Localization-Based Super-Resolution Light Microscopy

Kristin A. Gabor,[1,2,3] Mudalige S. Gunewardene,[1] David Santucci,[4] and Samuel T. Hess[1,3,*]

[1] Department of Physics and Astronomy, 120 Bennett Hall, University of Maine, Orono, ME 04469

[2] Department of Molecular and Biomedical Sciences, 5735 Hitchner Hall, University of Maine, Orono, ME 04469

[3] Graduate School of Biomedical Sciences, 263 ESRB/Barrows Hall, University of Maine, Orono, ME 04469

[4] Volen Center for Complex Systems, 415 South St., Brandeis University, Waltham, MA 02454

* sam.hess@umit.maine.edu

Introduction

Fluorescence microscopy is an essential and flexible tool for the study of biology, chemistry, and physics. It can provide information on a wide range of spatial and temporal scales. However, since the inception of light microscopy, diffraction has limited the size of the smallest details that could be imaged in any sample using light. Because much of biology occurs on molecular length scales, interest in circumventing the diffraction limit has been high for many years. Recently, several techniques have been introduced that can bend or break the diffraction limit. Localization-based methods introduced in 2006 have reached this goal and are now rapidly growing in popularity.

Principles of Localization-Based Super-Resolution Microscopy

Localization versus resolution. Diffraction blurs the details of objects imaged by a lens-based far-field microscope system. Objects closer together than $R_0 = 0.61\lambda/NA$ are unresolved according to the Rayleigh criterion, where NA is the numerical aperture, λ is the wavelength of detected light, and R_0 is ~200–250 nm for green light imaged by a high-NA objective. In contrast, localization, which is the determination of the position of an object using its image, has been achieved with precision of ~1 nm [1], limited in principle only by the number of detected photons.

Localization-based super-resolution microscopy [2–4] relies on both the imaging of single molecules and the stochastic activation of sparse subsets of such molecules. This is often achieved by optical control of molecular transitions between bright and dark states. When the number of emitting molecules is limited, and the distance between molecules is greater than R_0, the molecular images are distinguishable (Figure 1). By activating a subset, imaging it, photobleaching or deactivating it, and repeating this process for many subsets of molecules, coordinates of thousands of molecules can be obtained. Molecular positions are measured by fitting the molecular image with a two-dimensional Gaussian function or with the experimental point spread function (PSF). For reliable localization, no more than a few molecules per frame can be localized within any diffraction-limited area. Thus, many (for example, 100–10000) image frames are required to achieve a high density of molecules in the final rendered image. Once a sufficient number of these molecules are acquired and localized, the molecular positions are plotted to generate an image. The effective resolution of the image obtained depends

