can be used to isolate single molecules, such as spectrum [13], lifetime [14], and binding/dissociation [15] among others, photoswitching remains the most flexible strategy. While there is no substantive difference between PALM and FPALM, as implemented they differ from STORM in the mechanism of fluorophore photoswitching. (F)PALM imaging exploits photo-activatable fluorescent proteins (reviewed in Ref. [16]) as well as photoswitching YFP and GFP [17,18], while STORM imaging was demonstrated using pairs of switchable synthetic fluorophores. More recently direct STORM (dSTORM) [19] and ground state depletion microscopy followed by individual molecule return (GSDIM) [20] expanded the variety of synthetic fluorophores compatible with SR (reviewed in Ref. [2,21]). In summary, (F)PALM is compatible with genetically encoded labeling and live-cell imaging while (d)STORM has been realized primarily using antibody labeling and was thus limited to imaging in fixed cells. More recently, the use of self-labeling proteins [22,23] has brought some of the advantages of (F)PALM to (d)STORM, allowing more specific targeting of synthetic fluorophores [24] and live-cell imaging [25•]. In addition, extensive characterization of imaging and buffer conditions required to make standard dyes blink by transitioning into a reversible dark state or shifting spectrum [18,26]

Figure 1



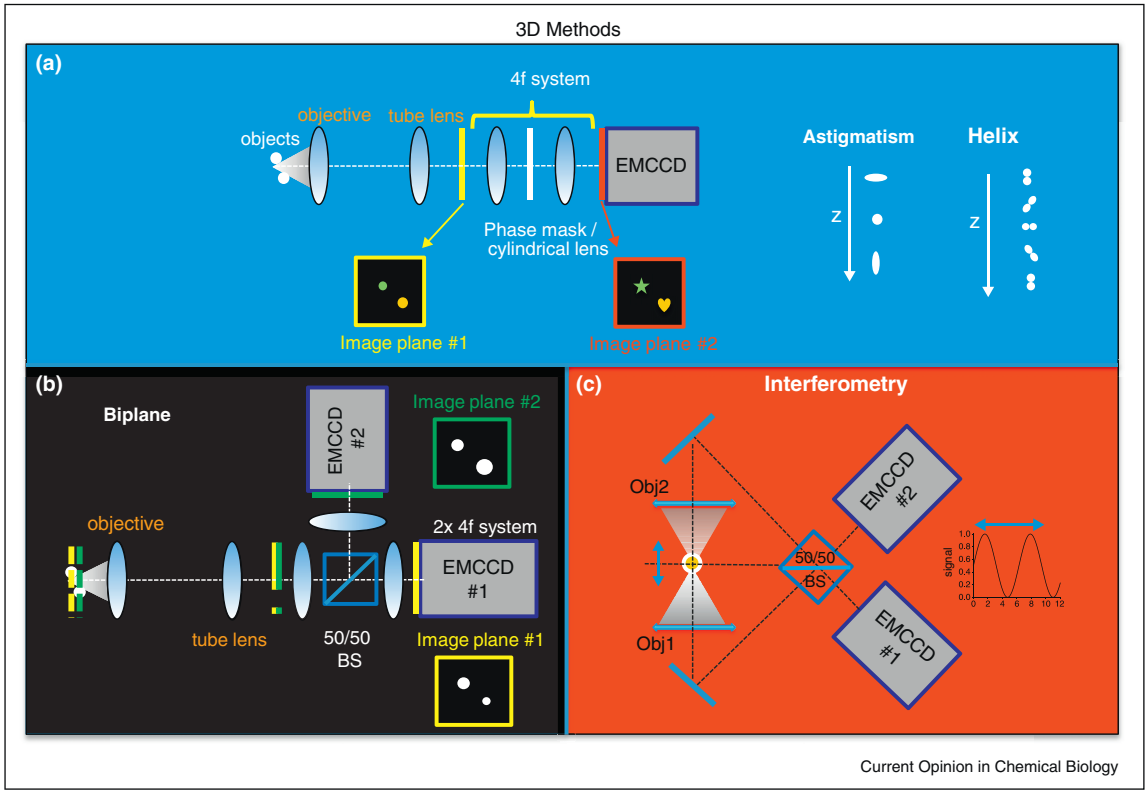
A super-resolution starter kit. SR requires a broad combination of competences; we present here several combinations of hardware, software, and sample preparation. On the hardware side, the requirements are: a sensitive and efficient detector, which implies using a high numerical aperture objective, an EMCCD camera, and ~10–100 mW lasers for excitation. A control software synchronizing the lasers with the camera can also be helpful [46,63]. For 3D imaging, several optical setups are possible and are described in Figure 2. For sample preparation, several options are presented since the requirements can be quite different depending on the application. We have noted here just a few of the most commonly used dye combinations, since there are many possibilities with new dyes being published frequently. For software, a good point-fitting algorithm is necessary, and several options are freely available, described in Table 1.

