Twenty-five years of confocal microscopy

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Introduction

This article was commissioned to describe the challenges that have been faced in the development of confocal microscopy and how they have been, and still are being overcome. The following is necessarily a personal account and will inevitably omit some important developments, but in order to put the discussion in context it’s important to remember why the confocal microscope has achieved its current prominence in light microscopy.

The basic problem in conventional optical microscopy has always been that the image produced by a fluorescence microscope, say, contains clear, in-focus information, from those parts of the specimen which lie close to the focal plane of the microscope, together with unwanted blur arising from other portions of the specimen lying in nearby but necessarily out-of-focus planes. It is the presence of this unwanted blur that degrades the contrast and ultimately the resolution of the image. Further, it means that a three-dimensional rendering of the specimen cannot be achieved. The critical advantage which the optical architecture of the confocal microscope provides is that it is able to reject, or greatly attenuate, the unwanted out-of-focus information and so reveal a sharp, in-focus, high-contrast image of a thin section of the specimen. In essence this is all the instrument does. What the confocal microscope does not do, indeed fundamentally cannot do, is to image structures in three dimensions. It does the absolute opposite. A well-designed confocal microscope is able to produce a high-resolution image of a very thin section within a thick specimen. In order to image the whole of a thick object it is necessary to generate a through-focus series of such optically sectioned images. It is then a matter of computer processing to decide how this set of two-dimensional thin (optical) sections should be combined.

The traditional explanation of the origin of optical sectioning is shown in Figure 1. The architecture of the confocal optical system is such that it is relatively insensitive to signals originating from features lying in out-of-focus planes. The strength with which a specimen feature is imaged depends on its position with respect to the focal plane. However it is also clear that the system of Figure 1 does not produce an image of the specimen. In order to produce an image it is, of course, necessary to probe many points in the specimen. In other words we must scan the focused spot with respect to the specimen. Scanning is necessary in both the lateral (x-y) plane in order to record the optical section and also in the axial direction so as to be able to record a through-focus series. The figure here has simply indicated that an illumination pinhole must be used. In practice this means a laser must be used since other light sources have traditionally been insufficiently bright.

How to scan in x and y? In the early days, the groups in Oxford, Stanford (Sheppard, 1984; Pawley, 1995; Wilson, 2011) and the reader might reasonably wonder why it is still necessary to go through this tedious process of recording through-focus series of images in order to achieve our goal. Why can’t we devise an optical system that will provide such three-dimensional renditions directly? As so often in human endeavour, we want it all.

Figure 1

The confocal optical system showing that light originating from object features lying in the focal plane is focused on the pinhole. Almost all of this light passes through the pinhole and reaches the photodetector. Light from regions of the object lying in planes away from focus arrives as a defocused spot at the pinhole. Much of this light is physically blocked by the pinhole and so is prevented from reaching the photodetector, which consequently measures a greatly reduced image signal for specimen features lying in this defocused plane.

Figure 2

A sketch of a very early Oxford scanning stage.
**Is there an easier way?**

The goal, therefore, is to devise an optical system that has the ability to produce a perfect magnified image of a thick object. The question of perfect imaging – and how it might be achieved – has unsurprisingly been a subject of interest for many years. Criteria have been devised which permit the design of optical systems to produce perfect images of subsets of objects that might be contained within an arbitrary specimen. For example, if we wish to produce a perfect image of two point objects lying close to the optic axis in a plane perpendicular to the axis, then the optical system must be designed according to the sine condition. Indeed, modern microscope objectives are usually manufactured so as to satisfy this requirement. On the other hand, a system having the ability to form a perfect image of two points slightly separated axially along the optic axis must be designed according to the Hirschel condition. Further, the ability to image two axially separated planes perfectly must obey the Maxwell conditions. And so it goes on. Unfortunately it turns out that these various conditions cannot all be met simultaneously except for the very special case when unity magnification is acceptable.  

Unity magnification, of course, is something of an anathema in microscopy where magnification (along with contrast and resolution) is a sine qua non. The requirement for magnification – which fundamentally prevents perfect imaging – has meant that alternative methods have to be employed.

What might be the options? Tomography is one possibility whereby an optical system with a large depth of field is used to record projections of the object at many different angles. Approaches based on geometrical optics as well as diffraction may then be used to calculate the images of sections through the object. These methods, although promising and important in certain areas, have not captured the general imagination, and indirect methods of obtaining three-dimensional representations of thick specimens have found favour by devising systems that possess optical sectioning. The design goals here are to design optical systems that produce as thin an optical section as possible as quickly as possible. The ability to tune the optical section thickness is also desirable. This led to the development of confocal systems employing pinholes of continuously variable diameters. The use of slit-shaped limiting apertures rather than circular pinholes was also considered, although the optical section width proved to be broader in slit-detector systems than with pinhole, point-detector approaches.