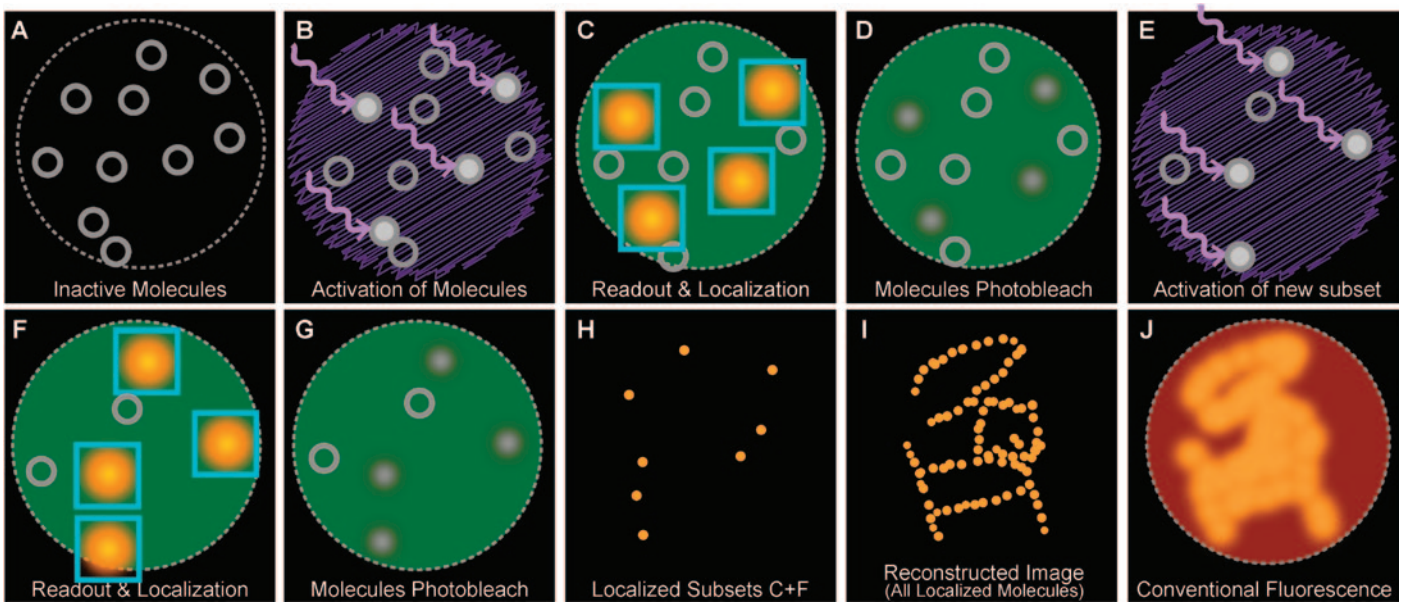

Figure 1: Principle of localization-based super-resolution microscopy. By limiting the number of fluorescent molecules visible at once, the images of the individual molecules become distinguishable. (A) Molecules are initially in an inactive (non-fluorescent) state. (B) Sparse subsets of molecules are converted into a fluorescent state by the activation beam (purple). When excited by the readout laser (green), they are imaged (C) until deactivated or photobleached (D). Molecules are localized by fitting the image with a two-dimensional Gaussian. Cycles of activation (B, E), readout and localization (C, F), and photobleaching (D, G) are repeated for many subsets of molecules. Rendered images with few (H) and large number (I) of localized molecules show buildup of structural detail as density increases. (J) Conventional image with diffraction-limited resolution.

 doi:10.1017/S1551929511000435

on both the precision with which molecules can be localized as well as the nearest-neighbor distance between molecules [5, 6]. Fluorescence photoactivation localization microscopy (FPALM) and similar techniques have achieved a resolution approximately ten-fold better than the diffraction limit. In general, any technique that is capable of creating a sparse subset of visible molecules, imaging that subset, and sampling many subsets can be used to generate a super-resolution image.

FPALM, PALM, STORM, and dSTORM. Conceptually, FPALM [2], photoactivated localization microscopy (PALM) [3], stochastic optical reconstruction microscopy (STORM) [4], and direct stochastic optical reconstruction microscopy (dSTORM) [7, 8] are very similar. Each technique relies on photophysical properties of probes to control the number of visible fluorescent molecules in the field of view. FPALM and PALM typically utilize photoactivatable fluorescent proteins (PA-FPs) to allow for direct optical control over the number of fluorescent molecules visible at a given time. This is achieved by adjusting the rates of photoactivation and photobleaching. Similarly, STORM uses pairs of combinations of organic fluorophores that photoswitch in the presence of thiol-containing reducing agents to control the number of molecules that are fluorescent at a given time. dSTORM uses single fluorophores that photoswitch in the presence of thiols and can be used in living cells, which contain reducing agents such as glutathione at micromolar to millimolar concentrations [8]. In all of these techniques, images are subsequently reconstructed from the coordinates and intensities of the localized molecules.

Probes. Localization-based techniques can be performed with either certain conventional fluorophores [8–10], PA-FPs, caged or photoswitchable organic dyes, or pairs of probes that act together as a molecular switch [11–15]. Typically, one wavelength (called the activation wavelength) is used to control the density of visible molecules while another wavelength (called the readout wavelength) is used to collect the fluorescence from the activated molecules. In some instances, one wavelength is sufficient to do both tasks; regardless, the choice of wavelength, intensity, and temporal illumination pattern enables active control over the number of visible molecules. In doing so, these methods rely on photophysical properties of the probes to control the number of molecules in the field of view at a given time [2, 16].

