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Microscopy in 3D: a biologist's toolbox

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Abstract

The power of fluorescence microscopy to study cellular structures and macromolecular complexes spans a wide range of size scales, from studies of cell behavior and function in physiological, three-dimensional (3D) environments, to understanding the molecular architecture of organelles. At each length scale, the challenge in 3D imaging is to extract the most spatial and temporal resolution possible while limiting photodamage/bleaching to living cells. A number of advancements in 3D fluorescence microscopy now offer higher resolution, improved speed, and reduced photobleaching relative to traditional point-scanning microscopy methods. Here, we discuss a few specific microscopy modalities that we believe will be particularly advantageous in imaging cells and subcellular structures in physiologically relevant 3D environments.

The Challenges of 3D Imaging

Imaging cells and subcellular structures in three-dimensional environments brings with it challenges that are not unique in biological microscopy, but are amplified relative to the 2D imaging of cells adhered to traditional glass coverslips *in vitro*. We will review a few of the major challenges for 3D and time-lapse 3D (4D) biological imaging, and discuss the weaknesses of conventional laser-scanning confocal microscopy in meeting these challenges. We then cover the strengths and weaknesses of newer microscopy modalities that have successfully addressed these challenges in recent years (see **Glossary** for introduction to terminology).

The 3D Point Spread Function and imaging in thick specimens

The first and most obvious challenge of 3D imaging is that in conventional, uni-axial microscopy arrangements, the resolution along the Z-axis of the microscope is substantially (~2x) worse than in the lateral dimensions due to the inherent shape of the point spread function (PSF; the three-dimensional diffraction pattern of a point source viewed through an objective lens; see Box 1 and Figure 1). The characteristic anisotropy of the PSF is seen as “Z-stretch” in XZ-projections of reconstructed 3D image sets obtained with uni-axial fluorescence microscopy. This reduced axial resolution and Z-stretch can introduce profound errors in tracking of objects in 4D and in co-localization in multichannel 3D imaging¹.

Adding to the problem of axial resolution is that the structures of interest are sometimes tens or hundreds of microns away from a coverslip or objective lens front element. Focusing at

these depths requires longer working distances for objective lenses, which comes at the cost of lowering the numerical aperture, thus light collection efficiency and resolving power are reduced. Furthermore, for fluorescence techniques, excitation light penetration in most biological samples is problematic due to photonic interaction with the sample, resulting in elastic scattering^{2,3}. The emitted light from a fluorophore also scatters within the sample, and thus contributes to out-of-focus fluorescence and background signal, thereby decreasing the image signal-to-noise ratio (SNR)⁴. In addition, at deeper focal planes in scattering samples, the observed PSF can degrade rapidly due to spherical aberration such that it becomes severely asymmetric along the Z axis (Figure 1). Refractive index mismatches between thick specimens, glass, and immersion media further add to spherical aberration, inducing errors in Z localization⁵.

Photobleaching and Speed

Another challenge for 3D fluorescence imaging, particularly over time (4D imaging), is that excessive light exposure to the sample is inherent in the need to collect several Z-planes for each time-point, leading to problems with photobleaching of fluorophores, phototoxicity, and slow 3D frame rate. Photobleaching and phototoxicity occur primarily as a result of the possibility that high energy electrons in excited fluorophores may not emit energy as fluorescence, but instead react with dissolved oxygen. This irreversibly bleaches the fluorophore and produces reactive oxygen species (ROS) which are highly reactive, causing phototoxic effects directly proportional to their diffusion and concentration. While ROS are a major contributor to phototoxicity, other ROS-independent photodamage may also occur, causing even small amounts of light absorption to produce toxicity and unintended effects on cell function⁶⁻⁸. Although photobleaching and phototoxicity can be abrogated in part by addition of oxygen scavenging systems or simple antioxidants such as L-ascorbic acid⁹, the effects of phototoxicity are cumulative and directly proportional to excitation light intensity and total photon load⁷. Thus, strategies to maximize light collection efficiency and minimize excitation of the total sample volume (e.g.^{6,10}) are critical in advancing 4D imaging.

In addition to photobleaching and phototoxicity, the need to collect several Z-planes increases the acquisition time for each time-point, thus slowing the rate of 4D image acquisition. This leads to trade-offs between time resolution, imaged volume, Z-resolution, and length of imaging period, and places further constraints on the total photon load. This is particularly true with some types of very dynamic processes such as rapid cell migration, membrane remodeling or microtubule dynamics that require both high temporal and spatial resolution in 4D for motion tracking analyses.

Limitations of Scanning Microscopy

Point-scanning methods such as CLSM and two-photon microscopy (2PM) have been a major boon to biological microscopy for decades, revolutionizing our ability to define the 3D structure of cells, tissues and whole animals. By rejecting out-of-focus fluorescence with a pinhole or limiting excitation to a diffraction-limited volume, CLSM and 2PM offer excellent optical sectioning, because only fluorescence emitted from the diffraction-limited volume in the focal plane can reach the photomultiplier tube detector (PMT; for review, see⁴).