has broadened the palette of dyes compatible with SR imaging. However, a challenge remaining for these strategies is to control the transition rates between dark and bright states to meet the isolation criteria required for point localization while maintaining a high enough density of molecules to satisfy the Nyquist criteria for SR.

Technological advances

The publications introducing (F)PALM and STORM demonstrated single color 2D SR imaging either in fixed cell samples [9••], *in vitro* DNA loops [11] or on glass and sapphire surfaces [10]. This was due to significant limitations imposed by both hardware and sample preparation. On the hardware side, TIRF illumination was used to excite a 2D section, so as to increase the signal to noise ratio and eliminate signal from out-of-focus molecules. Samples were therefore limited to thin sections (suitable for correlative electron) and membranes microscopy [9••], or *in vitro* objects adhered to a coverslip [10,11]. Moreover, due to long integration times to maximize signal, acquisition times were far too long to allow imaging of living cells, a limitation that has been proven unnecessary for select applications [12,17,27]. Since then, technical developments have enabled multicolor [28••,29–31] and three-dimensional [32••,33–35] imaging and the combination of both in fixed [36,37] and more recently living cells [38]. In Figure 2, we provide a guide to the most readily implemented technologies used for 3D SR imaging. Additional technological advances enable the extraction of information about polarization [39], thick sample imaging [40,41•] and super-resolved high density single particle tracking [42••].

Figure 2



There are three main methods to extract the *z* position of a particle. **(a)** The first family of methods relies on deforming the point spread function (PSF) so that its shape depends on the z position of the particle. In Ref. [32••], astigmatism was induced by a cylindrical lens as a shaping method, which resulted in a PSF whose ellipticity depended on axial position. Different refinements of this method have been proposed using more controllable shaping methods resulting in a spiral [64] or helical [35] PSF, which aim at increasing the range over which molecules can be localized and the isotropy of the localization precision. **(b)** The second method [33] relies on simultaneously imaging two different planes, and extracting the z position from the relative form of the PSF in the two planes. **(c)** The third method, called iPALM [34] relies on interferometry: two opposing lenses are used to make single photons interfere on three different cameras, and the intensity ratio of the images provides a measure of the z-position of the particle. For simplicity, two-way interferometry is shown here. This method provides the highest resolution with the *z* localization, even exceeding the *x*–*y* localization.

Guide to dyes for multicolor imaging

One area of significant interest is multicolor imaging, which presents unique challenges. The photoswitching mechanisms of dyes available for (F)PALM and STORM necessitate a compromise between control over blinking rates and spectral overlap between the different fluorophores. That overlap can be in the switching wavelength, or in the imaging wavelength, or even in the initial fluorescent state in the case of photoconvertible proteins. For example, a green-to-red protein such as Eos is difficult to combine with another dye since it occupies most of the visible spectrum: it is activated by UV or blue light, fluoresces initially in the green, and photoconverts into a red form. A few of the most common labeling schemes are outlined in Figure 1.