The photophysical properties of probes play a major role in the spatial resolution and rate of image acquisition. Resolution in localization microscopy is a function of the localization precision and density of localized molecules. As localization precision and single-molecule detection (against background) are greatly improved with more detected photons per molecule, probes that emit the maximum number of photons possible before bleaching are ideal. Desirable probes have a high rate of photon emission and a low rate of spontaneous or readout-induced activation, are small in size to minimize perturbation of cell structure or function, and have a large extinction coefficient to minimize the illumination intensity necessary for readout. Probes should also have large contrast (the ratio of brightness in active to inactive forms) so that background from inactive molecules is negligible. Probes with longer wavelength emission are beneficial to minimize autofluorescence background levels. For multicolor labeling, optimal probe combinations have these same characteristics and are typically spectrally well separated.

Fundamental limitations. One important limitation is the trade-off between spatial and temporal resolution. Sub-millisecond observation of molecular motions is limited by the intensity of the readout laser, the probe emission rate, and camera readout speed. Therefore, acquisition of super-resolution FPALM images on timescales faster than 0.1 s is currently difficult to achieve. The density of localized molecules is another limiting factor in image resolution. Because only one molecule (or at best a few molecules) can be localized per diffraction-limited area in any given frame, improved spatial resolution comes at the price of temporal resolution. Encouraging recent work using methods from astrophysics has enabled analysis of molecular images with at least ten-fold higher density, which in principle should allow imaging rates that are faster by the same factor [17].

Minimization of background is crucial both for identification of fluorescent probe molecules and for maximization of the localization precision. Thus, samples should not emit significant autofluorescence in the same spectral region as the probe being used. Use of UV-bleached water for imaging buffers, use of phenol-red-free media for cell growth, and use of a water-immersion objective beaded with bleached water can help. Additionally, if background levels are high initially, activation of probes can be delayed while the background is allowed to bleach.

Many available PA-FPs have overlapping emission spectra, limiting the number of species usable in a typical multicolor imaging application. Ultimately, the continued development of photoactivatable probes will extend the capabilities of localization microscopy. The improvement of probes relies on better understanding and control of their photophysical properties. The labeling efficiency of each probe is another factor to consider, as well as the expression level of the protein to which it is fused. As probes are developed with higher peak photon emission rates, localization microscopy will be possible with higher spatial and temporal resolution.

Methods and Applications

Recent progress in the field of localization-based super-resolution microscopy has led to extensions of these techniques to multicolor [8, 18–20], three-dimensional [21–24], and polarization imaging [25]. For instance, using modifications to the FPALM detection path has enabled multi-channel detection on the same camera chip as well as nanoscale imaging of single molecule anisotropies. A typical experimental geometry for two-color three-dimensional imaging is shown in Figure 2. These and other advances show great promise for improved understanding of biological systems at the molecular level.

Single-species localization. A typical localization microscopy setup is a fluorescence microscope with activation and readout lasers aligned so that they are collinear, a high-NA objective lens, and a sensitive charge-coupled device (camera) capable of imaging single fluorescent molecules. Illumination light is focused in the center (or at the edge) of the objective back aperture to illuminate the sample by widefield (or by TIRF) over an area of ~100–1000 μm^2.

