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Optical Sectioning and Confocal Microscopy

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Understanding Light Microscopy, First Edition. Jeremy Sanderson.

17.1 Introduction

There are many advantages to using fluorescence microscopy, which is why it is very widely used. Fluorophores are self-luminous, so they exhibit very high contrast; they are very sensitive; only small concentrations are required for successful labelling; they are very specific, discriminating individual proteins, organelles and components of cells very well. Fluorophores can be tolerated by living cells in low concentrations and can be produced endogenously within the cell as fluorescent proteins. Fluorophores also lend themselves to tracking protein diffusion kinetics (e.g. FRAP) and also measuring interactions with other biomolecules through FRET and ratio analysis. Nevertheless, to take maximum advantage of this powerful technique, there are three disadvantages that must be successfully managed:

- blurring;
- bleaching; and
- bleed-through.

Unless the sample is very thin, blurring is arguably the most serious disadvantage that must be addressed. Bleaching is – to a large extent – inevitable but can be managed through careful selection of fluorophores and restricting their exposure to light in a controlled manner. Bleed-through will occur only if the sample is stained with, or inherently possesses, more than one fluorophore. Whether the management of bleaching or bleed-through is more important really depends upon the nature of the specimen and the fluorescent markers used.

This chapter discusses the stratagems and approaches to imaging that are available to reduce blurring in the final image or are required to collect blur-free 2D and 3D image data sets. In order to acquire images that are in focus, some compromises must be made. There are several designs of optical sectioning microscope, and which one is best for your purposes will depend upon the nature of the specimen, most often its thickness, how labile it is and whether it is fixed or living. This chapter explains how the point-scanning confocal and other optical sectioning microscopes work. More information and guidance is also given later in Chapter 27 regarding core imaging facilities and on which microscope is the best choice to use.

The single-beam point-scanning confocal is the most widespread and popular of all the optical sectioning fluorescence microscopes, and several designs are available on the market. At the time of writing, typical microscopes are the Zeiss LSM 810 and the LSM 780, the Olympus FluoView FV-3000, the Nikon C2+ and A1R+/A1+ and the Leica SP8. Both Nikon and Leica also produce confocal macroscopes (the Nikon AZ-C1 and the Leica TCS LSI) for low-magnification examination of thick samples and entire organisms in vivo. Macroscopic optical sectioning can be performed on a widefield macroscope – the Zeiss Axio Zoom V16 macroscope is designed for use with the ApoTome structured illumination optical sectioning device.

Manufacturers patent their unique design features so that although the overall concept and design is identical, instruments from different manufacturers differ in small but sometimes significant respects. Of course, the sales staff of each instrument manufacturer will try to convince you that their particular microscope is best suited for your applications! Take advice from co-workers, read the Confocal Listserver[1] also post questions on it, and balance the information and opposing views that you may receive from different sales staff. As a starting point, refer to Appendix 1 if you intend to purchase a high-end research microscope.

17.2 Blurring and Optical Sectioning

Because, firstly, fluorescent molecules are self-luminous and are present throughout the mass of the stained cell or tissue and because, secondly, the depth of field (the distance between the upper and lower planes of the in-focus region along the optical or z-axis) of a high-quality microscope objective is generally much smaller than the thickness of the cell or tissue, that part of the image which is in focus will be degraded by light that is emitted or scattered by the tissue outside the narrow plane of focus (Figure 17.1). The image of a 'thick' specimen will only be wholly in focus throughout if the thickness of the specimen is less than the depth of field for the objective used. Usually, this is not the case.

If a typical cell or tissue section is, say, 10 µm thick and the depth of field of the microscope objective is 1 µm, then 90% of the image seen will be blurred because most of the light is contributed by regions that are not exactly in focus. The contribution of the blurred background light – from the out-of-focus regions – is superimposed over the weaker (less-intense) in-focus image. This will reduce the signal-to-noise (SNR) ratio, and thus the contrast, of the image. This out-of-focus haze will also reduce the resolution of detail in the image.

We can view the entire field of view at once, as we do in bright-field, DIC or phase-contrast microscopy and hence also see the out-of-focus information axially in z, but this does not help with depth discrimination of fluorescently stained cells and tissues, unless they are both thin and also flat. This situation is very restrictive. When cells divide (even those that spend much of the cell cycle in a flattened state) they tend to compress and round up during cell division. For those who wished to study such a fundamental process, this posed a problem, hence the practical development of the laser-scanning confocal microscope. Blurring can be prevented or eliminated

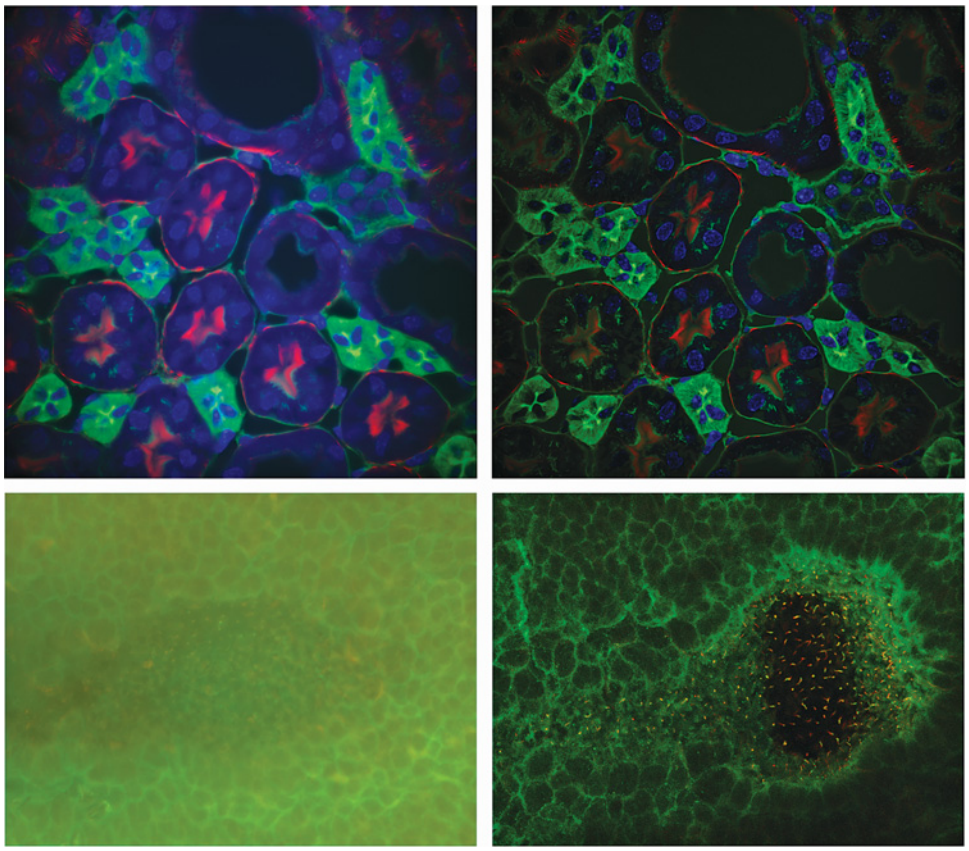


Figure 17.1 ***Why a confocal microscope is used*** *Showing blurring of signal in two different images in widefield microscopy and the result of optical sectioning. In the widefield images (left-hand panels) the in-focus part of the image is degraded by light emitted or scattered by the tissue outside the narrow plane of focus. The specimen in the top row is a 16 μm thick triple-stained cryostat section of mouse kidney, which is available (F24630) from Thermo Fisher Scientific and is widely used for confocal microscopy training. The bottom row shows double-stained cilia in the node of an 8.5 dpc mouse embryo. The thickness of the whole mount is about 20 μm, it scatters light very effectively and is therefore ideally suited to optical sectioning. Compare both of these images to Figure 18.5, which shows the respective maximum intensity projection as 2D representations of the 3D Z-stack data sets. Mouse images courtesy of Dr J. Keynton.*

by generating an 'optical section' through the sample, whereby only the image information that lies entirely within the objective's depth of field is displayed. Those parts of the sample that are out of focus are excluded from the image. Consequently the image has increased sharpness, increased contrast and increased resolution in the axial direction. As Inoué (2006) points out, the confocal can either increase or decrease the effective depth of field without loss of resolution.

Besides the confocal microscope – the most commonly used 'optical sectioner' – there have also been other approaches to optical sectioning[2] in order to 'clean up' the fluorescent image. These are listed in Table 17.1. In the point-scanning laser illumination confocal microscope, a single pinhole is used as a spatial filter to isolate the in-focus optical section from the out-of-focus portion of the image. The size of the pinhole determines how much of the Airy disc reaches the detector. In the multiple beam-scanning Nipkow spinning disc confocal microscope, a disc perforated with a series of pinholes of fixed diameter performs the same function. Optical sectioning can also be performed with a widefield microscope using the Optigrid or ApoTome (see Section 17.8). Parallel series of grid lines superimposed over the image act as the required spatial filter.

The ideal fluorescent sample prepared for confocal microscopy should be very well stained, to give a good signal with corresponding low noise. In this respect it is wise to use freshly prepared antibodies conjugated to modern 'second and third generation' fluorophores (e.g. Alexa,Cy cyanine and Atto or DyLight dyes) rather than earlier ones such as fluorescein (FITC) or rhodamine (TRITC).