The initial demonstrations of multicolor imaging relied in the case of STORM [28••] on two donor–acceptor pairs, Al405–Cy5 and Cy2–Cy5, where two different wavelengths were used sequentially for activation before bleaching both dyes with a single laser, yielding the additional advantage of removing the effects of chromatic aberration. In the case of PALM [30], a combination of irreversible green to red (tdEos) and reversible dark to green (Dronpa) proteins was used. The irreversibly switching tdEos was imaged first, and once completely bleached in both its green and red states allowed the imaging of Dronpa. However, even with the development of new red photoactivable proteins [29], which made multicolor imaging using two irreversibly switching proteins possible, issues associated with the use of different wavelengths exist, with potential unwanted activation and bleaching of fluorescent proteins yielding an effectively reduced labeling density.

A promising development for multicolor imaging is dSTORM/GSDIM [19] where common dyes based on rhodamine and cyanine are used, and blinking is obtained

Figure 3

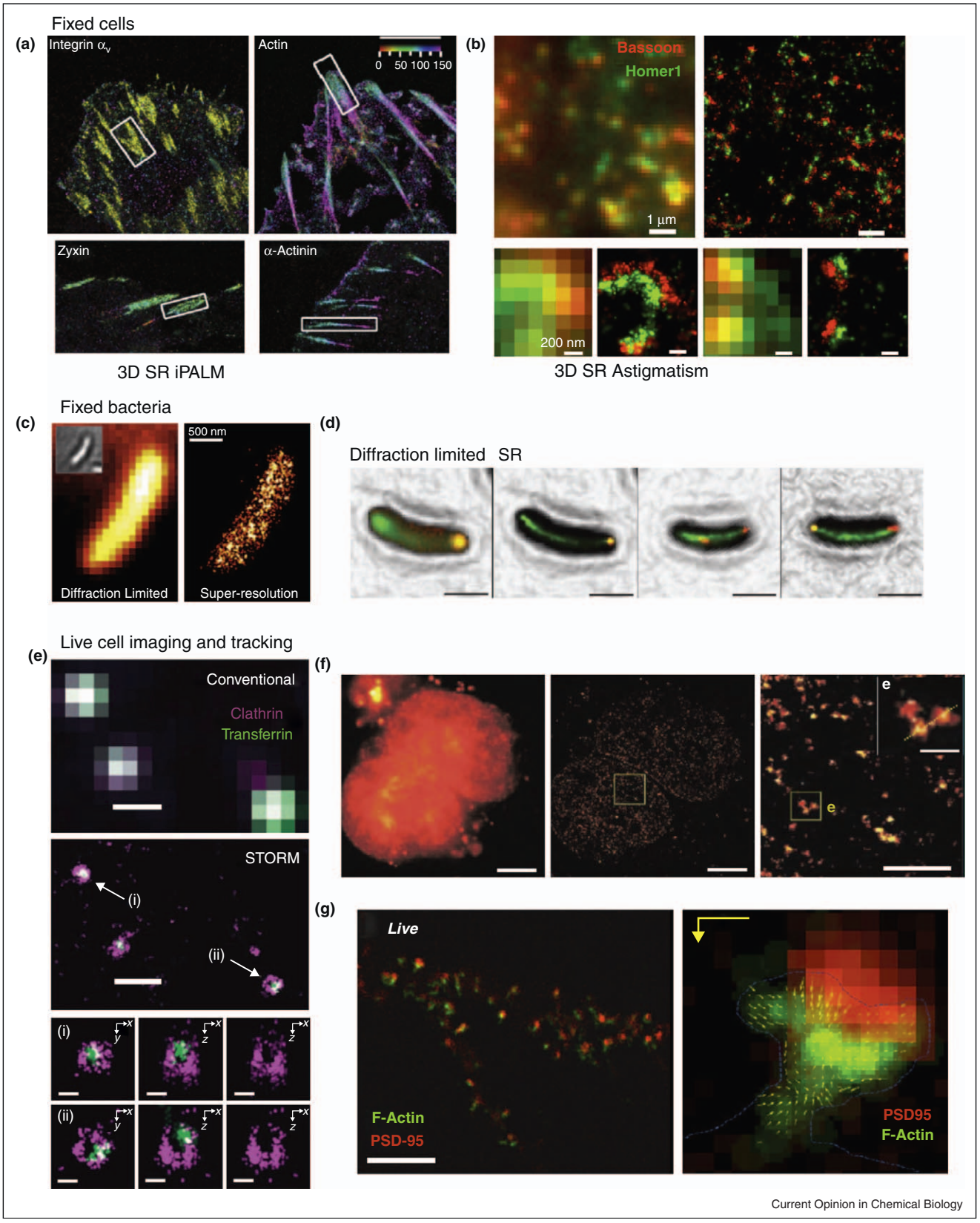

Fixed cells
(a)
Integrin α_v
Actin
0 50 100 150
Zyxin
α-Actinin
3D SR iPALM
(b)
Bassoon
Homer1
1 μm
200 nm
3D SR Astigmatism
Fixed bacteria
(c)
500 nm
Diffraction Limited
Super-resolution
(d)
Diffraction limited SR
Live cell imaging and tracking
(e)
Conventional
Clathrin
Transferrin
STORM
(i)
(ii)
(f)
e
e
(g)
Live
F-Actin
PSD-95
PSD95
F-Actin
Current Opinion in Chemical Biology

Table 1

The choice of an adapted software to convert the sparse and noisy raw data into a SR image is crucial, since it requires an efficient yet rapid way of localizing multiple peaks in each frame. We present here only open source software, since codes often have to be adapted for different dyes/applications and executable only software is therefore inconvenient or unusable.

Name	Language	Ref	Use friendly	3D	Misc
QuickPALM	(Java)	[49]	++ (ImageJ)	Astigmatism	Can be batch processed using ImageJ
PALM3D	(Python)	[41]	–	Astigmatism	3D imaging of thick samples
Rapid2STORM	(Java)	[51]	++	Astigmatism + biplane	Posttreatment + tracking
Octane	(Java)	[50]	++ (ImageJ)	2D only	Tracking