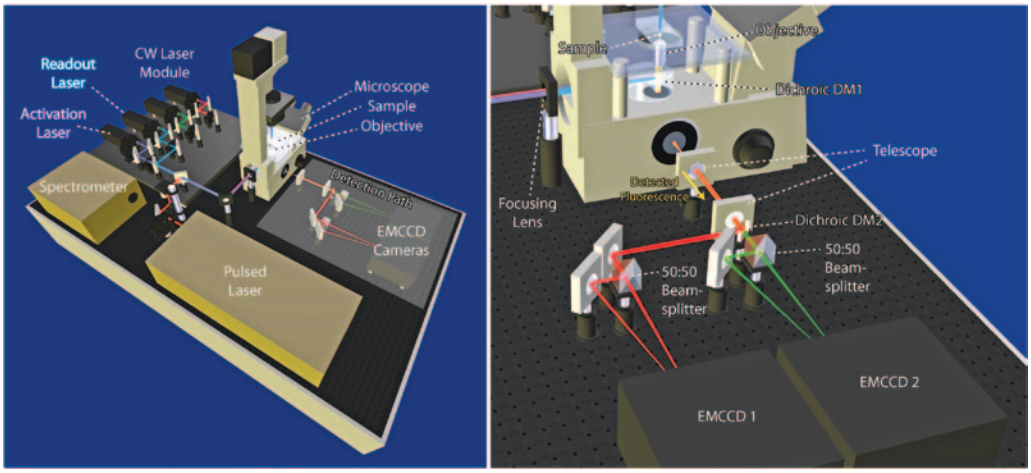

Figure 2: Experimental geometry for two-color bi-plane FPALM. (Left) A set of lasers is aligned into a collinear path to allow a choice of one activation and one readout illumination wavelength. These two beams are directed through a series of mirrors into a focusing lens positioned at one focal length from the back aperture of a microscope objective within a fluorescence microscope (here shown as inverted). (Right) The sample is illuminated on the microscope stage, and emitted fluorescence from single molecules is separated from illumination by a dichroic (DM1) and sent through the tube lens and out the microscope side port. Next, the fluorescence image is magnified by a telescope and then split by a second dichroic (DM2) into two detection channels. Each detection channel can then be passed through a 50:50 beamsplitter to form separate images on each of two EMCCD cameras. Note that the two longer arms of the red and green paths should have the same distance between sample and camera, as should the two shorter arms of the red and green paths. For illustrative purposes, the figure does not show the optics arranged in this way. Also shown are an optional spectrometer, for confirmation of molecular species, and a pulsed laser for multi-photon activation and/or readout.

Activation laser intensity is regulated, either by pulsing with progressively higher frequency or gradually increasing intensity as the pool of inactive molecules is depleted, to maintain a small number of activated molecules within the field of view. Readout laser intensity is controlled such that the active fluorescent molecules are clearly visible above the background. Typically, hundreds to thousands of frames are acquired and then analyzed to localize visible molecules. Images are rendered by plotting each molecule as a Gaussian spot with a weight and size proportional to the number of photons detected and the localization precision, respectively. An example of single-color imaging of dendritic spines is shown in Figure 3.

The first published example of localization microscopy in living cells used single-color FPALM of the viral surface protein hemagglutinin (HA) to distinguish between several competing models of membrane organization [26]. A wide variety of biological applications have been presented [27–30]; localization microscopy is applicable to virtually any subcellular structure, as long as the structure can be labeled and the molecular millieu is compatible with imaging and localizing single molecules.

In addition to molecular coordinates, a direct count of the numbers of localized molecules (and thus, with careful analysis, the densities of molecules) is obtained, and sub-populations of molecules can be identified based on brightness, or other spectroscopic properties, which would otherwise be averaged out in ensemble imaging methods. Correlation of localization microscopy results with other methods, such as confocal or widefield fluorescence, atomic-force microcsopy, or electron microscopy, is extremely valuable as a way to provide context for the molecular positions.

Multicolor imaging. Multicolor super-resolution imaging is invaluable for the direct visualization of molecular interactions at the nanoscale level [8, 18–20]. Multicolor imaging and analysis has been performed as an extension of standard FPALM using a detection scheme similar to that reported previously for multicolor single molecule imaging [19]. With a detection path divided by a dichroic into two wavelength ranges [19], camera frames containing two spatially distinct (but simultaneous) images representing the two emission wavelength ranges are obtained. The two images are correlated and superimposed for localization, while the intensity ratio of one channel over the sum of both channels is used to distinguish the multiple molecular species.