17.3 Confocal Microscopy

The *confocal* microscope is so-called because the illumination pinhole, the plane of focus within the specimen and the detector pinhole are all situated at *con*jugate *focal* planes. Every laser-scanning confocal (a generic design is shown in Figure 17.2) has the following features in common:

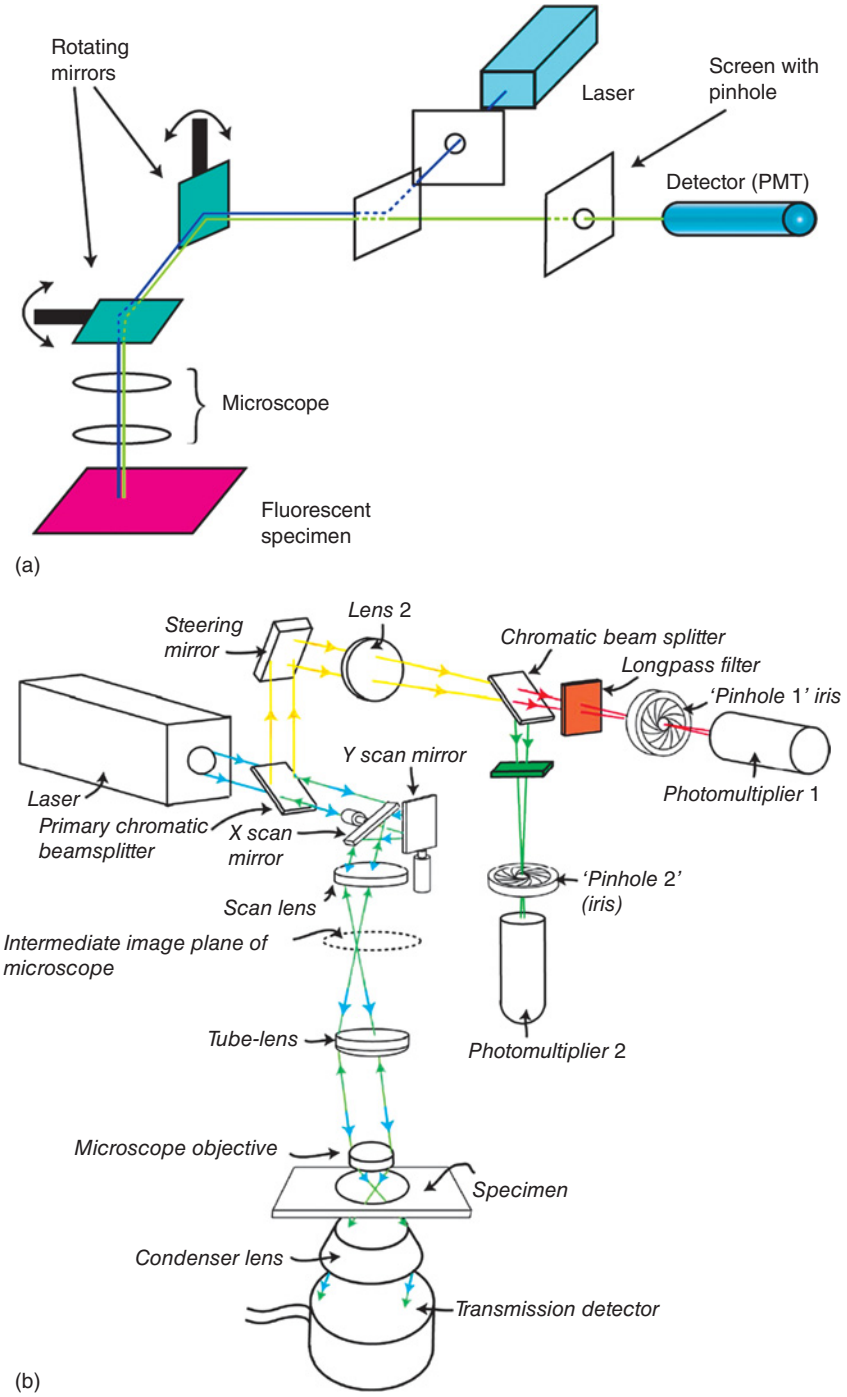


Figure 17.2 ***Generic design of single-beam point-scanning confocal*** *Features of the confocal microscope. The essential components are shown in (a) and are: the laser for illumination, the illumination pinhole in front of the laser, the galvanometer mirrors and the confocal pinhole in front of the detector. The lower panel (b) shows how the actual components are arranged within a confocal point-scanning microscope. Ideally an iris diaphragm comprises the pinhole, but it may in some designs be as simple as two sliding triangular cut-outs forming a diamond shape, which approximates a 'pinhole'. Source: (a) Semwogere and Weeks 2005. Reproduced with permission from Taylor & Francis. (b) Amos et al. 2012. Reproduced with permission from Elsevier.*

- one or more gas or diode laser(s) providing illumination of specific wavelengths;
- fluorescence filters and a dichromatic beam-splitting mirror as a filter set to direct the illumination onto the sample and direct the emitted fluorescence of specific bandwidth towards the detector;
- a mirror and galvanometer-based raster scanning mechanism;
- one, or more, pinholes; and
- photomultiplier tube (PMT) detectors for each channel.

Table 17.1 *Optical Sectioning Methods*

Microscopy type	Illumination	Detector/camera	When to use	Advantages	Disadvantages
Point-scanning confocal	Laser	PMT	**With thick tissues**	Variable & precise optical sectioning	Lasers photo-bleach. PMT & circuitry noisier than widefield.
Multi-beam Nipkow spinning disc confocal	Mostly laser (can be white light)	EM-CCD or CCD	**For acquisition speed**	For photo-sensitive living samples. High temporal resolution.	Fixed pinhole: fixed optical section thickness. No optical zoom.
Structured illumination	White light	CCD	**For photo-sensitive samples**	Better than confocal at lower mags.	Ronchi grid can burn into image.
Widefield deconvolution	White light	CCD or sCMOS	**For thin fluorescent cells;** for photo-sensitive samples	Good SNR, less noise than confocals.	Requires post-processing. Sensitive to spherical aberration.
Multi-photon (two-photon)	Pulsed laser	PMT	**For very thick tissues**	For very thick, living tissues.	Expensive. Requires dark for NDD detector.
TIRF	Laser	EM-CCD	**For imaging cell membrane**	Very thin optical section 100–200 nm.	Can only image in the evanescent field.

The merits of each type of optical sectioning microscope are shown in further detail in Tables 27.1 and 27.2 in chapter 27.

For those who prefer a more objective approach as to which optical sectioning microscope to use, John Murray, who has written an excellent chapter (Murray, 2011) on imaging fluorescent specimens, suggests the Haziness index, H. The metric H is the ratio of background to signal in a widefield image. The intensity of out-of-focus background is divided by the intensity of signal from a very small in-focus object. For practical purposes, with $H \leq 20$, widefield with deconvolution is appropriate. Where H lies in the range $20 < H < 200$, spinning disc confocals are best, and where $200 < H < 1000$, point-scanning confocals are the microscope of choice. For values of H above 1000, a multi-photon microscope should be used.

Ultimately, scientists wish to carry out 'in toto' imaging; that is, acquiring images and tracking every single cell movement and division that forms a tissue or organ. This approach is powerful for understanding how cell lineage, shape changes and movements control the morphogenesis of a tissue (Megason, 2010). A suitable objective is a water- or multi-immersion objective with a numerical aperture >1.0, yet with sufficient working distance (200–600 µm) to reach and image through the entire cell layer. Zeiss makes two 40× objectives that meet these criteria – the 40× C-Apochromat NA 1.2 (WD = 290 µm) and the 40× LD C-Apochromat NA 1.1 (WD = 600 µm). Zeiss also manufacture the 25x/NA 0.8 multi-immersion objective, which, with a fully open pinhole, can image a monolayer of adherent cells in an optical slice thicker than the cell layer. Zeiss, Leica and Olympus have all manufactured 20x objectives with NAs of 0.95 to 1.05 and long working distances for multi-photon and cleared tissue imaging.

References

Megason, S.G. (2010). 'In toto imaging of embryogenesis with confocal time-lapse microscopy'. *Methods in Molecular Biology 546*: 317–332.

Murray, J.M. (2011). 'Methods for imaging thick specimens: confocal microscopy, deconvolution, and structured illumination'. *Cold Spring Harbor Protocols 2011*/12: 1399–1437.

Presley, J.F. (2006). 'Measurement of protein motion by photobleaching'. In: Stephens, D. (ed.), *Cell Imaging*, Chapter 6, Protocol 2, note c. Bloxham: Scion Publishing, p. 135.

By restricting the illumination to a pinhole rather than illuminating the entire field of view at once, it becomes possible to build up a blur-free image in three dimensions (Figure 17.3). Point-by-point illumination of a small volume of the sample and corresponding synchronous (identical small volume) point-by-point detection provides good spatial discrimination in all three axes. The PMT does not 'see' the entire image as a CCD detector does but very rapidly produces a voltage equivalent to the photon intensity emitted at each sampling point in the object, which is then digitised to form a corresponding pixel in the image.

The entire image is built up point-by-point in each frame, as the diffraction-limited spot of the illuminating beam rasters across the frame, like reading words in lines along a page of script in a book. Each frame corresponds to a single plane in the z-axis. This may be equivalent to, or thinner than, the depth of field of the objective, but sharp discrimination of detail is provided in the z-axis, as well as laterally in the x and y axes. To continue the analogy, each frame can be regarded as a separate page, distinct from those on either side. By using a stepper motor to drive either the stage or the nosepiece axially, through z, a three-dimensional image, which is in-focus throughout, can be acquired of the specimen.

With only a single point illuminated (rather than the entire field of view as in 'widefield' mode) the illumination intensity rapidly falls off above and below the plane of focus as the beam converges and diverges to a point (Figure 17.4). This reduces excitation of fluorescence of these objects situated out of the focal plane, improving depth discrimination. This provides the necessary selective axial illumination along the z-axis, thus giving a sharp, blur-free result. The confocal microscope differs from widefield fluorescence microscopy in that the microscope configuration for widefield (both bright-field and fluorescence) microscopy is designed to be used with Köhler illumination where the excitation illumination is maximally out-of-focus at the specimen plane (i.e. the lamp filament is not imaged at the specimen plane but at a different conjugate plane: the entire purpose of Köhler illumination). In the confocal microscope the illumination *is* focused onto the specimen plane as a diffraction-limited point.

The Airy disc, named after Sir George Biddell Airy, who studied diffraction patterns and image formation in stars, describes the *central* intensity maximum of the two-dimensional pattern of the point spread function, the PSF. The Airy pattern is the PSF projected onto a flat surface[3]. An example is shown in Figure 17.5. In confocal microscopy the Airy unit is used to set the diameter of the pinhole and the thickness of the optical slice relative to the Airy disc. When the Airy unit (AU) is set equal to 1, only the central intensity maximum of the PSF reaches the PMT; this is optimum.