In spite of their advantages and major contributions to 3D imaging, these traditional scanning techniques are particularly poor at overcoming the challenges to 4D imaging discussed above. First, neither CLSM nor 2PM point-scanning methods abrogate the axial stretch of the PSF discussed above (Box 1). Second, because these methods raster the excitation and detection to build an image one voxel position at a time over the entire X-Y-Z

imaged volume, they suffer from slow 3D frame rates and excessive light exposure to the specimen. This contrasts with traditional transmitted light and wide-field epifluorescence modes in which entire X-Y focal planes are imaged, reducing image frame rate considerably for 4D imaging. Furthermore, since PMT detectors used in CLSM and 2PM are noisy and have poor quantum efficiency (QE; ~10-15%), in order to get sufficient fluorescence photons to the detector in dim or highly scattering samples, either the scan speed must be lowered to allow longer excitation dwell times, or the intensity of the excitation must be increased to accumulate more fluorescence signal¹¹. Typically, the combined effects of scanning and PMT properties result in a SNR that is several times lower than other imaging modalities¹². Furthermore, the need to increase excitation intensity can result in fluorophore saturation¹³. In this regime, the number of fluorescent photons produced does not increase as excitation input increases, but the chance for triplet state ROS production continues to increase⁸. In addition, for CLSM, although fluorescence emission collection is limited to the in-focus image volume, the entire Z-axis of the specimen is exposed to excitation throughout scanning. Coupled with the fact that peak excitation intensity with CLSM is quite high at ~1 mW/ μm^2 , CLSM can cause extensive photodamage and photobleaching in 4D imaging.

In 2PM, the volume of excitation is limited to a diffraction-limited region within the specimen where pulsed, long-wavelength laser intensity is high enough to produce fluorescence excitation via the non-linear two-photon effect (Figure 2)¹⁴. The use of long-wavelength illumination improves penetration of the sample and the limited excitation volume eliminates photobleaching in out-of-focus areas of the specimen. In addition, pulsed lasers allow for second harmonic generation imaging of collagen fibers and other non-centrosymmetric polymers without sample labeling¹⁵⁻¹⁷. In spite of these advantages over CLSM, the photobleaching rate with two-photon excitation increases at a much faster rate with excitation intensity than with single-photon excitation¹⁸. Furthermore, the maximal rate at which fluorescence can be emitted from a single fluorophore is lower with two-photon excitation than single-photon excitation¹⁹. Even more importantly, phototoxicity observed with two-photon excitation may actually be worse than single-photon excitation in some cases^{19,20}. While the average power of the input beam over time may be relatively low, the instantaneous power during an excitation pulse can be as high as 10W/ μm^2 ¹¹. Nevertheless, because of its ability to limit excitation volume and penetrate deep into tissues, 2PM will continue to be a primary tool for 3D and 4D imaging, particularly intravitaly in adult animals²¹⁻²³. Furthermore, 2PM will be particularly advantageous if used in conjunction with other optical sectioning techniques (see below)^{24,25}.

Solutions for 3D Imaging

Breaking the Resolution Barrier: Super-resolution in 3D

The most fundamental challenges to 3D optical imaging are the resolution limit imposed by diffraction and the anisotropic nature of the PSF, which reduces resolution in the axial dimension compared to the lateral dimensions. These challenges have been overcome recently by structured illumination microscopy (SIM) as well as point source localization techniques that hold great promise for the future.

Structured Illumination Microscopy (SIM)

While optical sectioning of 3D samples has been most often obtained through either CLSM or 2PM, optical sectioning can also be performed using a wide-field microscope with laterally structured illumination²⁶. Structured illumination microscopy (SIM) was first introduced to enhance lateral resolution beyond the limits of diffraction²⁷⁻²⁹. In SIM, the sample is illuminated with patterned light, producing Moire fringes or a 'beat pattern' that moves otherwise unresolvable high-frequency information into the passband of the

microscope objective. By taking multiple images with phase-shifted patterned illumination, it is possible to computationally remove the excitation pattern and reassemble an image with approximately twice the resolution of the original images.

Gustafsson and coworkers have expanded the initial 2D technique by using three mutually coherent beams to generate interferometrically patterned excitation in both axial and lateral dimensions²⁸, offering resolution doubling in all three dimensions (Figure 2). This version of 3D SIM can be improved further by employing two opposed objective lenses in a ‘4Pi geometry’ and a beam splitter that makes 6 coherent beams³⁰. In the 4Pi implementation, the resultant images do not suffer from the same Z-stretch observed with confocal images^{28,30,31}, as 100 nm near-isotropic resolution is obtained. In addition to the significant increase in resolution, 3D SIM does not rely on a pinhole, thus enabling efficient light collection, good sensitivity, and the ability to capture widefield images on a high-QE, low-noise array detector such as charge-coupled device (CCD) or complimentary metal-oxide semiconductor (CMOS) cameras. In addition, SIM is relatively easy and inexpensive to implement in the context of a traditional widefield epifluorescence microscope, and several manufacturers already have commercialized 2D SIM systems on the market. The power of SIM to study processes in cells in 3D has been beautifully demonstrated in the study of nuclear structure and chromatin organization³², as well as the dynamics of the cell cortex at the cytokinetic furrow^{33,34}.