**Spinning Discs**

A further requirement was for speed. Imaging of living samples adds an additional layer of difficulty as these samples may also be moving rapidly. This means the optical sections have to be acquired at frame rates high enough to completely resolve the dynamics of the process to be analysed. One of the very early forms of confocal microscope was constructed by Mojmir Petran and Milan Hadravsky (Figure 3a). Instead of building an instrument with one source pinhole and one detector pinhole, they built an instrument containing a large number of source/detector pinhole combinations, arranged on a disc so as to probe neighbouring parts of the specimen [Petran et al., 1968]. In this reciprocal geometry the same pinhole was used as both source and detector. However, in order to prevent defocused light from one confocal system being detected by a neighbouring system, it was necessary to place the pinholes far apart – typically by ten pinhole diameters. This resulted in a low light-budget instrument together with sparse probing of the specimen. In order to probe the entire specimen, the pinholes were arranged on an Archimedian spiral and the disc rotated to cause the entire specimen to be imaged. In this way this real-time confocal system was able to acquire images at high speed as well as to image large, thick samples in vivo (Figure 3c).

It might be mentioned that when Petran and Hadravsky built their first microscope in late October 1965, the weather in Plzen was rather cloudy, so they took the microscope to the top of the Tatra mountains, 2632 meters above sea level, and used the sun as a light source. Today arc lamps and lasers are typically employed and other techniques such as the use of microlens arrays have been used by the Yokogawa company [Ichi-hara, 1996]. Reflective arrays are used by Till Photonics so as to ensure that a significant amount of the available illumination light is used to excite fluorescence in the specimen. In a further attempt to improve the light-budget, a different approach...
Figure 4
(a) A through-focus series of images taken of the eye of a fly.
(b) A three-dimensional rendering of the eye of the fly constructed from the through-focus series of images presented in (a).

Contrast
The discussion up to now has been concerned with the development of optically-sectioning microscopes based around the confocal (point source/point detector) optical architecture. We have implicitly assumed that the contrast mechanism was either reflection from the specimen or the excitation of (single-photon) fluorescence within the specimen. If we concentrate on the fluorescence case, we find that the image formation, in the ideal confocal case, may be described by an effective intensity point spread function (PSF) that – if we ignore the Stokes shift between the excitation and fluorescence – is given by the square of the PSF of the conventional microscope. This observation also explains the (slightly) higher lateral resolution of the confocal microscope. However, other contrast mechanisms have proved to be very useful. In particular, two-photon fluorescence [Denk et al., 1990] has proved to be a rich vein to explore. The contrast mechanism here is such that fluorescence is generated in proportion to the square of the incident intensity and hence is generated efficiently only in the region around the focus where the excitation has been taken [Juskaitis et al., 1996]. The reasoning here was that in order to improve the light-budget, it is necessary to place the neighbouring confocal systems as close together as possible. The challenge then is to find a method of preventing cross-talk between other neighbouring channels. The solution was to use aperture correlation. The Nipkow disc of the tandem scanning microscope (Figure 3a) was replaced with an aperture mask consisting of many pinholes placed as close together as possible. This aperture mask has the property that any of its pinholes can be opened and closed independently of the others in any desired time sequence. This might be achieved, for example, by using a liquid crystal spatial light modulator. Since we require that there be no cross-talk between the many parallel confocal systems, it is necessary to use a sequence of openings and closings of each pinhole which is completely uncorrelated with the openings and closings of all the other pinholes. As an alternative to a liquid crystal spatial light modulator, the correlation codes may be impressed photolithographically onto a disc that may be rotated so as to effect the required time-dependent correlation. Figure 4 shows a set of images from such an instrument.

Figure 5
(a) THG images of a 5.5-day-old live mouse embryo with correction of only system aberrations (left) and after additional correction of specimen-induced aberrations (right). The dashed lines show where the x-y and x-z planes intersect.
(b) Intensity profiles along the solid lines A–B and C–D as drawn in (a).
beam is tightly confined. This leads to an intrinsic optical sectioning property without the need for a confocal pinhole. Formally, the effective point spread function in this case is given by the square of the excitation intensity PSF. In this sense the imaging is identical to that found in a confocal microscope, but we must remember that the PSF in the two-photon case must be evaluated at the excitation wavelength, which is typically twice that of the single-photon case, and leads to resolution twice as poor as in the single-photon case. In spite of this, there are tremendous advantages to the two-photon approach which are, by now, well known. Other contrast mechanisms have also started to find favour, including those based on second- and third-harmonic generation [Barad et al., 1997; Yelin and Silberberg, 1999]. Third-harmonic imaging is particularly appealing since the contrast mechanism is both intrinsic – no label is required – and parametric in the sense that, unlike the two-photon case, energy is not dissipated in the specimen and hence is an attractive candidate for live cell imaging [see, e.g. Jesacher, 2009] (Figure 5).