Since the confocal microscope depends upon a pinhole to exclude out-of-focus light, lasers are used because they have sufficient collimation and power to provide the necessary intensity, after passing through the illumination pinhole, to induce sufficient emission of signal[4] from fluorescent markers, which must then pass through the confocal detection pinhole in front of the detector. Lasers produce very intense, coherent, highly directional light beams of very narrow wavelength (usually of 1 nm bandwidth) that are ideal for illumination in a microscope where 90% or more of the emitted signal may (intentionally, due to the pinhole) not reach the detector. However, the use of lasers as illuminants all too easily causes photobleaching of fluorophores and photo-toxicity of biological tissue,

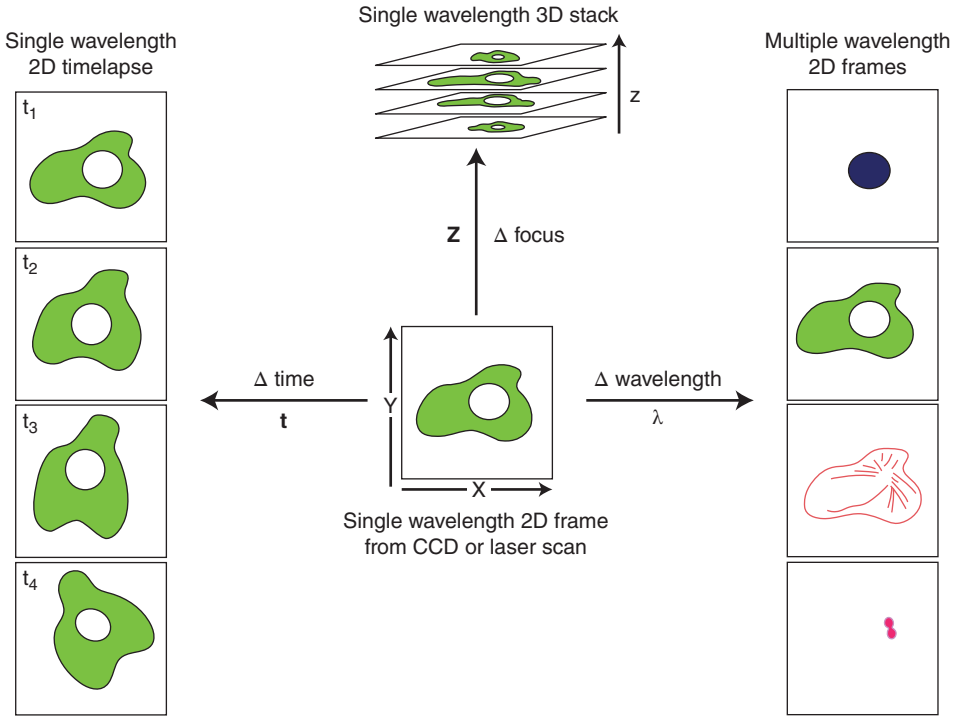


Figure 17.3a ***Image acquisition by confocal*** *Figure 17.3a summarises the image collection possibilities with the confocal microscope. Source: Andrews et al. 2002. Reproduced with permission from John Wiley & Sons.*

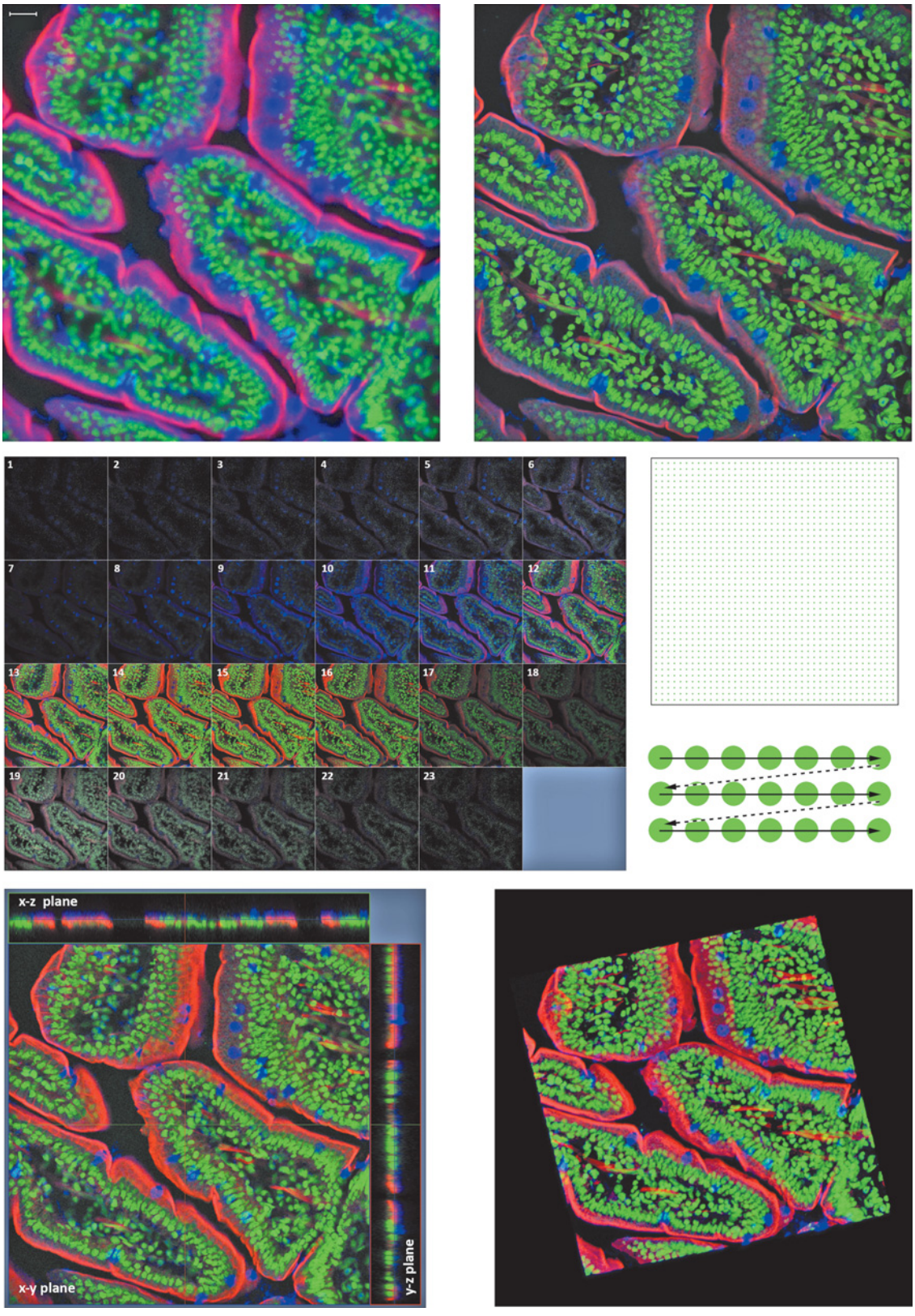


Figure 17.3b ***Pinhole illumination and detection*** *Figure 17.3b shows a 16 μm thick section of triple-stained mouse intestine (Thermo Fisher Scientific F24631) acquired as a widefield image (top left) with consequent blurring, followed by (top right) a single optical section. A series of these optical sections are formed as the laser is rastered in outwards and flyback mode across the field of view, effecting point illumination and point detection. It is possible to collect a second image on the flyback, which increases the collection speed, but the sample must not move, and the scanning mirrors must absolutely be in phase. The Z-stack can be presented as a gallery, orthogonal projection or 3D projected movie.*