While SIM holds much promise for future studies of cells and cellular architecture in 3D below the diffraction barrier, several caveats apply. First, since multiple exposures with varying phase need to be captured for each Z-plane (15 for 3D SIM), SIM has intrinsic speed limitations. While increases in camera sensitivity and speed have enabled live cells to be studied with high spatial and temporal resolution using 2D SIM, movements of structures by more than one resolution length in the time it takes to acquire the requisite phased images result in image reconstruction artifacts³⁵. While 2D SIM can be performed at relatively high speeds (up to 11Hz), multicolor 3D-SIM is more practical at speeds of one z-plane every 2-6 seconds^{6,32}. For 3D applications this is an important limitation to consider when one is studying dynamic processes. Second, while SIM effectively removes out-of-focus light computationally, it still leaves behind the associated shot noise²⁸. Thus, in practice, in very thick samples with a large amount of out-of-focus light, 3D SIM may produce lower contrast images compared to confocal approaches. Finally, current implementations of 3D SIM rely on interferometric pattern generation, and so very thick or highly scattering samples will likely degrade the pattern enough that reconstructions will suffer. Future use of non-linear optics with 3D-SIM may extend its useful working distance and reduce photobleaching through the volume of the specimen. In any case, because of its relatively inexpensive implementation on existing microscope platforms and greatly improved resolution, 3D SIM will likely contribute to important advances in understanding organelle structure and function within cells.

STORM & PALM in 3D

Another group of approaches that overcomes the limitations of diffraction are the ‘pointillist’ super-resolution localization techniques including (fluorescence) photoactivated localization microscopy, (f)PALM^{36,37} (referred to henceforth as PALM), and stochastic optical reconstruction microscopy, STORM³⁸, which in some cases offer localization precision of less than 20 nm. These techniques rely on iterative cycles of activation, excitation, photobleaching coupled with image acquisition, and subsequent localization with subdiffraction accuracy of small numbers of labeled molecules that are separated in space by greater than the Rayleigh limit within a densely labeled specimen (Figure 3). To achieve spatially separated excitation/emission events, PALM uses genetically photoswitchable fluorescent proteins to reversibly switch between emission wavelengths or between a dark

and fluorescent state³⁹, while STORM utilizes reducing buffers and small molecule fluorophores to similar effect⁴⁰. Localization in both cases is achieved by fitting the image of individual molecules to a model PSF⁴¹, computing its centroid⁴², or by performing a cross correlation to an experimental PSF²⁵. As localizations of many isolated fluorophores are collected over hundreds to many thousands of frames to build up a localization image of fluorophores at high density, PALM images are typically acquired in a few tens of seconds to tens of minutes.

PALM and STORM have been extended to the third dimension, either by optically altering the shape of the PSF as a function of axial depth^{40, 43} or by simultaneous collection of two planes to collect 3D data which is fit to a 3D PSF³⁷, thereby enhancing subdiffraction localization capability along the Z-axis. In STORM, use of a cylindrical lens in the optical path creates a controlled astigmatism to distort the PSF as a function of Z position (Figure 4A)^{40, 43}. By comparing the PSF of a fluorophore to a calibration, a localization precision of ~50-100 nm in the Z direction can be achieved, over an axial range of several microns. This approach has enabled the first super-resolution images of the clathrin-coated pit structure^{40, 44, 45}. However, imaging thicker 3D samples is difficult, as conventional wide-field illumination activates and excites many out-of-focus probe molecules, increasing background and impeding isolation of single molecules in the imaging plane. Out-of-focus activation and excitation has the additional disadvantage that it potentially wastes localizations, decreasing the effective label density and reducing image resolution. Use of two photon activation⁴⁶ in “3D-PALM” can be used to avoid this problem by limiting photoactivation to the focal plane, and has been demonstrated up to ~8 μm deep in the sample while retaining high localization densities²⁵. Future technical improvements may better confine the illumination, extending the per-molecule photon budget and permitting 3D super-resolution at greater depths.