Both PALM and STORM have also reported multicolor super-resolution images obtained using combinations of cyanine dyes or PA-FPs [18, 20]. Advantages of PA-FPs for localization microscopy include the ability to perform live-cell imaging (that is, without thiol or oxygen-scavenging reagents),

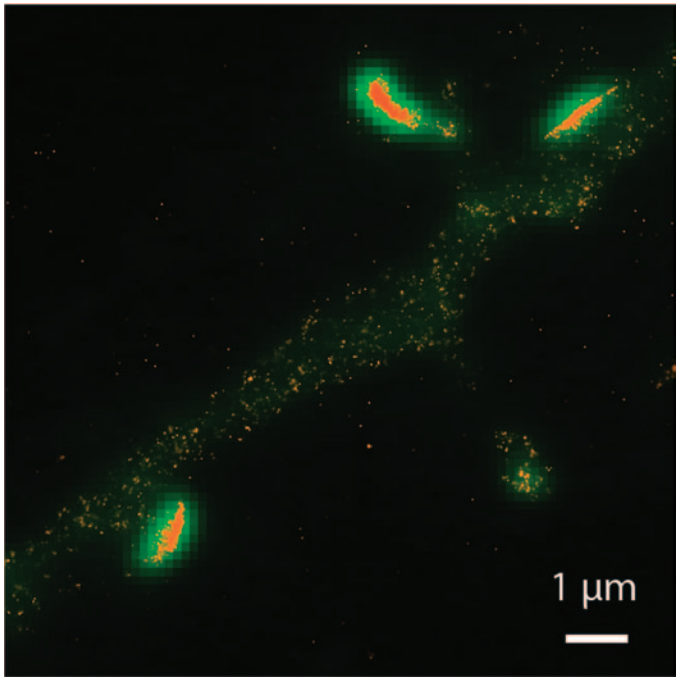

Figure 3: FPALM image of dendrites of fixed rat hippocampal neurons in a culture labeled with Dendra2-PSD95. Green: convention fluorescence. Orange: FPALM image. Credit: D. Santucci, T. Gould, T. Newpher, A. Mabb, M. Ehlers, J. Lisman, and S. Hess.

the use of relatively non-invasive labeling procedures (that is, transfection), and high specificity due to genetic encoding of PA-FP-protein chimeras.

Time-lapse imaging. Temporally-resolved super-resolution imaging is useful for live or dynamic specimens. Time resolution depends on desired resolution: 50-nm resolution requires a nearest-neighbor distance of 25–50 nm, or approximately 70–280 molecules per diffraction-limited area, which requires (for one rendered image) at least 70–280 frames of acquisition, or 0.2–0.7 seconds per acquisition at 400 Hz. Such frame rates are achievable using a number of commercially available EMCCD cameras, such as the Andor iXon+ DU-897. Time resolution of 25 nm requires nearest-neighbor distance of 12.5–25 nm, or 280–1150 frames, requiring 0.7–3 seconds per rendered image. Intensity should be adjusted to bleach molecules within approximately one frame. Note that the structure of interest should not rearrange significantly during this time period, although the individual molecules can move within the structure as long as their motion samples or is meaningfully representative of the structure overall. For example, the dynamics of actin structures at the cell leading edge (motion toward the cell periphery at 0.1 to 2 µm/min) [31] is compatible with live-cell FPALM. The dynamics of membrane protein clusters is also well-suited to time-lapse imaging [26]. Of course, control experiments must always be performed to confirm that addition of labels to the sample does not perturb structure or function. Figures 4 and 5 show examples of two-color live-cell and fixed-cell FPALM, respectively.

Molecular trajectories. Imaging conditions can be adjusted to allow probe molecules to remain fluorescent for several frames before photobleaching. Thus, several consecutive images of the same molecule can be analyzed to construct a short trajectory, which can be equivalent to a few milliseconds or as much as a few seconds. A trade-off results between trajectory length and localization precision per step from the limited photon budget per molecule: for a thousand photons detected from a single molecule before bleaching, 10 steps with 100 photons per frame results in a localization precision of ~20–30 nm at best, whereas 40 steps would yield 50–60 nm localization precision. On the other hand, trajectories may be obtained for hundreds of thousands of molecules, allowing higher density sampling of structures [32]. Clearly, such methods are not well-suited to situations where long-term tracking of the same molecule(s) is important. However, for many applications, such as tracking of membrane proteins and lipids within small clusters, or measurement of the local velocity of cytoskeletal structures, trajectory imaging can be quite useful. Probes that are particularly resistant to photobleaching, allowing detection of >10^3 photons, are highly desirable.