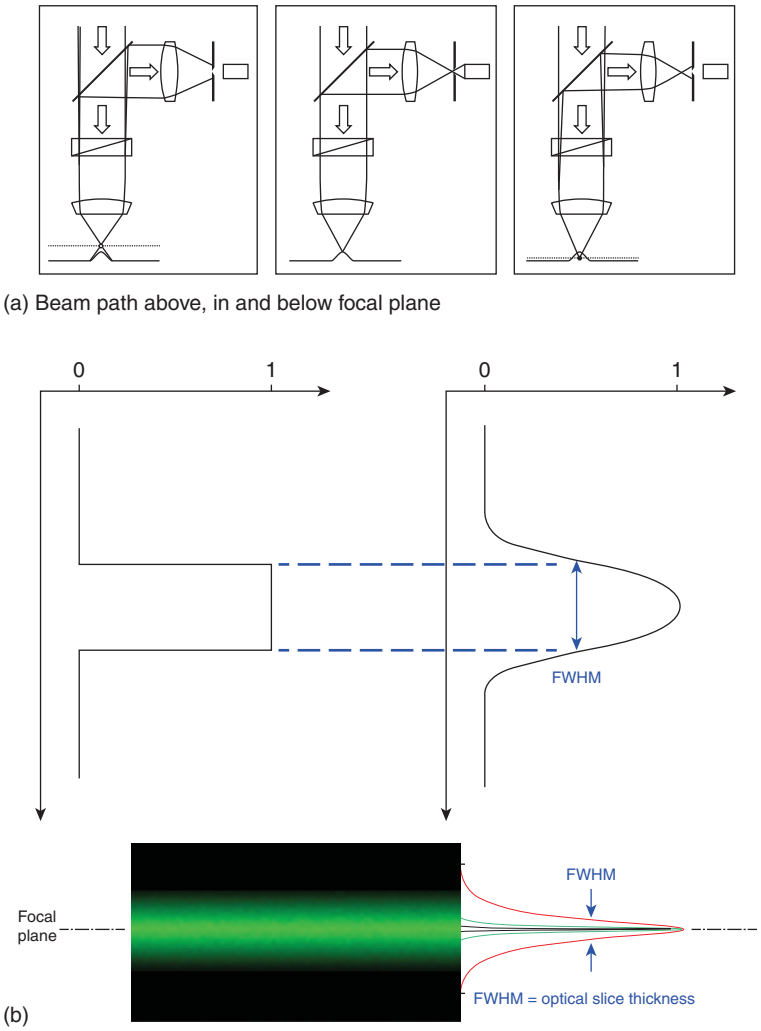


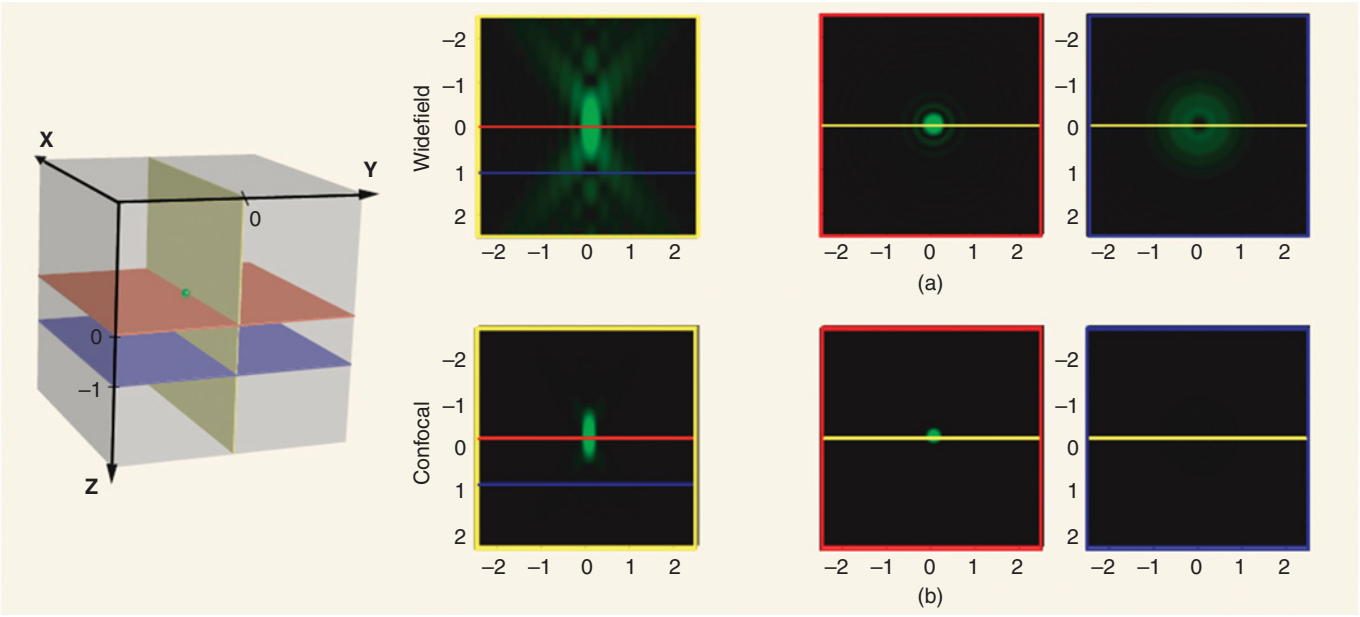
Figure 17.4 ***Illumination intensity fall-off above and below focus*** *Ray diagram showing the intensity fall-off above and below the plane of focus in the confocal microscope as an optical section is collected. Ideally, the optical section would have sharp edges, but this is not the case, and the optical section forms a horizontal Gaussian distribution of fluorescence with the Full Width Half Maximum, a measure of the optical slice set in the software. Source: Adapted from Isenberg 1997 with permission from Springer.*

in particular of living cells. For advice on the safe use of lasers and UK law (Artificial Optical Radiation at Work Regulations 2010) regarding the operation of lasers and intense lamps, see Section 18.2, in the next chapter.

It is essential to be able to adjust the laser intensity so that fluorophores are not saturated and bleached. Lasers are generally used at fractions of a percent of their maximum output power unless, of course, they are intentionally used to bleach the sample. The lasers can be switched very rapidly and adjusted in intensity by the use of an Acousto-Optical Tuneable Filter (AOTF) (Figure 17.6) or neutral-density filters. AOTFs have an advantage over neutral-density filters and are now used exclusively for this purpose, because they allow the intensity of individual laser lines in multi-line lasers to be individually adjusted. AOTFs also permit blanking of the laser beam on the flyback during the raster scan, to reduce exposure. They also allow specific regions of interest to be scanned in the field of view. Furthermore, neutral-density filters tend to permit a significant amount of bleed-through.

Photomultiplier tubes (PMTs) are used as detectors in point-scanning confocal microscopes because these enhance the weak signal and can also very rapidly collect single points of light emitted as signal, whereas using a CCD, suited to widefield microscopy, would be far too slow. PMT technology (Figure 17.7) is dated but still dominates microscopy as the detector of choice at low light levels with a fast response time, despite introducing multiplicative noise.

However, speed in detection comes at the price of efficiency. PMTs acquire the diffraction-limited spot signal at each pixel rather like speed-reading the words on the page and missing several in haste. A PMT detector has a quantum efficiency (QE) of 12–15% at best and so only detects 1 in every 7 or 8 photons of fluorescent signal. By comparison (see Figure 17.8) a charge-coupled device (CCD) camera that is used on a widefield microscope has a QE of 60–70%. Nonetheless, CCDs have lower gain, higher background noise and cannot read the point signal as rapidly as a PMT, because they read out their signal once the entire frame is collected. Signal gains of up to 10^7 are possible with PMTs; however, they amplify noise in the signal at high gain settings. Therefore **to secure the best possible image, the controls for laser strength, gain and pinhole size must all be adjusted and balanced relative to one another**.



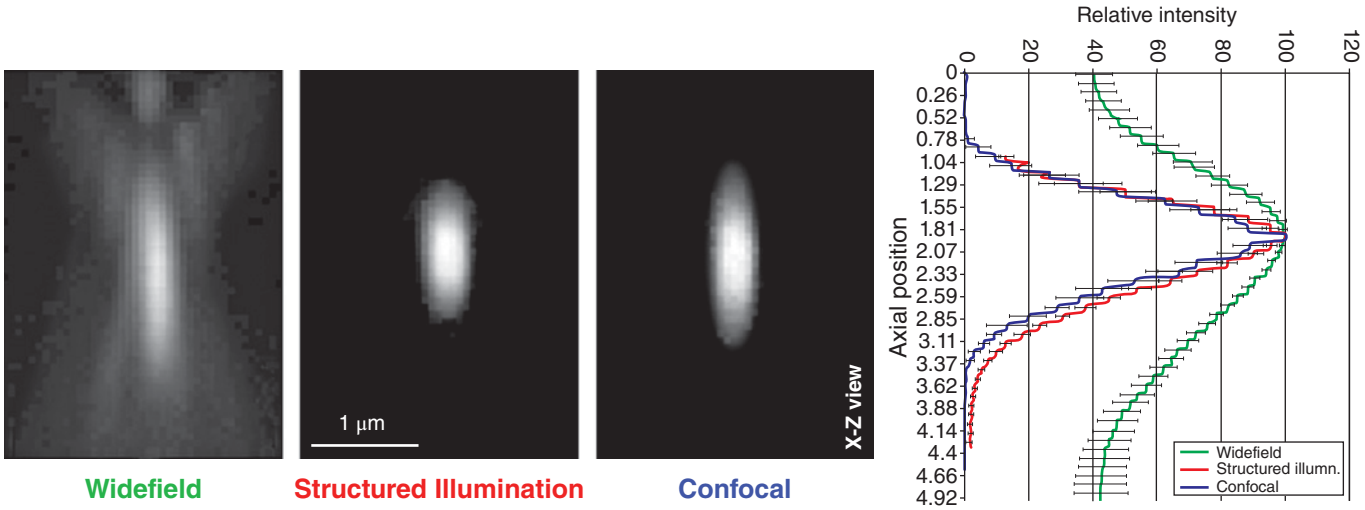


Figure 17.5 ***Confocal PSF*** *Comparison of the relative size and shape of the PSF between the widefield, the point-scanning laser confocal microscope and the structured illumination optical sectioning microscope. Source: (top) Vonesch et al. 2006, Reproduced with permission from IEEE and Dr C. Vonesch. (bottom) Puche 2005.*

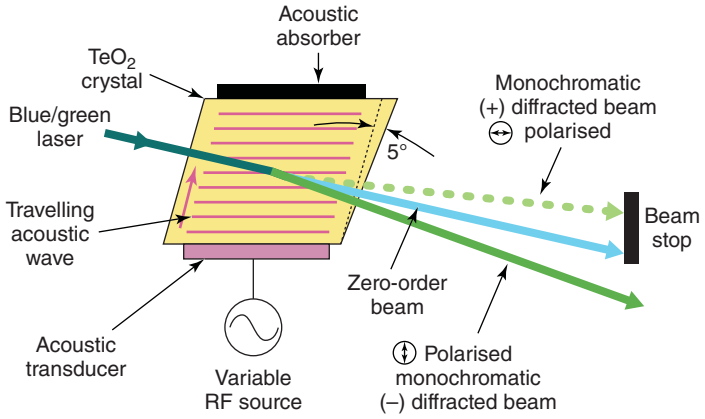


Figure 17.6 ***How the Acousto-Optical Tuneable Filter works*** *The AOTF is a solid state device with no moving parts. Radio signals propagate an acoustic wave through the anisotropic crystal causing it to act as a diffraction grating with a bandpass characteristic, enabling very rapid wavelength tuning. Source: Rietdorf and Stelzer 2006. Reproduced with permission from Springer.*

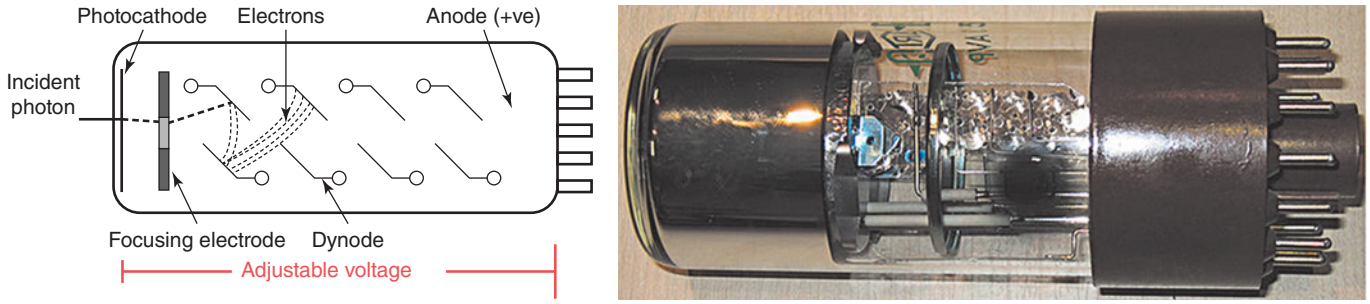


Figure 17.7 ***The design of the photomultiplier tube, and how it works*** *The PMT consists of a photo-sensitive surface, the photocathode, electron-multiplying dynodes and a collection anode all within an evacuated glass envelope. Light enters the input window and is absorbed by the photocathode, which emits electrons. These electrons are accelerated to the first dynode by an applied voltage with a potential sufficient to produce secondary electrons upon hitting the dynode. These secondary electrons are in turn accelerated to the next dynode, and the process repeats itself. The resulting electron cloud is collected at the anode to form an amplified signal. Source: (left) Adapted from Harp, via Wikimedia Commons. (right) Poll. Reproduced under the terms of the Creative Commons Attribution Share-Alike licence, CC-BY-SA 3.0 (http://creativecommons.org/licenses/by-sa/3.0/), via Wikimedia Commons.*