For 3D imaging of samples up to ~300 nm in thickness, interferometric photoactivated localization microscopy (iPALM) offers unmatched localization precision. iPALM uses localization of single molecules for super-resolution in the lateral (X-Y) dimension, and simultaneous multiphase interferometry for super-resolution in the axial (Z) dimension. Like its 2D counterparts, iPALM depends on photoswitching of small populations of fluorophores to determine X-Y-coordinates (Figure 3). However, in iPALM, the enhanced resolution along the Z axis is achieved by using dual objectives in an opposed, 4Pi configuration (Figure 4), thus allowing each emitted photon to propagate through both top and bottom objectives and be recombined in a beamsplitter. The optical path-length difference between the top and bottom emission beams, and hence the phase of the recombined signal, is directly proportional to the Z coordinate (Figure 4B). Due to the greater collection efficiency of the 4Pi geometry in iPALM, X-Y localization precision is typically higher than in conventional PALM. Additionally, the high sensitivity afforded by interferometry can result in an axial localization precision better than 10 nm⁴⁷. Indeed, in iPALM, axial resolution is approximately two times better than the lateral resolution, unlike single-objective-based techniques where axial resolution is typically 2-3x worse than lateral. Due to the high resolution, iPALM is thus advantageous for investigating molecular organization at the ultrastructural level. For example, iPALM was deployed to study focal adhesions⁴⁸, integrin-based adhesive organelles that play important roles in cell migration, matrix-remodeling, and mechanotransduction. Due to their density and complex composition, molecular organization within focal adhesions has long been difficult to access by techniques such as electron microscopy (EM). iPALM revealed for the first time that focal adhesion proteins are stratified along the axial dimension⁴⁸.

While iPALM provides high spatial resolution approaching EM, a number of limitations remain. Since direct optical access is required from both sides, the sample must be relatively

thin (less than 15-20 μm). Likewise, the axial range of imaging depth is limited to $\sim 250\text{--}600\text{ nm}$ ⁴⁷. Also, iPALM instrumentation is technically complex and demands very high mechanical and thermal stability. In contrast, the instrumentation for conventional PALM and STORM (Figure 4A) are relatively simple, free localization software⁴² is available, and several major manufacturers now offer turnkey commercial platforms with some 3D capability (based on biplane imaging and astigmatism) that are compatible with suitable dyes, making these technologies even more accessible. While PALM and STORM offer superb resolution for 3D imaging, the need to collect hundreds to tens of thousands of raw frames for a single reconstructed image places a severe limitation on the imaging speed. Progress in dye brightness, contrast and attachment chemistry⁴⁹ suggests that the field will continue to evolve, with the promise of faster live cell applications, in multicolor and 3D⁵⁰. In spite of their limitations in speed, PALM, STORM and iPALM offer near-ultrastructural 3D resolution together with the molecular specificity of fluorescent labeling, and will continue to answer important questions about molecular-scale biological architecture that have eluded electron imaging-based approaches.

Breaking the Speed Limit: Spinning Disks to Light Sheets—Another major set of challenges for 4D imaging has been the inherent speed limitation of point-scanning techniques for optical sectioning, along with the photobleaching that accompanies 4D imaging, as discussed above. Two basic approaches have been used to address these challenges, which are multiplexing of confocal pinholes, such as in spinning disk confocal microscopy (SDCM) and use of orthogonal plane illumination in light sheet fluorescence microscopy (LSFM).

Spinning Disk Confocal Microscopy (SDCM)

Several methods have been developed for taking advantage of the confocal principle of pinhole-based out-of-focus light rejection while maintaining the ability to generate wide-field images to overcome the speed limitations of point-scanning microscopy. These include slit scanning⁵¹ and pinhole multiplexing methods, including swept-field and spinning Nipkow disk confocal techniques. Of these, the most robust method that has gained wide acceptance among biologists is SDCM. This method uses an array of excitation and emission pinhole apertures on a rapidly spinning disk, such that the pinhole array sweeps the entire field of view over 1000 times per second (reviewed in⁵²). The high scan speed not only improves image acquisition rate (up to 360 frames per second⁵³), it also has the effect of lowering the peak excitation light density down to a few $\mu\text{W}/\mu\text{m}^2$, thereby increasing fluorescence efficiency and decreasing photobleaching and photodamage effects compared with point scanning¹³. Perhaps most important, since the entire confocal field of view can be captured by a high-QE, low-noise camera instead of a PMT, SDCM systems have more than 50-fold increase in light capture efficiency, resulting in several-fold increase in SNR relative to CLSM or 2PM^{12, 13}. Because of these advantages, SDCM has been well suited to the study of cells in 3D extracellular matrices⁵⁴, engineered tissue in matrices⁵⁵, as well as intravital imaging^{56, 57}. The ability to rapidly collect entire frames at once also enables the quantitative study of very dynamic processes, such as microtubule dynamics in cells in 3D matrices⁵⁸.

While SDCM has many powerful features for 4D imaging, there are a few caveats and limitations. First, like other linear excitation imaging methods, SDCM suffers from problems with illumination penetration of the sample and photobleaching of the entire axial column of the specimen at each Z-plane for each time-point. Second, imaging thick specimens suffers from an effect known as ‘pinhole crosstalk’. As light from a point in a focal plane expands over distance, portions of this light can enter into adjacent pinholes, and scattering of light in the sample increases this effect. Third, since pinhole size is fixed in

current implementations of SDCM, this limits its use to high-magnification, high-resolution objective lenses. Finally, SDCM suffers from the same axial resolution problems of other confocal methods (Table 1). However, because of its high speed, low noise and reduced photobleaching compared to CLSM, SDCM will continue to be extremely important for imaging fast cell and organelle dynamics in 3D.