Three-dimensional imaging. Because most biological structures are three-dimensional (3D), methods that can image beyond the diffraction limit in the axial direction (z) are essential for advancement of our understanding of biological systems. However, the diffraction-limited resolution in z is typically worse than the lateral resolution. Advances in 3D localization microscopy have been demonstrated in several incarnations, including biplane detection [21], optical astigmatism [22], interferometry [23], and double-helix point-spread function [24].

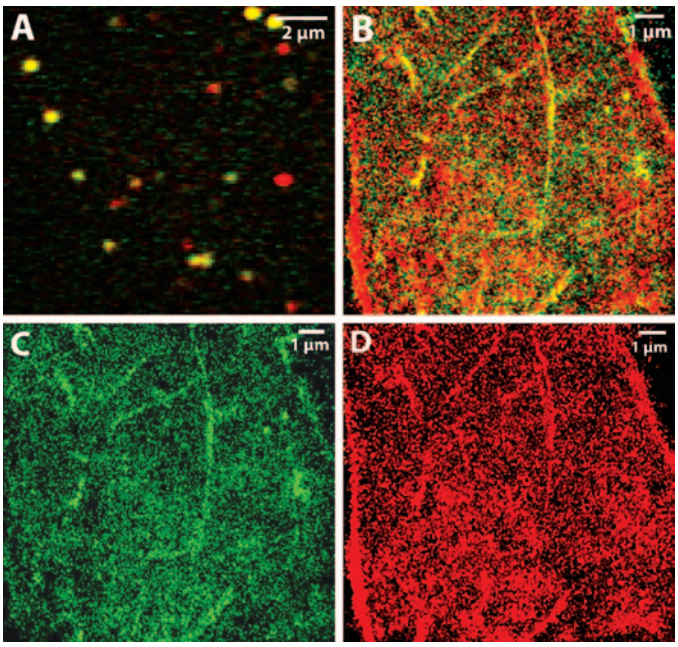

Figure 4: Multicolor live-cell FPALM. (A) Pseudocolor image of individual PA-FP molecules (30 ms exposure with Andor iXon+ EMCCD) illustrating FPALM with simultaneous two-channel detection. (B) Two-color FPALM image of Dendra2-HA (green) and PAmCherry actin (red) in a live HAb2 fibroblast cell at 37° C (8000 frames total). (C) Dendra2-HA only. (D) PAmCherry actin only. Credit: M. Gudheti, M. Siyath Gunewardene, T. Gould, and S. Hess.