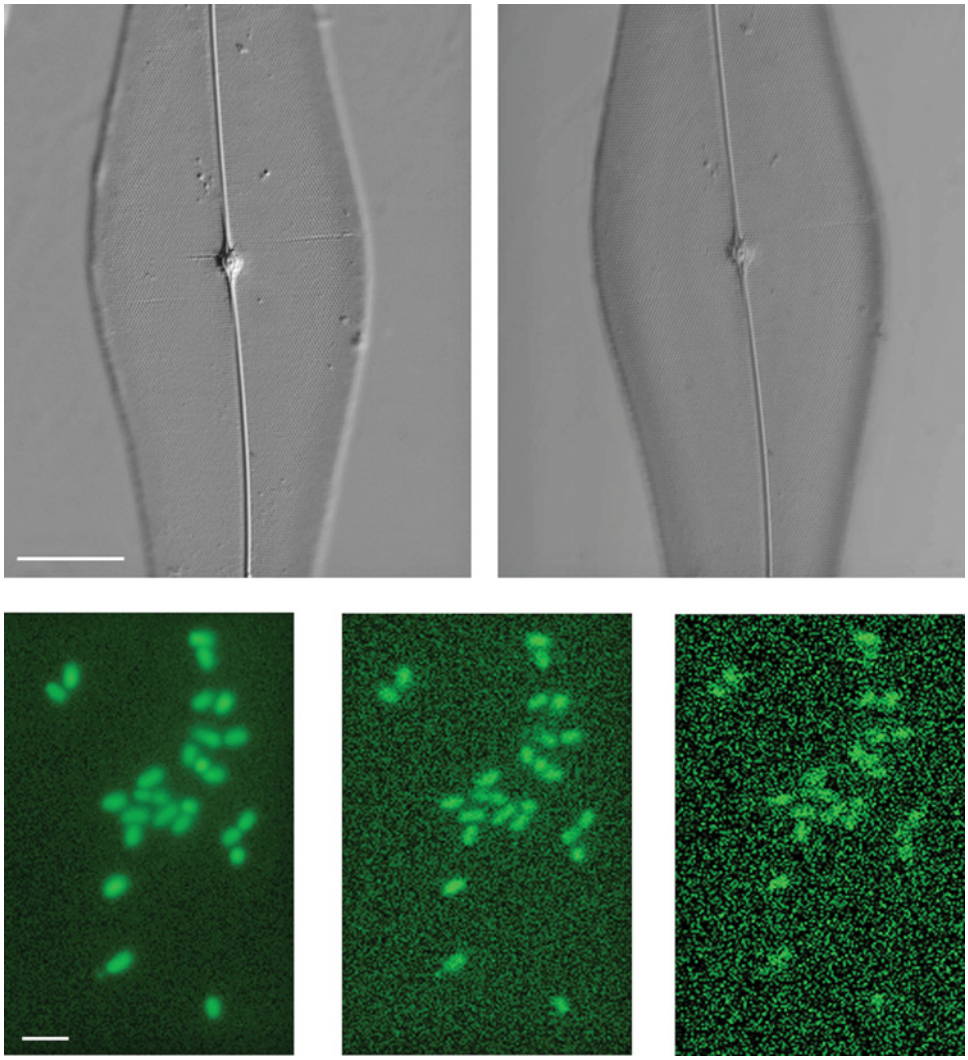


Figure 17.8 ***Comparing the quantum efficiency between PMT and CCD camera detectors*** *Comparing the quantum efficiency between the PMT and CCD camera detectors. A live-cell time-lapse microscope fitted with both lasers for point-scanning confocal and a widefield CCD camera with 6.45 μm pixels has been used to acquire these images. In the upper two panels is an image of the diatom* Pleurosigma angulatum. *On the left is a widefield image and on the right a non-confocal image taken with the 488 nm laser using the confocal software and the transmission PMT. In the lower three panels is an image of GFP-expressing NTHi* Haemophilus influenzae *bacteria. The widefield image is on the left; the single-scan confocal image on the right. The middle image is a confocal image taken with 16 average scans – the best possible. Although improved, it is still noisier than the widefield image. The scale bar for the* Pleurosigma *images is 20 μm and for the NTHi* H. influenzae, *2 μm.*

The requirement to illuminate living samples with minimum radiant intensity or else to detect weakly emitting samples and still image both scenarios with good signal-to-noise ratios means that improved detectors are always being developed. The first significant development was the avalanche photodioide, or APD. It is basically a silicon-doped PMT designed for photon-counting; the APD has an increased quantum efficiency (about 45%) particularly at the red end of the spectrum, as well as an increased amplification range. Their spectral response range is typically within 200–1100 nm. APDs deliver a higher signal-to-noise ratio than regular photodiodes and also have fast time response, low dark current and high sensitivity. Recently very sensitive gallium arsenide phosphide (GaAsP) and related hybrid PMT (HyD) detectors have entered the marketplace. These combine the advantages of PMTs and silicon-based photodiodes and are now offered by all major manufacturers.

17.4 Development of the Confocal Microscope

The evolution of the confocal microscope has a long history. A 'critical mass' for effective development required the development of fluorophores resistant to photobleaching, high-quality and sufficiently economical lasers, fast computers with enough memory to control the laser scan and the optical expertise to build these instruments. The first system was developed from the flying spot microscope (Amos et al., 2012) by Marvin Minsky[5] in 1957, and was a stage-scanning system, but it gained little interest because stage-scanning confocals possessed inherent disadvantages, not least their slow scan speed. It was not until images were published from the first practical single-beam laser-scanning confocal microscope that interest in the confocal microscope really took off. This design (which kept the specimen stationary and utilised raster-scanning of the illuminating beam) was developed (see Amos and White 2003) by William 'Brad' Amos, John White, Mick Fordham and Richard Durbin at the Medical Research Council laboratory in Cambridge in 1986. It followed earlier crucial development work by Brakenhoff in the Netherlands and by Wilson & Sheppard in Oxford. Stage-scanning confocal microscopes are better suited to non-living, inert, materials specimens. This is because stage movement can perturb living cells, particularly at high speeds. Also, crucially, for work requiring immersion objectives for high-resolution studies (i.e. most life science applications), stage-scanning instruments would disrupt the oil or water connection between objective and microscope preparation. Beam-scanning confocals present more technical challenges to overcome optical aberrations but are faster and better suited to living cells.

The generic features of a typical laser-scanning confocal microscope are shown in Figure 17.2. Fluorescent light emitted by the specimen travels by the reverse beam path through the microscope. Unlike the raster scanning pattern of excitation light passing over the specimen, the fluorescence emission remains in a steady position at the pinhole but fluctuates with respect to intensity over time as the illumination spot traverses the specimen producing variations in signal. Because in the exceedingly brief time required for fluorescence excitation and emission, the scanning mirrors have (for all intents and purposes) not moved, the beam of emitted light is stationary and in register with its point of origin. This return of the emitted signal through the confocal beam path to the pinhole detector is referred to as 'descanning' of the beam. Without the descanning of the emitted signal by the galvanometer mirrors on the return path, the signal would only pass through the pinhole at one position of the raster scan. By descanning the signal in the beam-scanning design of confocal, only one pinhole is required. If the emitted beam of light were not descanned, the emitted signal would move relative to the pinhole. This would necessitate a separate pinhole for each position of the laser during the raster scan, which would be impractical.

The size of the adjustable pinhole in front of the PMT detector governs how much in-focus signal (and hence the thickness of the optical section, or 'slice') is recorded in the image. The thickness of the optical section depends upon the diameter of the detection pinhole. It is less than, or equal to, the depth of field – the focal layer. As the pinhole is opened, a thicker optical section is collected. However, the limited depth of field of high numerical aperture objectives also defines the maximum thickness of the in-focus optical section. See also Protocol 18.1 in Section 18.4 and endnote 2 of Chapter 18.

The first designs of confocal microscope were equipped with individual pinholes in front of each PMT in order to compensate for poorer detection in the red part of the spectrum. There is also a good theoretical reason to have individual pinholes: the PSF alters with wavelength and so the ability to set different pinhole diameters at the UV-blue, the central green-yellow and the far-red part of the spectrum means that a constant thickness for the optical slice can be maintained throughout. The disadvantage of having multiple pinholes means that there is 'more to go wrong'. It is easier for the pinholes to become misaligned and so collect poor optical sections. Most pinhole adjustment protocols run on automated macros in software, but it is possible to make manual adjustments. A brief description of how to do this is given in Chapter 27, Section 27.6. The Zeiss LSM 510 has multiple pinholes in its design, but the design of modern point-scanning confocals, including the LSM 710 and its successor, has only one pinhole. This can be kept constant, or (better) adjusted between scans for the particular wavelength associated with each channel.

Altering the size of the pinhole will proportionally alter the thickness of the optical slice that is collected. The optimum diameter is related to the numerical aperture of the objective and wavelength of the illuminating radiation.

- The larger the pinhole, the thicker – less confocal – is the optical section.
- The higher the NA of the objective, the thinner the optical section.
- The shorter the wavelength of the illuminating radiation, the thinner the optical section.
- A pinhole set at 1 AU collects only the central intensity maximum (the Airy disc) of the PSF.
- The maximum size of the pinhole at which the system is still confocal is ≈1.2 AU.

The graph in Table 18.2 shows graphically how altering the pinhole diameter will determine the thickness of the optical section. These optical sections through the cell or tissue (i.e. frames; 'pages') can be collected non-invasively. It is not necessary to physically

slice up the specimen to reconstruct it in three dimensions. Because light is used to image each optical section, these will be in perfect register, so they can be reconstructed into a three-dimensional model with ease. A computer is used to control the sequential scanning of the sample, building up the image point-by-point for display onto a monitor.