Light Sheet based Fluorescence Microscopy (LSFM)

In LSFM⁵⁹, known in different implementations as ‘selective plane illumination’, SPIM⁶⁰, or ‘digital scanned laser light sheet fluorescence microscopy’, DSLM⁶¹, the sample is illuminated with a thin sheet of light from the side of the specimen to illuminate a single XY plane, and fluorescence detection occurs in the direction perpendicular to excitation (Figure 2). This geometry leads to major advantages over confocal and two-photon approaches. First, acquisition speed is greatly increased relative to point-scanning methods, as the entire imaging plane is detected simultaneously. A volume is recorded by scanning the light sheet in one dimension, instead of the 3D scan required for point-scanning. Second, excitation is confined to the focal plane, providing optical sectioning without pinholes, thus boosting detection efficiency while reducing photobleaching and photodamage to rates far below other techniques. Finally, as acquisition is parallelized, each pixel is exposed for the full integration time of a high QE, low noise widefield camera, resulting in ~10-1000-fold higher SNR than point-scanning techniques operating at similar frame rates.

The high SNR and very fast acquisition afforded by LSFM have enabled ground-breaking observations of vertebrate embryonic development^{60, 62, 63} and photomanipulation of cardiac pace-making function *in vivo*⁶⁴. Improving the axial resolution of LSFM by using a Bessel beam and structured illumination (see below) has provided high-speed, isotropically resolved 4D imaging of cellular dynamics²⁴. Three-dimensional cell culture can likewise be imaged over long times using LSFM⁶⁵.

LSFM does possess drawbacks compared to other 4D imaging techniques. First, the axial range of LSFM is reduced compared to 2PM, as the effect of scattering degrades the excitation sheet and is more pronounced on the emission side due to wide-field detection. Using a femtosecond laser for two-photon light-sheet excitation mitigates some of this disadvantage, providing an interesting hybrid technique that combines advantages of 2PM and LSFM⁶⁶. Second, the spatial resolution of LSFM is generally lower than other techniques, which is why most applications have targeted larger systems such as whole embryos or tissue slices^{60-62, 67}, instead of single cells. Lateral resolution is determined by the NA of the detection lens, while axial resolution is determined by both the detection objective and the light sheet thickness. The perpendicular excitation/detection geometry required by LSFM forces the use of long working distance, somewhat low NA (typically 1.0 or less) objective lenses. In almost all LSFM implementations, the light sheet is created from a Gaussian beam. Gaussian beams undergo widening at increasing distances from the beam waist, coupling the quality of optical sectioning to the position within the field of view and degrading the effective axial resolution at the sample edges. Exciting a fluorescent sample with a scanned Bessel beam⁶⁸ in an LSFM geometry mitigates this problem and improves axial resolution²⁴. Such a resolution improvement comes at a cost, however, as “side lobes” in the excitation profile cause significant out-of-plane illumination. Removing the contaminating effects of the side lobes requires either structured illumination or two-photon excitation. Finally, implementing LSFM is nontrivial, so in spite of its exceptional promise, until LSFM becomes commercially available, it is likely to remain the province of relatively few labs. However, once commercialized, LSFM promises to be extremely important for imaging cellular dynamics in larger, thick specimens such as whole animals during early development.

Concluding remarks—The array of imaging modalities available for study of animals, tissues, cells and cellular components in 3D has increased dramatically over the last decade. We have only covered a few promising modalities for 3D cell biology here, but there are others which are also able to give super-resolution images of biological processes in 3D⁶⁹, such as stimulated emission-depletion microscopy (STED), which are well covered elsewhere^{70, 71}. As more of these modalities become commercially available, this will expand the imaging toolbox for a much wider audience of biologists (Table 1). As the audience broadens, more robust reagents and components will inevitably follow. However, each imaging modality has relative strengths and weaknesses that need to be taken into account for a given biological problem; as with any technology, there is no single tool for all jobs. As in the past, the best results will be obtained when the imaging modality is ideally matched to the biological question at hand.