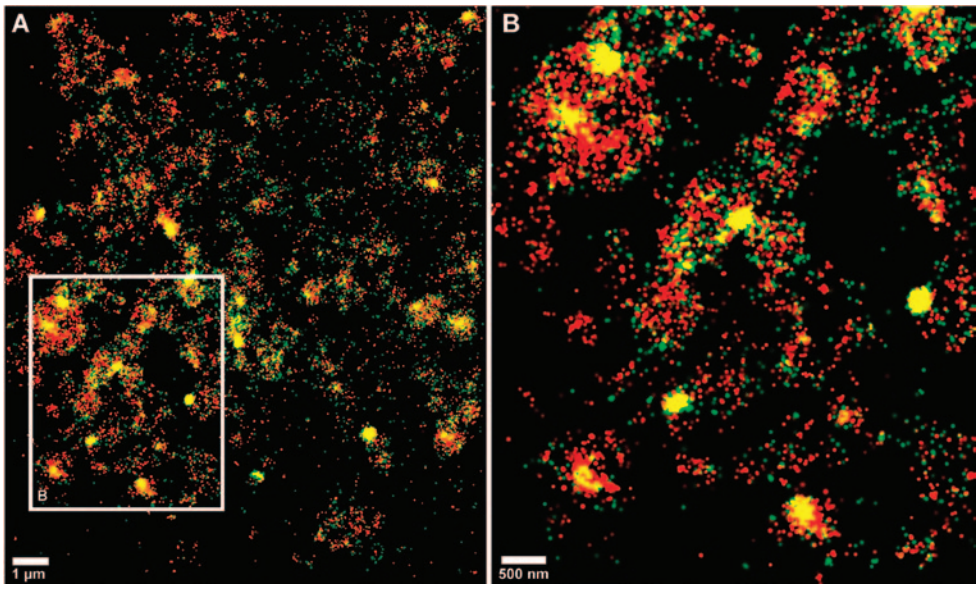

Figure 5: Multicolor FPALM image showing colocalization of PAmCherry-caveolin-1 (red) with Dendra2-interferon receptor molecules (green) in fixed ZFL cells. Credit: K. Gabor, T. Gould, C. Kim, and S. Hess.

Biplane FPALM (BPFPALM) is an extension of FPALM to 3D, which works by splitting the detected fluorescence into two separate paths with different overall lengths. The two images simultaneously formed on separate regions of the camera correspond to two different focal planes and are later fitted with the measured PSF to determine the *x*, *y*, and *z* coordinates of each molecule. BPFPALM of samples was demonstrated with an axial resolution of ~50–75 nm [21, 33], approximately 10-fold improved over conventional fluorescence microscopy.

Optical astigmatism is used to determine both axial and lateral positions of single molecules in 3D STORM [22]. By inserting a cylindrical lens into the detection path of the microscope, two slightly different focal planes result for the *x* and *y* dimensions of each molecular image. As a result, fluorophores appear elliptical in *x* or *y* depending on their axial position, allowing that axial position to be determined from the molecular image.

Interferometric PALM (iPALM) resolves 3D molecular coordinates of individual PA-FP tagged proteins with ~10 nm axial resolution and ~20 nm lateral resolution using a 4Pi (two-objective) geometry [23]. The detected fluorescence is divided by a three-way beam splitter and imaged with three cameras. The relative intensities of the three images of each molecule are then analyzed to determine the axial position of the molecule. iPALM was recently used to deduce the structure of the focal adhesion complex [34].

In the double-helix point spread function (DH-PSF) method, a phase modulation pattern in the detection path yields a detected PSF with two lobes whose orientation rotates as a function of the axial position of the detected molecule. DH-PSF has a demonstrated resolution of ~10 nm laterally and 20 nm axially over a sample depth of ~2 μm [24].

Polarization FPALM: imaging molecular orientations at the nanoscale level. The localization-based imaging techniques described above are crucial for studies of biological structures, but they do not provide information about the orientation or rotational freedom of single molecules. Polarization FPALM (PolPALM) [25] has extended FPALM to image single molecule anisotropies by simultaneously detecting two spatially separated images of the fluorescence, polarized parallel and perpendicular to a specific axis at the sample. To measure the anisotropy for each localized molecule, PolPALM analyzes the relative intensities of molecules in the two images. The demonstrated resolution of ~17 nm using PolPALM allows quantification of structure on length scales inaccessible to diffraction-limited techniques. The anisotropy obtained reflects the range of accessible probe orientations and can also be used to quantify binding between molecules.

Conclusion

Localization microscopy provides nanoscale spatial resolution in living fluorescent specimens using far-field optics. Such capabilities suggest that a multitude of fundamental but previously inaccessible biological questions can now be addressed. Current limitations such as number of simultaneously distinguishable species and the time resolution of imaging are likely to improve with refined experimental methods and improved probes. Furthermore, using existing localization microscopy methods, the number of compatible biological systems is continuously increasing. New biological insights hopefully are imminent.

Acknowledgments

The authors thank M. V. Gudheti and T. J. Gould for FPALM images included within the article; S.-R. Yin and Vladislav Verkhusha for constructs; and A. Gosse, J. Lake, and J. Gosse for support. This work was funded by NIH Career Award K25 AI65459, NIH R15 GM094713, NIH R01 GM094713, NSF MRI CHE-0722759, Maine Technology Institute MTAF 1106 and 2061, UMaine V.P. for Research, and the Maine Economic Improvement Fund (MEIF).

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