Because the image is collected from an (analogue) microscope and encoded as a digital signal, the final displayed image can be contrast-enhanced, and image collection can be seamlessly integrated with electronic triggers (e.g. in a physiological experiment) or else a time-lapse movie taken of living cells. The confocal microscope is therefore an integrated electronic microscope system, with sophisticated electronic circuitry, under computer control, synchronising the raster sweep of the point of the laser illumination across the sample in the field of view of the microscope.

17.5 Advantages, Disadvantages and Resolving Power of the Confocal Microscope

Confocal microscope technology has been very widely adopted, and considerable numbers of new instruments continue to be sold worldwide. Confocal microscopes clearly offer considerable advantages over widefield fluorescence microscopy:

- First and foremost, the confocal microscope is a very efficient optical sectioner, providing very clear image slices from thick cells and tissues where finely stained detail would otherwise be lost within the noise arising from the out-of-focus blur.
- Secondly, because the lasers used exhibit very narrow wavelengths, the confocal microscope is ideally suited to imaging multiply-labelled specimens with minimal bleed-through.
- Thirdly, the illumination is rastered across the specimen under computer control. This allows the single-beam laser scanning confocal microscope to acquire images from precisely defined specific areas, or regions of interest, of the specimen.
- Fourthly, computer control of the laser illumination permits controlled bleaching in protein mobility studies employing FRAP and similar techniques.

From a strictly theoretical point of view and under perfect conditions, the confocal microscope offers *at best* slightly better resolving power than the widefield fluorescence microscope. This is because the ray path is focused *twice* by the objective: first the excitation light is focused to a point, then the emitted signal is detected point-by-point: **point illumination** and **point detection**. The total point spread function (PSF) is therefore the *product* of both the excitation PSF and the emission PSF[6]. The resolution is therefore equal to the *square* of the intensity of the PSF. However, the position of the minima of the two Airy discs remains unaltered (so, in a strict sense, the resolution according to the Rayleigh criterion does not change), but the full width at half maximum (FWHM) of the PSF is improved by a factor of $\sqrt{2}$, or 1.41.

Thus the classical equation $d_{x,y} = 0.61\lambda/NA$ (multiplied by $1/\sqrt{2} \approx 0.71$) becomes $d_{x,y} \approx 0.43\lambda/NA$, with the focal spot being 60% of the diameter of that in widefield microscopy. In the axial direction, the PSF of the confocal microscope is also slimmer, and thus smaller, than for the widefield fluorescence microscope, and $d_z = 2\lambda n/NA^2$ becomes $d_z = 1.4\lambda n/NA^2$. Confocal optics do not improve the Rayleigh resolution criterion at all but confers a 1.41x ($\sqrt{2}$) resolving power advantage over the Sparrow criterion as applied to widefield microscopes in both lateral and axial directions.

However, to utilise this advantage in practice, the pinhole must be closed to an extent (< 0.5 AU) where most of the emitted signal is rejected; this is very rarely done. In practice, the primary advantage of the confocal microscope is optical sectioning rather than making possible an increase in resolving power.

There are several different formulae cited in the literature to calculate resolving power in the confocal microscope, and this has led to confusion about which is the 'correct' formula. The formulae turn out to differ according to whether a large or small pinhole or a large or small objective aperture is under consideration. The formulae may also differ depending upon whether the Full Width Half Maximum (FWHM) of the Gaussian distribution of the PSF intensity is measured experimentally using a fluorescent point or a fluorescent sheet. Naredi-Rainer et al. (2013), Amos et al. (2012) and Wilhelm et al. (2010) cite for lateral and axial resolution, respectively:

$$\text{for a pinhole} > 1\ \text{AU: } d_{x,y} \approx 0.51\lambda_{ex}/NA \quad \text{and} \quad d_z = 0.88\lambda_{ex}/[n - \sqrt{(n^2 - NA^2)}]$$

Besides the advantages listed above in the bullet-point list, the single-point laser-scanning confocal microscope has some inherent disadvantages:

- confocal microscopes are relatively noisy compared to widefield microscopes, exhibiting a lower signal-to-noise ratio;
- the laser illumination can easily photo-bleach the sample to a much greater extent than widefield fluorescence microscopes or other designs of optical-sectioning microscopes; and
- confocal microscopes are relatively slow at acquiring full-frame images.

Widefield microscope images are much less noisy for three reasons. Firstly, the illumination is delivered in parallel to all regions of the sample simultaneously, not pixel by pixel, thus eliminating pixel-to-pixel differences across the field of view. Secondly, the CCD has approximately a four-fold higher detection quantum efficiency than the photomultiplier tubes of the laser-scanning confocal microscope. This increases the number of detected photons by four, thus reducing the Poisson shot noise by a factor of two for the same total illumination (the available photon flux emitted for detection is limited in living samples by photobleaching and photo-toxicity). Thirdly, the CCD read-out electronics add less noise to the signal than does the laser-scanning confocal microscope (LSCM) electronic circuitry.

There are three principal sources of noise in the laser-scanning confocal: (a) the gas lasers themselves, (b) the PMT detector electronics, and (c) the fibre optic coupling of laser to microscope. The 2–5% power deviation of LSCMs acts on *each* individual pixel, whereas fluctuations in arc lamps illuminating the widefield microscope act *equally on all* the pixels, or sampling points that form the image. The noise variation can obscure the true signal, especially in weakly stained samples. This was investigated, and is further explained in an important paper by Swedlow et al. (2002); see also Figure 25 on page 275 in Murray (2010) which unfortunately cannot be reproduced here for copyright reasons.

A typical 2D frame scan representing a single optical slice within the thickness of tissue may consist of 512 lines each comprised of 512 points, that is: 262,144 points overall, which are represented as digitised pixels. In order to scan this 2D frame in 1–2 seconds, the laser must dwell over each point – and have the emitted signal detected – in under 4 microseconds. As explained previously in this chapter, a photomultiplier tube is used to enhance the weak signal and read it very rapidly. However, speed in detection comes at the price of efficiency: a PMT has a QE of 12–15%. Furthermore, the shorter the 'dwell time' of the laser over each part of the specimen, the greater the noise.

Because the PMT detector is quantum-inefficient, the resulting image may – unless the sample is well stained – also be noisy unless the dwell time is increased and/or successive images are taken and summed to average[7] out such noise (see Figure 18.12). Both stratagems carry the risk of extra photobleaching. An image taken with the confocal microscope may well suffer from photobleaching (see Figure 15.3) unless care is taken to operate the laser judiciously at the minimum power necessary. For these reasons, the confocal is best suited to collecting images from relatively thick (20–80 µm) well-stained cells and tissue slices: these will survive the effects of photobleaching better than thin samples. It is very easy to forget this minimum power requirement: if the eyepieces of the confocal are not adjusted to be parfocal with the PMT detector, it is possible to secure an initial in-focus image at the start of a microscopy session with the microscope in widefield visual mode, as usual, then switch to digital confocal acquisition only to find very little or no evidence of fluorescence on the monitor. Many users are tempted to open the pinhole, or worse, turn up the laser, at this point to get a smattering of signal to help them refocus an image on the monitor. This will inevitably lead to premature bleaching of the sample.

Single-beam laser scanning confocal microscopes have a limited acquisition speed for two reasons. Firstly, the lasers are used at a high intensity very near the saturation point of most fluorophores, as explained in Section 18.5. A high illumination intensity is required because the pixel dwell time is very rapid – each point in the raster scan being sampled in microseconds. Secondly, the galvanometer mirrors are driven very hard indeed, particularly the X-galvanometer. This is mechanically challenging, and it is not possible to collect full frames at video frame rates of 30 fps. Even so, to scan a 512 × 512 frame in a second or so, each point must be sampled in under 4 µs, and the galvanometer mirrors must oscillate at over 15 000 rotations per second. If they are driven faster, they are heat-distorted[8].

Despite the fact that the PMT is a very rapid detector, the laser beam must still be scanned over the entire sample within the field of view point-by-point. This is done by galvanometer mirrors, which are relatively slow in operation. Since a full frame can take 1–2 seconds to be collected, the point-scanning laser confocal using a single laser beam is not fast enough to capture dynamic events at anything much more than 5–10 frames per second, even when strategies such as pixel binning (using larger pixels at the expense of resolution) and reducing the boundaries of the scanned area are employed to speed up image acquisition. The answer has been either to use a CCD camera on a widefield microscope and subsequently employ mathematical deconvolution techniques or use a multiple laser beam spinning disc confocal scanning microscope to collect the entire field of view almost instantaneously with a CCD camera instead of a PMT detector.

17.6 The Spinning Disc Microscope

In 1884, Paul Nipkow patented a solution to scan a 2D object sequentially, so that the signal could be transmitted via a selenium photocell as a one-dimensional serial signal through a wire and the 2D image reconstructed in a remote location – an utterly revolutionary concept at the time. The so-called 'Nipkow disc' is a rotating circular disc with a helical array of pinholes and is a simple mechanical method to scan a light beam over the photocell. The intensity of the signal detected by the selenium cell was amplified and used to power a remote lamp placed in front of a second Nipkow disc. The image is reconstructed point-by-point by this second disc spinning in phase with the first, with each illuminating pinhole corresponding synchronously with a conjugate imaging pinhole (hence tandem-scanning of both illumination and detection). This was the basis upon which John Logie Baird devised the first mechanical television set.

The fundamental difference between the Nipkow disc and the first tandem-scanning microscope developed in 1965 by Mojmír Petráň was that the latter had a spinning disc containing thousands of pinholes (part of such a disc is shown in Figure 17.9), which were illuminated *in parallel*. Petráň's instrument was, in effect, thousands of confocal optical systems operating simultaneously. Each beam formed, by an individual pinhole, is confocal with the same aperture serving as the excitation pinhole as well as the emission pinhole (upon the return of the emitted signal)[9]. The colloquial name 'Nipkow' spinning disc microscope is therefore – strictly speaking – a misnomer.

This early spinning disc microscope worked well for reflection imaging of opaque materials (e.g. in dentistry); it was also very fast. Also, the image built up in real-time could be viewed by eye or recorded by camera directly. However, the pinholes in this design only occupied 1% of the area of the disc: the pinholes have to be spaced at least some distance apart in order to prevent cross-talk between adjacent pinholes. Petráň's microscope could not be used for studying fluorescently labelled samples.