Glossary

2PM	Two-photon microscopy. Utilizes the near simultaneous absorption of two low-energy photons by a fluorophore, resulting in a fluorescence intensity that depends quadratically on the excitation intensity. Often employs a femtosecond pulsed laser as an illumination source. The wavelength of the two photons is roughly twice that of single-photon absorption/excitation, but can occur over a range of wavelengths. See ⁷² for an excellent discussion of two-photon absorption.
4Pi	A microscopy technique where diametrically opposed objective lenses are focused on the same sample plane. It is called 4 η or 4Pi because unlike the partially spherical (solid angle of 2 η) wavefront collected from a single objective, two opposing objectives can capture a nearly completely spherical wavefront of a solid angle of 4 η . Likewise, if coherent excitation beams are delivered through the opposing lenses so that they constructively interfere at the focal plane, axial resolution may be improved 3 to 7-fold relative to microscopes with single objectives.
CCD	Charge-coupled device. Uses the photoelectric effect to generate electrons from photons that strike a layer of photoactive silicon, and transfers them to a capacitor array to comprise pixels, which are ‘read out’ in lines. Since the array is two-dimensional, a wide-field image plane can be captured rapidly (10-20 MHz). The pixel size of most current CCD cameras varies between 3 μ and 20 μ . EM-CCDs are electron multiplying CCDs which have on-chip signal amplification that effectively eliminates the effect of read noise, and better sensitivity than conventional CCDs.
CLSM	Confocal laser scanning microscopy. Uses a single diffraction-limited spot scanned in X and Y dimensions to excite fluorophores, and an adjustable pinhole to block out-of-focus fluorescent emission from fluorophores above and below the plane of focus.
CMOS	Complementary metal-oxide-semiconductor technology, used in wide-field imaging detectors. Offers much faster read-out than CCD-based imaging devices, but typically at a lower quantum efficiency, greater read-noise, and higher pixel-to-pixel nonuniformity.
iPALM	Interferometric photoactivated localization microscopy. Uses a 4Pi geometry and single-photon interferometry for enhanced signal

	collection and better axial localization precision than single-objective-based PALM or STORM.
Isotropic resolution	The resolution of a microscope system with a PSF that has the same extent in all three dimensions.
LSFM (SPIM, DSLM)	Light-sheet-based fluorescence microscopy (including selective plane illumination microscopy and digital scanned laser light sheet fluorescence microscopy). Uses ‘sheets’ of excitation light delivered to the sample in an axis orthogonal to the imaging axis.
PA-FP	Photoactivatable fluorescent proteins. Photoactivation generally occurs by absorption of short wavelength light by a fluorescent protein, which allows the otherwise poorly excited fluorescent species to convert to an efficiently excited fluorescent conformation. ‘Photoconvertible’ proteins are similar, but shift their emission wavelength upon conversion with short-wavelength light. In the text, we use PA-FP to refer both to photoconvertible and photoactivatable proteins.
PALM	Photoactivated localization microscopy. Photoactivation is used to repetitively isolate sparse subsets of molecules that are separated by distances larger than the diffraction limit; subsequent localization of each fluorescent emission results in a super-resolution image.
Photobleaching	The irreversible quenching of fluorescence, often due to the reaction between the excited fluorophore and oxygen. Such a reaction may also release reactive oxygen species, which can lead to further damage to other molecules in the vicinity of the dye. PMT : Photomultiplier tube. A light detector that uses the photoelectric effect to generate voltage from a detected photon, and then multiplies this voltage as much as 10^8 -fold. Used as a ‘point-detector’ for collecting photons from a single spatial location. In their standard configuration in CLSM microscopes, they have relatively low QE (10-15%), but are very fast. PMTs can be run in ‘photon counting mode’, which can raise the QE substantially, but this requires voltage resets after each detection event, decreasing the speed dramatically.
PSF	Point spread function. The three-dimensional diffraction pattern of a point source viewed through an objective lens.
QE	Quantum efficiency. The probability that photons interacting with a detector generate electrons. QE is wavelength-dependent, and can vary greatly for a given detector at different wavelengths. Current CCD cameras usually are most efficient (~60 - 90%) between 450 nm and 650 nm, but drop off substantially over 700 nm.
SDCM	Spinning-disk confocal microscopy. Uses an array of pinholes on a rapidly spinning disk that revolves on a much faster timescale than the image acquisition rate, parallelizing confocal imaging and enabling optical sectioning at a much faster rate than CLSM.
SIM	Structured illumination microscopy. Uses structured excitation light to generate Moire fringes between the object and illumination pattern, thus enabling otherwise uncaptured high-frequency information to be

	collected by the objective and computationally resolved in Fourier space.
SNR	Signal-to-noise ratio. Here, we use this term to mean the ratio of fluorescent signal originating from a region of interest to the total noise (resulting from Poisson statistics and imaging system/detector).
Spherical aberration	The differential focusing of light along the optical axis of a lens, such that rays parallel to the optical axis but at different radial distances from the axis do not form a perfect focal point in space. Present to some extent with all spherical lenses, but is worsened when the index of refraction changes between lens, immersion media, and sample.
STORM	Stochastic optical reconstruction microscopy. Like PALM, uses repetitive cycles of activation and localization to assemble a super-resolution image.

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Box 1**Comparison of 3D images of a point source with different imaging modalities**

A 3D image of a diffraction-limited point spread function (applies to wide-field epifluorescence, confocal, two-photon, and light-sheet microscopies). Once excited, a fluorophore behaves as a point source of light. When collected by a microscope, the spread of the light waves from the point forms a diffraction pattern (Figure 1A). At high magnification in the image plane, the pattern has a central spot, the Airy disk, and diffraction rings around it. The size of the Airy disk is given by the equation

$$d = 1.22 \lambda / \text{NA}$$

where d is the diameter of the Airy disk, λ is the wavelength of light, and NA is the numerical aperture of the objective lens (which is determined by the index of refraction of the immersion medium and the maximum angle of light cone that can enter the lens). Thus, the higher the NA, the smaller the Airy disk becomes. The size of this Airy disk (defined as the distance between first intensity minima) limits the ability of the microscope system to resolve two point sources emitting at the same time.