through reversible transitions into a dark state. This technique is therefore doubly multicolor friendly: not only do a large number of compatible dyes already exist (including Atto-dyes and Alexa-dyes), but since only one laser is used for both switching and excitation unwanted overlap is reduced. The downside comes from the need for 'blinking buffers', as the chemical environment has to be controlled to maintain reversible dark states, and different fluorophores often have different blinking rates in a given buffer. Moreover, even a small overlap in excitation and emission spectra can render pairs of dyes incompatible for simultaneous imaging, either through increased noise, STED-like de-excitation, or promoting different triplet state pathways leading to increased photobleaching. Nevertheless, this approach has enabled imaging with up to six different colors [21] although not simultaneously, and combined with spectral unmixing up to four dyes have been imaged simultaneously [43•]. Several reviews and articles are helpful in this quite complex field [26,44–47]. We draw your attention to [48•], where the blinking properties for a large number of dyes in several different buffers are compiled and the results are used to perform 4-color imaging.

Guide to software packages

The raw data for point-localization SR consist of a stack of images containing a few molecules in each frame. Thus, software for point localization and image reconstruction from molecular locations is indispensable. A number of open source software packages are now available [41•,49–51], so it is no longer necessary to write one's own analysis package. We present a summary of open source software that will as a minimum requirement process raw data and render SR images in Table 1. Although several freeware packages are also available, they are rife with limitations in terms of both their adaptability for new applications and their compatibility with new platform versions.

Biological applications

Most biological applications of point-localization SR use cellular systems as their subject of study. Cells are very crowded environments, with a mean spacing between proteins of ~10 nm, far below the diffraction limit. Previously, information on protein organization *in vivo* at the nanoscale could only be extracted for highly dilute proteins, or the spatial resolution for denser structures could be improved by deconvolving the obtained images, but without single molecule information. Extracting single molecule locations and dynamics with nanoscale accuracy in dense structures is now possible with point-localization SR. In this section we present select proof-of-principle measurements, but focus on examples where biologically significant information was obtained from nanoscale protein organization.