Engineers at the Yokogawa Institute Corporation solved the poor light budget of the spinning disc by incorporating a second disc (Figure 17.9) rotating in parallel fashion above the first disc, carrying a series of microlenses that focus the illuminating laser light onto the pinholes of the first disc. The emitted fluorescent signal returns through the first disc and is directed via a dichromatic beam splitter

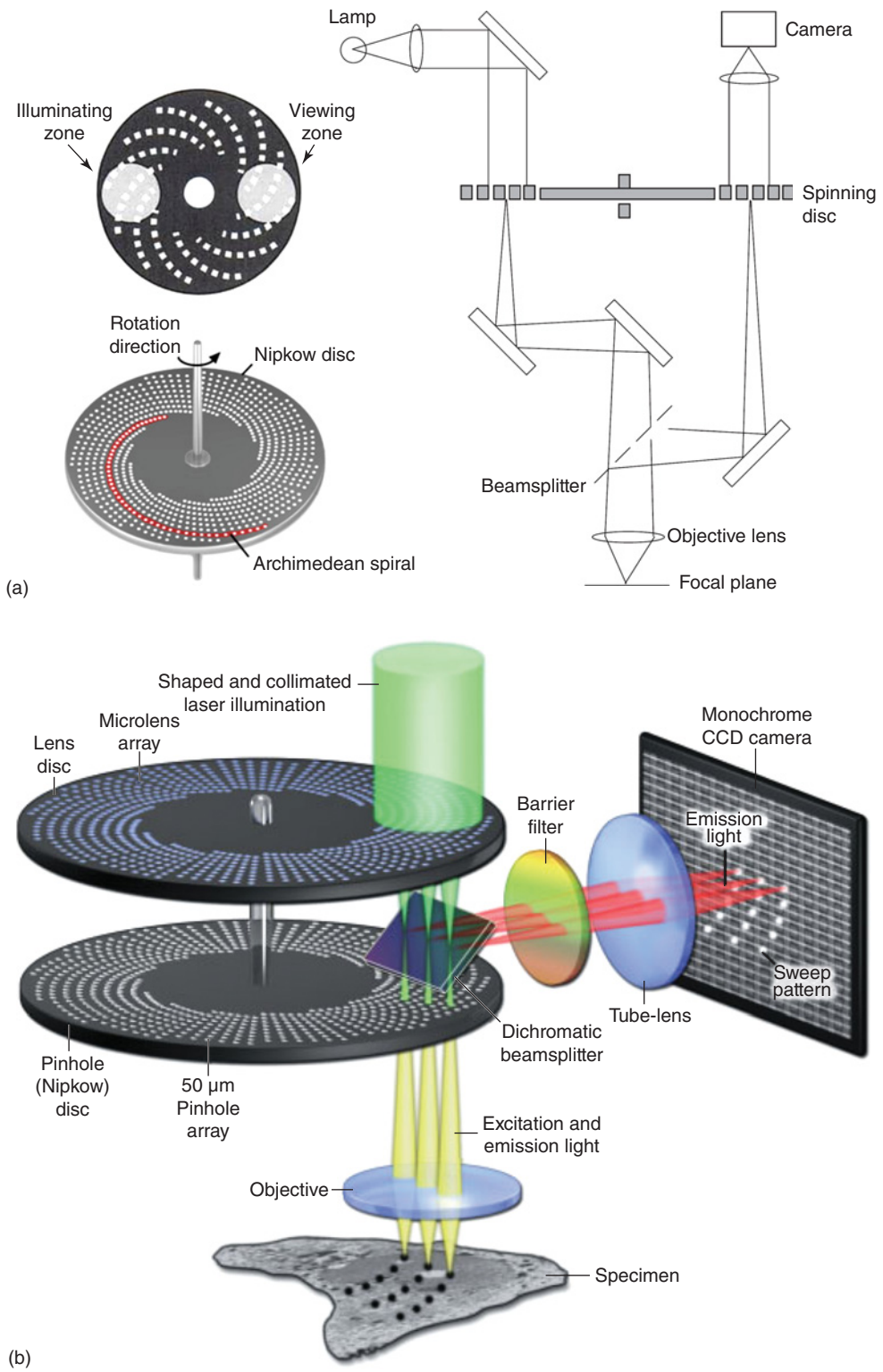


Figure 17.9 ***The Spinning disc microscope design, how it works*** *Panel (a) shows the Nipkow disc and the Petráň design of the original design of spinning disc microscope. Panel (b) shows the modern tandem-scanning design incorporating the Yokogawa double disc arrangement with the microlenses that significantly improve light throughput. Source: (a) Nipkow disc, reproduced with permission from Carl Zeiss Ltd; Dean 1998. Reproduced with permission from John Wiley & Sons. (b) Murphy and Davidson 2013. Reproduced with permission from John Wiley & Sons.*

to a very sensitive EM-CCD camera (which allows very low exposures of weak[10] signals). Since the image is observed in real time, it can be detected with a higher-QE (quantum-efficiency) sCMOS, CCD or an EM-CCD camera instead of a lower-QE PMT. The frame rate is essentially limited to the camera read-out speed.

Very fast z-sectioning is possible through the use of a high-quality piezo-actuator attached to the stage or objective lens. The spinning disc confocal therefore has a much higher temporal resolution than the single-beam laser-scanning confocal microscope. A high-quality three colour Z-stack, which could take 70 minutes to collect on the point-scanning confocal, is likely to take 70 seconds on the spinning disc – sixty times faster. There are several commercial spinning disc microscopes built around the Yokogawa CSU-X1 unit. These include the Perkin Elmer UltraVIEW VoX, the Zeiss Cell Observer SD and the Andor Revolution XD. Yokogawa have also developed the CSU-W1 with a slower frame rate, but a 4x larger field of view.

The multi-beam spinning disc confocal microscope therefore offers the following advantages over the single-beam LSCM:

- (EM) CCD and sCMOS cameras have a much higher quantum efficiency (65–95%) over PMTs (approximately 12–15%) or avalanche photodiodes (approximately 40%). The signal is collected more efficiently, leading to
- shorter exposure times;
- higher frame rates (for equivalent SNRs);
- more efficient fluorescence detection (lower intensity individual beam over longer exposure); and
- less photobleaching.

The thickness of the optical section in a single-beam laser-scanning confocal is controlled by altering the size of the pinhole. However, in the spinning disc microscope, the size of the pinholes are fixed and are calculated to give confocality only for one objective, usually a 100x/NA 1.4 objective, yielding an optical slice of 0.8 µm. The spinning disc microscope generally has less axial resolution than the point-scanning confocal. It is also not possible to zoom in and out, as can easily be done with a single-beam point-scanning confocal microscope. This means that the optical resolution cannot be matched to the Nyquist sampling requirements of the object and thus can only be optimised for a single magnification.

Although the total light flux may be similar to a point scanner, the spinning disc confocal has a much lower light intensity *per individual scanning spot*, which reduces photobleaching and cellular photo-toxicity. Furthermore, the pixel dwell time of the spinning disc microscope is much higher than a single-beam point-scanning confocal. These two factors, together with the higher quantum efficiency of the EM-CCD detector, cause less photobleaching and the ability to image cells longer over time without saturating the fluorophore. However, bleaching is proportional to both the laser intensity and the frame rate. It is worth remembering that although spinning disc scanners generally use lower laser intensities, they can bleach just as much as a point-scanning confocal *if* images are collected with too high a frame rate.

The multi-beam spinning disc microscope does have a few disadvantages when compared to the single-beam laser-scanning confocal:

- the size of the pinholes are fixed and cannot be altered to match the NA of the objective in use or the wavelength of the excitation illumination in use;
- lack of scan zoom to match the sampling rate to the Nyquist criterion;
- only able to use very simple ROI overlays, unlike the single-beam confocal;
- the field of view is non-uniformly illuminated; and
- pinhole cross-talk, which gives rise to background haze and reduced SNR, as well as reduced axial resolving power.

Just as with the single-beam confocal, the image acquisition rate can be increased by reducing the lateral resolving power and/or reducing the image area by using a region of interest or CCD subarray readout to capture a smaller field of view. Using the zoom function and pinhole diameter to change the size of the voxels[11] imaged is very easy in the single-beam confocal, since the zoom and pinhole functions are very flexible continuously variable controls. Likewise, selecting a reduced field of view in the single-beam confocal by means of drawing a region of interest to restrict the sample area that is rastered by the laser(s) to form the image is also very easy to manage. The spinning disc confocal, however, has no zoom function, and the size of the pinholes is fixed, so adjusting the frame rate by changing the image size can only be done stepwise by changing the objective magnification (and thus resolving power) or by increasing the image pixel size by binning the CCD camera. Sub-array readout is still possible, and this can also increase the frame rate. It is also more difficult to apply a complex region of interest (e.g. for bleaching) using the spinning disc microscope, whereas this is very easily and quickly done with the single-beam point-scanning confocal.

The spinning disc confocal is ideally suited to imaging single-labelled samples stained with just one fluorophore. It is not possible to switch lasers pinhole by pinhole, which would be the equivalent of line-scanning on the single-beam confocal; the stratagem of line-by-line switching using the LSCM single-beam confocal avoids mismatches in register between channels, which could otherwise happen when collecting images frame by frame. Sequential imaging – to avoid bleed-through – whilst using the spinning disc microscope has to be done by switching lasers and filter sets between frames. This will reduce the frame rate to a certain extent, because although switching lasers with an AOTF is fast (2–5 msec), changing filters is an order of magnitude slower (≈ 20 msec) depending on the angular travel (position of filters within the filter wheel) and velocity of the filter wheel.