Above and below the focal plane, the light spreads out from the point source, and the extent of this in 3D is known as the Point Spread Function (PSF). When viewed perpendicular to the axis of imaging (in an XZ plane), it has a longer distribution than it does laterally (Figure 1B). An important aspect of this is that the immersion medium in which the objective lens contacts the specimen, as well as the specimen itself, both contribute to the optics of the system. Due to differences in refraction index of the immersion medium with that of the sample, increased spherical aberration is created as one goes deeper into samples. This causes the effective PSF (and hence the light pattern produced by it) to be asymmetric in Z and the Z-stretch to become larger (Figure 1C).

Figure 1. The effect of diffraction on imaging in 3D

(A) An idealized diffraction pattern of a point source observed in a microscope. The inner spot is the so-called Airy disk. (B) A slice in the XZ plane from a three dimensional image stack of a fluorescent collected in an aqueous medium, centered at 5 μm from the coverslip. Note side flanges on the distribution are symmetric in X and in Z. (C) A similar XZ-plane to that shown in (B), but taken from a three dimensional image stack of a fluorescent bead collected in a collagen gel at 150 μm from the coverslip. Note elongated distribution and asymmetry in the Z axis. (D) Effective PSFs of various microscopy methods, in lateral dimensions (X-Y, top) and axial dimensions (X-Z, bottom). The observed PSF of each is drawn to scale, scale bar = 0.25 μm. For super-resolution techniques, practical 'resolution volumes' are shown instead, since these techniques are not limited by the diffraction-limited PSF of the microscope system.

Figure 2. Optical paths of 3D microscopy modes

In all panels, illumination light is green, dichromatic mirror is purple, specimen is gray, sample excitation is red, in-focus fluorescence emission is orange, lenses are blue, detector is yellow. (A) Confocal laser scanning microscopy light path. Excitation laser light is focused through the objective lens to a diffraction-limited spot in the focal plane of the sample. Note that fluorescence is excited above and below the focal plane. Out-of-focus fluorescence (pink) is rejected by the pinhole, while in-focus light is allowed through the pinhole (black) and collected by the photomultiplier (PMT). (B) Two-photon microscopy light path. Infrared pulsed-laser illumination is focused to a diffraction-limited volume such that two-photon excitation is confined to this small volume (red). Since all fluorescence that is created by this excitation originates in the focal plane, no pinhole is needed, and all fluorescence captured by the objective lens is focused to the point source detector (PMT). (C) Structured illumination microscopy. Laser illumination is collimated onto a phase grating, creating three alternate phases that are recombined through the objective lens into the sample where they interfere to create 3D patterned illumination. Because excitation over all collected phases is essentially equivalent to wide-field excitation the entire axial volume is excited. Resulting fluorescence from each phase collection is reflected by the dichroic mirror and detected by a CCD (D) Spinning disk confocal microscopy light path. Laser excitation light passes through dual pinhole-array disks (black) that scan the excitation across the specimen by spinning at high speed. The fluorescence from points in the focal plane pass through the proximal disc pinholes, and are reflected by a dichroic mirror to an array detector such as a charge-coupled device (CCD) camera. For clarity, only four pinhole

paths are shown. The sum of all scanning pinhole illumination over time excites the full axial volume of the sample. (E)

Light-sheet fluorescence microscopy light path. Excitation light is focused into a sheet that is delivered to the specimen orthogonally to the axis of imaging. Thus, only the plane of focus is excited. Since all fluorescence is from the focal plane, no pinhole is needed, and the field of fluorescence is collected by an array detector, such as a CCD camera.

Figure 3.

Photoactivated point source localization. In PALM and STORM, cells express proteins tagged with photoactivatable or photoconvertible fluorophores (green), random subsets of which are converted to fluoresce (red). Each of these single molecule point sources creates an Airy disk-like distribution of fluorescence at the detector, and the center of each such distribution is found computationally (red points in cell image on right). Since the molecules in the random subset are separated in space, overlap of the fluorescent emissions does not occur. Each fluorophore is imaged until it bleaches (grey), and then a new round of photoconversion and excitation occurs. As the cycles continue, point sources are imaged, localized, and bleached to build a dense localization map (bottom right).