Fixed cell SR: from 2D, one color to 3D and multicolor

In fixed cells, the correlative single molecule information obtained with 2D multicolor PALM and dSTORM allowed the imaging of the nanoscale colocalization pattern of transferrin receptors and clathrin light chain (CLC) [29] and revealed heterogeneities in the distribution of kinases at the plasma membrane [52]. The high spatial resolution of 2D multicolor PALM also allowed the nanoscale structure of adhesion complexes to be resolved, revealing the relationship between different pairs of focal adhesion proteins [30]. Several pairs of these proteins that seem to colocalize in conventional images were found to form spatially distinct nanoscale structures.

(Figure 3 Legend) Biological applications of point-localization SR. **(a)** 3D iPALM image of integrin αV-tdEos, actin-mEos2, zyxin-mEos2, and α-actinin-mEos2 forming focal adhesions. The super-resolved 3D structural information obtained from these images and four other focal adhesion proteins allowed building a schematic model of focal adhesion molecular architecture [53••]. **(b)** 3D STORM imaging based on astigmatism of the presynaptic protein Bassoon and the postsynaptic protein Homer1. Proteins were labeled by immunohistochemistry using Cy3–A647 and A405–A647 conjugated antibodies, respectively. Left hand side images correspond to the diffraction limited images. The lower panels show individual synapses [54•]. **(c)** PALM imaging of the nucleoid-associated protein HU2-eYFP in fixed *C. crescentus* [58]. **(d)** Diffraction-limited and PALM images of ATPase ParA and centromere-binding protein ParB in *C. crescentus* tagged with eYFP and mCherry, respectively. A spindle-like structure involved in bacterial chromosome segregation could be resolved [57•]. **(e)** Diffraction-limited and 3D multicolor STORM image based on astigmatism of CCPs labeled with Alexa647 via a SNAP tag (magenta) and transferrin labeled directly with Alexa 568 (green) in live cells. Lower panels show the 3D view of individual CCPs [38]. **(f)** Diffraction-limited and dSTORM image of H2B labeled with ATTO655 via an eDHFR tag in living cells [25•]. **(g)** (Left side) Local density of actin-mEos2 molecules (green) with overlaid diffraction limited image of the postsynaptic density marker PSD-95-cerulean (red) in live neurons. (Right side) Local actin density and averaged molecular movement of actin within a spine as obtained by sptPALM [62••].

These results were recently extended to 3D using a composite of multiple one-color iPALM images (Figure 3a), providing the basis for a structural model of adhesion complexes at the molecular level [53••].

In neurons, 3D multicolor STORM imaging of immunostained fixed mouse neurons allowed the nanostructure of scaffolding proteins and receptors (Figure 3b) in chemical subdiffraction sized synapses to be resolved [54•]. Here, the three-dimensional super-resolved images show the postsynaptic and presynaptic protein distributions of single spines as well separated, and also resolve their correlation from side and top views. This information is the basis for creating a 3D model of synaptic protein distribution dynamics and could not have been obtained with conventional imaging.

In a cell-free membrane system, multicolor 3D STORM allowed the resolution and quantification of the subdiffraction axial elevation of clathrin-coated pits from the basal membrane before budding and fission [55].

The biological applications described here are essentially limited to thin structures near the cell surface, but the recent demonstration of confined activation for whole-cell PALM presents a promising way to resolve ultrastructure in mitochondria, the ER, or the nucleus [41•].

