

Developments in 3D microscopy

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A variety of alternative approaches to 3D microscopic imaging can overcome various shortcomings and expand the range of areas and applications.

Even a single simple lens can be used to obtain 3D imaging, with different planes of the object imaged at discrete distances from the lens. Of course, as James Maxwell demonstrated in the 19th century, no optical system can produce a geometrically perfect 3D image of a 3D object. In practical microscopy, however, this is not a limitation. We scan axially to bring each plane of the object successively into the focal plane. Thus, an aberration-free 3D imaging system is in principle achievable.

There are two distinct ways of generating 3D images. In tomography we use an optical system with a large depth of field (DOF) to record projections of the object at different angles. From these we can calculate sections. An alternative approach employs a system that has an 'optical sectioning' property, with a small DOF, to image a series of sections. We can then calculate projections at different angles. Optical sectioning has the additional advantage of rejecting stray scattered light that originates mainly from outside the section. This 'gating' approach allows imaging through scattering media such as biological tissue.

One popular way to obtain optical sectioning is through use of the confocal gate in confocal microscopy (CM). The object is illuminated with a focused laser spot, and the light from the object is refocused to a small pinhole placed in front of the light detector.¹ The thickness of this optical section can be submicron if an objective of high numerical aperture is used. Its height, when profiling a surface, can be located within nanometers, and inasmuch as two parallel interfaces cannot be resolved to this level, we rather refer to this as precision. CMs can be constructed with a reflectance mode for a variety of industrial metrology applications, or for imaging through scattering tissue for medical diagnosis. However, biomedical applications are more often performed in a fluorescent mode, using either autofluorescence or labeling with a fluorescent dye.

The basic principle of confocal imaging has been reinvented many times in different areas and applications.²⁻⁵ CM uses a single illuminating spot, which is a major limitation for live cell imaging. Fluorescence, however, suffers from saturation such

that if we want to image quickly, the strategy of increasing laser power does not result in proportionally more fluorescent photons. As a consequence, the solution to imaging at near TV rates is illumination with a multitude of spots. These may be arranged geometrically, either in the form of line illumination⁶ or, alternatively, as an array of spots, as in the spinning disc microscope.⁷ Although both approaches can dramatically improve imaging speed, they suffer from cross-talk between adjacent illumination and detection channels. For line illumination, this results in an axial response that decays as $1/z$, rather than the $1/z^2$ of the point illumination microscope.⁸ For the disc scanning microscope, the axial response decays to a constant nonzero value.⁸ In both cases this limits the imaging of thick objects.

CM and array illumination, as with the spinning disc microscope, are both examples of structured illumination microscopy (SIM). Fringe projection is another example. The basic principle of SIM was first described by Lukosz.⁹ Modulating the object with a fringe pattern shifts the spatial frequencies in Fourier space so that some frequencies, normally outside the system's passband, are transmitted. Lukosz employed a grating to demodulate the signal, but digital techniques are also used. The grating can be oriented in the longitudinal direction, which has the effect of improving the axial resolution.^{10,11} If the grating is oriented in a transverse direction, the transverse spatial frequency cutoff is increased, eliminating the missing cone.¹²⁻¹⁴ SIM has the advantages over CM of being potentially simpler and cheaper. It is also fast because it records complete 2D fields and can achieve superior resolution.

Another approach to 3D imaging is to use interferometry or holography. Illumination of the object by a plane wave gives limited 3D information, but the use of low spatial coherence effects a correlation that results in optical sectioning (correlation gate) analogous to that found in CM. Interference microscopy can also be performed in a confocal mode.^{15,16} Optical sectioning in this case can be achieved by using a source of low temporal coherence (coherence gate). Interference occurs only for the parts of the object at a distance such that the optical path length in the two arms of the interferometer are equal within the coherence length.¹⁷⁻¹⁹ Optical coherence tomography (OCT)¹⁹ uses a confocal interference system with coherence gating. If a lens of high

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numerical aperture is employed, a correlation or confocal gating effect is superposed on the coherence gating effect. Known as optical coherence microscopy (OCM),²⁰ it offers improved optical sectioning and improved detection efficiency.

A variety of imaging modes based on nonlinear optics also have been proposed.²¹ In two-photon microscopy, the signal is proportional to the square of the illumination intensity, providing optical sectioning without the need for a pinhole.^{22,23} There are two different forms of two-photon microscopy. Second harmonic generation (SHG) microscopy relies on SHG of photons at twice the frequency of the illuminating beam.²² Only materials that lack central symmetry can give SHG, with collagen an important example.

Two-photon fluorescence microscopy (TPFM) uses two-photon excitation.^{21,23} Photobleaching occurs only in the plane of focus, greatly reducing bleaching effects during the recording of a 3D data set. This technique is performed near the infrared spectrum, and it is not necessary to image the fluorescent light, only to detect it. A major advantage of TPFM is thus its penetration into thick tissue. A disadvantage is that it requires an expensive pulsed laser.

CM will probably continue to be widely used across a range of different application areas. But for deep imaging of tissue, two-photon microscopy has distinct advantages. We would expect its use to increase greatly once cheaper light sources become available. For in vivo imaging at high speed, structured illumination of some type is preferable, using fringe projection or array illumination.²⁴

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