A problem encountered in all forms of optical-sectioning microscopy is that one has to focus deep within the specimen in order to record a through-focus series of optical sections. This causes two immediate problems. The first is that one is using the objective lens in a regime for which it is not designed – objective lenses are usually designed to image well just below the coverslip – and the second is that there is often a refractive-index mismatch between the immersion fluid and the specimen. Both effects, together with any optical system imperfections, lead to an aberrated focal spot together with the inevitable accompanying loss of resolution and signal strength. These effects are arguably more pronounced when the contrast mechanism relies on a tightly focused focal spot as in the case of multi-photon or higher-harmonic imaging. In these cases adaptive optics techniques may be employed in such a way as to pre-condition the imaging beam – often using a deformable mirror – such that its phase front is equal and opposite in shape to that caused by focusing deep into the specimen. In this way diffraction-limited performance can, in principle, be restored. Figure 5 shows the effective of adaptive correction in the case of third-harmonic generation imaging.

**Is confocal best?**

In our discussion so far we have concentrated on the confocal optical system of Figure 1 as being the system of choice to obtain optical sectioning. But is it optimum? The confocal microscope is a scanning system that requires (single-wavelength) laser illumination and, in many cases, does not provide real-time imaging. Further, such systems are often built around conventional microscopes. In many ways the conventional microscope is the ideal instrument. It produces an image in real time and uses multi-wavelength standard illumination sources. However, as we have said, it possesses one serious drawback. It does not exhibit optical sectioning. A simple modification of the illumination system, together with simple image processing, overcomes this problem. The fundamental observation that leads to a simple method of introducing optical sectioning into the conventional microscope may be seen in Figure 6a, where we show the image of a one-dimensional grid as a function of defocus. As the defocus level increases the grid pattern becomes more and more difficult to see. This suggests another approach to obtain optical sectioning. We modify the illumination system of a conventional microscope so as to project a one-dimensional grid pattern onto the specimen. The image observed in the microscope now consists of the specimen with the grid pattern superimposed. However, since the grid pattern is imaged well only when it lies near the focal, we may use the presence of the grid pattern in the composite image as a label of the region of the specimen lying near the focal plane. In essence the grid pattern delineates the optical section, and the frequency of the grid determines the optical-sectioning strength [Neil et al., 1997; Wilson, 2011]. It is then a matter of computer processing to remove the unwanted grid pattern and reveal the optical section. Figure 6b shows images obtained with such a system (available commercially as Optigrid from Qioptic, while the Zeiss copy is marketed as ApoTome). Finally, we note that the removal of the grid pattern (the demodulation) may also be carried out in an arguably more elegant fashion using an optical equivalent of lock-in detection. Such an instrument, manufactured by Aurox Ltd, is marketed by Zeiss as the VivaTome and by Andor as the Revolution DSD.

The approach we have just described has essentially introduced structure to the optical field that excites the fluorescence and, in the application just described, has introduced optical sectioning to the conventional microscope. However, it is a much more powerful technique than our previous discussion may imply. Modulating the object with a fringe pattern shifts the spatial frequencies in Fourier space so that some spatial frequencies normally outside the system’s passband are trans-
and confocal microscopy have taken resolution out of the optics laboratory – localisation techniques and methods to inhibit super-resolution – the various methods toward super-resolution – the various methods of rapid focusing which permit images to be obtained along arbitrary three-dimensional trajectories. The use of adaptive optics when focusing deep within specimens is mentioned as are new methods of rapid focusing which permit images to be obtained along arbitrary three-dimensional trajectories.

References


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BIOGRAPHY

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ABSTRACT

The review begins by questioning why one cannot directly image thick specimens in three-dimensions at high resolution and hence why indirect methods based on optical sectioning, such as is provided by the confocal microscope, must be employed. A variety of techniques are discussed along with the use of structured illumination both to achieve optical sectioning as well as super-resolution. The use of adaptive optics when focusing deep within specimens is mentioned as are new methods of rapid focusing which permit images to be obtained along arbitrary three-dimensional trajectories.

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Figure 8
Schematic of a scanning laser microscope. The lateral (LSU) and axial (ASU) scan units produce changes in tilt and defocus of the illumination wavefront, which permits a diffraction-limited spot to be positioned at speed at any point within the specimen volume.