Imaging bacteria: small size, big challenge

Studying bacterial architecture is challenging due to their small size and high density of proteins. In this respect, SR provides a promising tool to resolve functional structures involving protein organization in bacteria. In *Escherichia coli*, PALM imaging of the chemotaxis network revealed that signaling proteins form clusters via stochastic self-assembly with no need for active transport [56•]. In *Caulobacter crescentus*, PALM imaging contributed to work that identified an unexpected chromosome partitioning apparatus similar to eukaryotic spindles [57•] (Figure 3d) and has been presented as a promising tool to study the structural organization of nucleoid-associated proteins [58] (Figure 3c). Most recently, nucleoid-associating proteins have been imaged in live bacteria using PALM [59] which identified the global transcriptional silencer H-NS as a key player in bacterial chromosomal organization.

Live-cell imaging and high density tracking: resolving dynamics

A tremendous step in the improvement of SR, toward making it more flexible for biological investigations, was its implementation in live-cell imaging. Although the acquisition time of localization-based SR techniques is still long relative to conventional wide-field imaging, it is now comparable to typical point-scanning microscopies (~10 s/frame). This has permitted the SR study of biological processes that take place on the time scale of minutes.

Live-cell dSTORM imaging enabled the study of the mobility of histone H2B proteins in the nucleus [25•] (Figure 3f), as well as the dynamic 3D colocalization of transferrin and CLC at the plasma membrane [38] (Figure 3e). In combination with the structural information obtained from fixed cells, this dynamic view may allow more light to be shed on signaling processes involving clathrin-coated pits and receptors. In neurons, live-cell PALM was used to study the long-term dynamics of the spine cytoskeleton and allowed the resolution of morphological changes in response to synaptic activity [60].

In live samples, the combination of PALM and single particle tracking (sptPALM) [42••] opened a new area of possible biological applications, by enabling the study of single molecule dynamics in highly dense structures at nanoscale resolution. This method has been demonstrated by tracking HIV Gag and VSVG proteins at the membrane [42••], and was used to study the actin dynamics in neuronal spines as well as the dynamics of prokaryotic cytoskeletal proteins [50,61,62••] (Figure 3g).

Conclusions and outlook

With point-localization SR, a wide array of imaging methods are possible, and have now been applied to address fundamental biological questions. It now remains for researchers to choose an imaging setup, fluorescent dye(s), and software to match their specific needs. This broad set of competences from molecular biology to chemistry to physics and computer science can be difficult for individual groups to achieve, but as the field of SR advances, more resources become available for users of the technology. Further innovations in multicolor and multimode imaging, probe design, and flexible, user-friendly analysis packages will result in even more widespread use of SR in the future. New fluorescent probes with better quantum yield (fluorescent proteins), better cell permeability (synthetic dyes), and optimized control for photoswitching and blinking will further open the possibilities for combining live-cell and multicolor imaging. Growth in this area will also depend on distribution and commercialization of new dyes.

An important challenge for the field is to agree upon quality and disclosure standards for dyes, software, and biological images. For fluorescent proteins, a histogram of single-molecule photon yields is already standard, and should become standard for synthetic dyes. Dyes for (d)STORM should be characterized for their photon yield during their 'on' periods, as well as their ability to recover which can be shown by plotting the number of localizations per raw image over the acquisition period, or the equivalent. An excellent example of quantitative characterization for (d)STORM is found in Ref. [48]. Better benchmarking of software, through open-source access and shared standard data sets will help to allow clear

comparisons between different analysis approaches. We have made available one biological and one synthetic data set, http://bigwww.epfl.ch/palm/, for such a purpose. Similarly, standards should be enforced for resolution as determined by localization accuracy and molecular density; this should be integrated into software to help users better understand their data quality. Specifically, it should be verified that published images contain points localized with high enough precision, and at sufficient densities (as determined by Nyquist) to match the resolutions claimed. Related to this, the number of raw images and localized molecules that go into creating each PALM image should be specified. These standards are exemplified well in Ref. [12]. Finally, the algorithms used for rendering molecules to create an image should be specified or described. The default rendering should be as similar as possible to what one would expect from a 'normal' image: the summed intensity of molecules individually considered as Gaussian point sources, whose width reflects the uncertainty in their position. Other choices for rendering should be explained and described, as would be required for altered or manipulated 'normal' images.

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