Figure 4. 3D super-resolution microscopy approaches

(A) 3D-PALM/STORM. Small subsets of molecules are activated to fluoresce, isolated in space and time enough that they can be captured as distinct point sources. A weak cylindrical lens is inserted into the optical path (dark blue), which creates astigmatism such that point sources in the center focal plane (orange) appear laterally symmetric, while point sources at other Z-planes (pink) are distorted in either the X or Y direction (depending on depth), to appear elliptical on the CCD detector. Using known calibration curves of point sources enables Z-localization below the diffraction-limited resolution based on the shapes of the point sources observed in the image. (B) iPALM. Opposed high numerical aperture objective lenses are used to collect fluorescent light waves from photoactivated fluorescent point sources. Since a given fluorophore emits in all directions, both objectives capture the fluorescence, but the difference in the path traveled by each portion of the emitted fluorescence wave depend on the Z-plane of the original point source. The collected fluorescence is recombined with a three-way beam splitter, which causes self-interference of each fluorescent photon. Such interference creates different intensities in the three output beams from the beam splitter, which vary according to the pathlength differences in the axial direction. Measurement of the relative intensities across the three detectors enables axial resolution better than 20 nm. (B) was reprinted with permission from ⁴⁷.

Table 1

Practical comparison of several 3D microscopy modes

Mode	Best suited applications	Advantages for 3D Biology	Limitations	Practical Imaging Depth (μ)	Resolution	Commercially Available	References
CLSM *	Cell structure analyses <i>in vitro</i> and <i>in vivo</i> with bright, fixed samples, live cell FRAP* analysis	Widely available, 2D ROI* photo-manipulation, good axial sectioning performance	High photobleaching and photodamage Slow speed in 4D, poor SNR*	200	250 nm XY, >600 nm Z	Yes	4
2PM *	intravital imaging, SHG* imaging of collagen & fluorescence together, bright samples	3D ROI photo-manipulation, SHG*, deepest sample penetration	Nonlinear photodamage Slow speed in 4D, pulsed lasers expensive, poor SNR*	600-800	250 nm XY, >600 nm Z	Yes	14, 15, 72-74
SDCM *	High resolution live cell and organelle dynamics, cytoskeletal and membrane dynamics, dim samples (FSM*)	Widely available, high speed, good SNR* with good CCD* camera, high sensitivity	Pinhole cross-talk, no ROI* control No low magnification imaging	150	250 nm XY, >600 nm Z	Yes	53, 56, 75-77
LSFM *	Whole cell dynamics during embryo development or other long time scale dynamics, cell surface dynamics with Bessel beam, e.g. filopodial dynamics on single cells	Very high speed, very low photodamage, good SNR*, higher resolution possible combined w/ 2P, Bessel, SI*, or DLSM* excitation	No high NA* objectives, poor light collection, lower resolution, sample mounting issues	200-500	500 nm XY, 1-3 μm Z; 300 nm X,Y,Z for Bessel	No	24, 59, 62, 64, 66, 67
3D SIM *	Macromolecular assemblies and organelles with relatively slow dynamics, e.g. chromatin remodeling. Low scattering samples best.	Resolution ~2x better than diffraction-limited, light efficient	Slow speed, photodamage possible, limited imaging depth, multiple raw images needed per 3D-SIM* image, shot noise not removed, complex optical configuration	20-40	100 nm XY, 250 nm Z, 100 nm XYZ in with 3DSIM (4η configuration)	Yes	6, 28, 29, 33, 35
3D STORM* / 3D PALM	Macromolecular assemblies and organelles in fixed difficult to image by diffraction limited imaging, e.g., microbial structure, membranous vesicles.	Resolution ~10x better than diffraction limited, amenable to whole cell imaging, simple optical configuration	Limited imaging depth, slow speed, reducing out-of-focus activation requires either TPM* or light sheet illumination, limited subset of dyes/PA-FPs*, mostly fixed cells	10	20-40 nm XY, 40-80 nm Z	Yes	25, 37, 38, 42, 43, 45, 46, 50, 78-80
iPALM *	Cell surface-associated macromolecular assemblies and organelles with complex structure in fixed samples; e.g., membrane-bound protein complexes; e.g., adhesion organelles, immune synapses.	Ultra-high (< 20 nm) resolution in 3D, possible to determine protein orientation or conformational change.	Very limited imaging depth, slow speed, limited subset of dyes/PA-FPs*, fixed samples only, complex optical configuration	0.5	10-20 nm XY, 5-10 nm Z	No	47, 48

* Abbreviations: CLSM: confocal laser scanning microscopy; FRAP: fluorescence recovery after photobleaching; ROI: region of interest; SNR: signal-to-noise ratio; TPM: two-photon microscopy; SHG: second harmonic generation; FPs: fluorescent proteins; SDCM: spinning disk confocal microscopy; FSM: fluorescent speckle microscopy; CCD: charge-coupled device; LSFM: light sheet fluorescence microscopy; SI: structured illumination microscopy; DLSM: dynamic light scattering microscopy; NA: numerical aperture; 3D-SIM: three dimensional structured illumination microscopy; 3D STORM:

three dimensional stochastic optical reconstruction microscopy; 3D PALM; three dimensional photoactivation localization microscopy; PA-FPs; photoactivatable fluorescent proteins; iPALM; interferometric photoactivation localization microscopy.