One way of imaging two multi-channel fields of view simultaneously is to use a beam splitter. There are three options on the market. The first is the DualView and QuadView from Photometrics or Olympus; the second is the Optosplit III from Cairn Research or Andor,

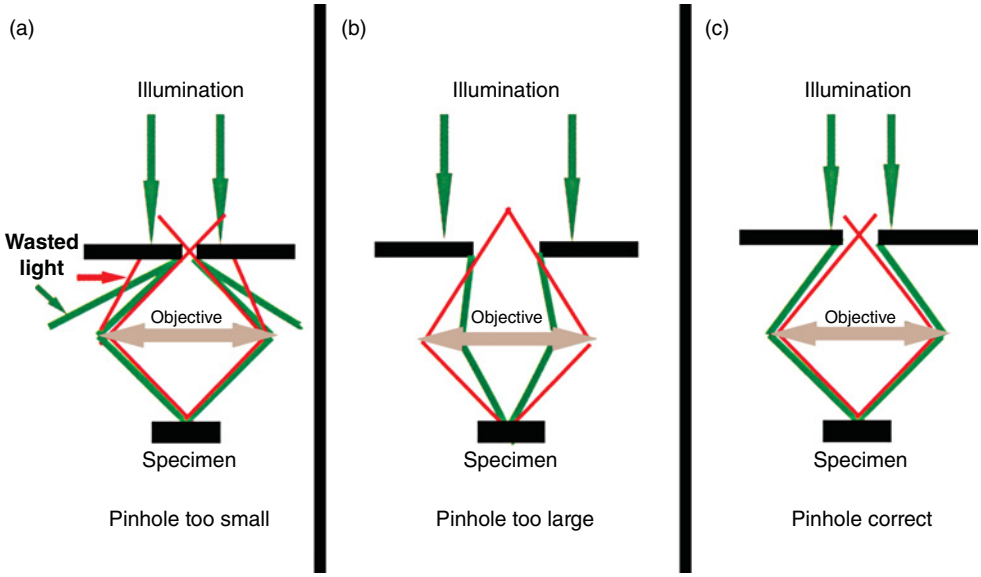


Figure 17.10 ***The Spinning disc microscope, cross-talk*** *Where the size of the pinhole is too small (a) light is wasted, and some higher-order diffracted light may not be collected. Where the pinhole is too large (b) the diffracted light may not adequately fill the back focal plane of the objective. Also, bleed-through will occur as signal returns to the detector through the wrong pinhole. Where the pinhole is of the correct size, all the light diffracted by the specimen is collected, and the proper Nyquist sampling occurs without bleed-through. Source: Fuseler 2011. Reproduced with permission from Springer.*

and the third the W-view from Hamamatsu. An ImageJ plugin is available to display and analyse split fields of view on a single monitor. Beam splitters such as these are also useful for displaying the two different polarisation states (crossed, uncrossed) on a single monitor simultaneously or for FRET and calcium ratio-imaging applications.

Because some light reflected, or emitted, from the sample will reach the detector via pinholes other than the one intended, a small amount of cross-talk (Figure 17.10) will also occur with the spinning disc microscope. Cross-talk will increase as the thickness of the sample increases, say above 50 µm. Cross-talk from one pinhole to adjacent pinholes will also occur if there is a significant degree of refractive index mismatch. Thus the Olympus silicone-immersion objectives or a similar multi-immersion objective (see Figure 7.4), are particularly suited for live-cell imaging of watery samples on the spinning disc microscope.

The camera of choice for the spinning disc confocal has been the EM-CCD, since these designs allow efficient collection of low-light level signals encountered in live-cell imaging, thus enabling fast frame-acquisition rate. Recently the performance of sCMOS cameras has improved to such an extent that, unless the emitted fluorescent signal intensity is 50 photo-electrons or less, the sCMOS is a better detector than the EM-CCD (Oreopoulos et al., 2014). In the summer of 2016, Andor released their Dragonfly microscopy platform, a multi-point confocal, with two selectable fixed pinhole sizes of 25 and 40 µm designed for high speed and high SNR with up to 200 fps, taking advantage of the latest Zyla sCMOS cameras.

An alternative to the Yokogawa spinning design, popular because of the cost benefit, is the aperture correlation microscope sold by Zeiss as the VivaTome II (Figure 17.11), by Andor as the DSD (differential spinning disc) and Aurox – the developer of the technology – as Clarity. Instead of a series of pinholes in the spinning disc, a grid pattern etched into both sides of the disc is used. It is the same structured illumination technology (Section 17.8) as the ApoTome and Optigrid, which are alternatives for the single-beam scanning confocal. As with the ApoTome and Optigrid, the VivaTome, DSD and Clarity use a white-light source. This is cheaper than lasers and provides for specific wavelength illumination, depending on the filter sets used. The double-sided spinning disc collects a transmitted- and reflected-emission image. Computer subtraction of these images differentiates between the in-focus and out-of-focus light from the fluorescence signal to give a confocal optical section. These aperture correlation microscopes can collect signals from a single fluorophore imaged at full frame at between 20–30 frames a second (e.g. at video rate). On some newer models it is also possible to increase the frame rate by binning and/or using a region of interest (ROI) to collect an image from a sub-array of the detector.

It is also possible to use white light for illuminating the spinning disc, using metal-halide, mercury or (best) xenon arc lamps, which latter have a relatively even profile across the visible spectrum. The advantage of white light systems is that the illumination can be better tuned to a greater number of fluorophores than merely with single-line laser illumination, although the introduction of several feasible diode laser sources to cover the near-UV, visible and infrared spectrum makes this less of an advantage than was formerly the case. The chief advantage of white-light spinning disc systems is cost: they can be added very cheaply and easily to an existing widefield fluorescence microscope. The Olympus DSU (disc scanning unit), Andor DSD (differential spinning disc), Clarity^{HS} the Crisel CrEST X-light and the BD Carv II are examples of white-light confocal designs. Single-beam laser scanning confocal microscopes and parallel-beam spinning disc confocal microscopes are complementary technologies, not alternatives to one another.

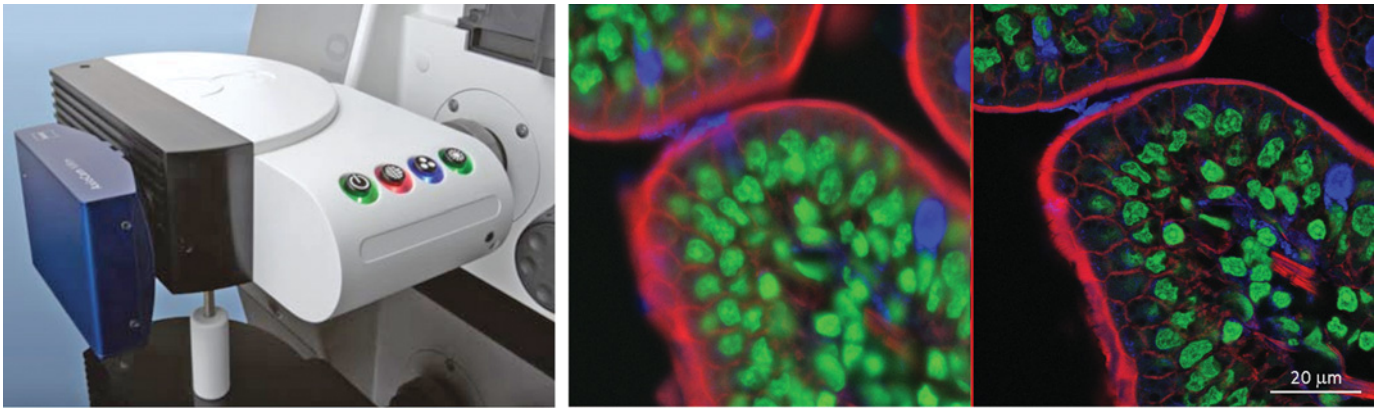


Figure 17.11 **The VivaTome** *The Zeiss VivaTome attached to the side port of a widefield microscope, together with an example of an optical section collected from a triple-stained mouse intestine preparation. Source: Reproduced with permission from Carl Zeiss Ltd.*

17.7 Line-Scanners and Array-Scanning Confocals

Programmable array and line-scanning microscopes are designs that attempt to increase the frame rate, and thus the temporal resolution, of the laser-scanning confocal microscope. Instead of using a diffraction-limited spot formed by a pinhole[12], these microscopes use either slit apertures, and sacrifice some confocality in so doing, or else use spatial grids or resonant scanning devices. In a single-beam point-scanning confocal, the X-galvanometer mirror ('X-galvo') works faster, and is exposed to greater mechanical stress, than the Y-galvo. This is because it must raster the point of the laser beam across the entire line scan, whereas the Y-galvo merely has to 'drop' the beam to the next line. Since the X-galvo is more prone to wear and eventual failure, if we do away with it and replace the mirror by a slit, we can scan each line in one go and merely use the single remaining mirror to raster the entire line across the frame. Since the slit used to illuminate the sample is aligned in the x-direction, some out-of-focus light is also detected, so confocality is partially lost in the x-direction but retained in the y-direction.

Slit scanners, such as the 7-Live from Zeiss or the Nikon LiveScan SFC, use a slit of light to sweep the field of view and so build up an image much faster than a single-beam point-scanning confocal. The Zeiss system uses beam-shaping lenses to create a 1-pixel-wide scanning beam that is detected and read out by a linear-array CCD line detector with 512 elements, which naturally has a very fast read-out time. The Nikon swept-field design is a hybrid model, combining both slits and pinholes, which can be exchanged by the user to suit the application. The scan head is made by Prairie Technologies, and the image is detected with a regular full-frame CCD. Maximum frames rates of over 1000 fps are possible with both models, provided that a sub-array ROI is scanned (also with binning on the full-frame CCD). Slit scanners may be inferior in optical sectioning to a point-scanning confocal, especially when a large fluorescence volume is scanned. They are as expensive, or more so, than point scanners, and they tend to bleach very quickly.

Instead of a disc with pinholes, another possible design is to use a rectangular microlens array where the illuminating beam is scanned across the sample with a single galvanometer mirror, and the emitted signal descanned using the reverse side of the same mirror. The Visitech VT-infinity is based on this design. A further solution, again from Visitech, is the VT-eye. This design uses an acousto-optical deflector (AOD) and since this is a solid-state device, it does not suffer from wear like a galvanometer mirror does. The AOD is wavelength dependent, functioning in a similar manner to an AOTF by passing a sound wave through a crystal in order to isolate a particular wavelength, so the longer-wavelength beam cannot be descanned through the return optics (as in a single-beam point-scanning confocal). Hence, point-scanning excitation and slit-scanning detection are used, resulting in less confocality than for a single-beam point-scanning system. AOD microscopes have not made point-scanners obsolete because of the greater technical challenges of maintaining stability at high speeds and high data transfer rates.

An alternative method of increasing temporal resolution is to retain the single-beam point-scanning design and to increase the rate at which the galvanometer mirrors are driven. Unlike the spinning disc, swept-field or line-scanning confocals, resonant scanning single-beam point-scanning confocal microscopes are able to alter magnification without changing objectives by retaining the versatile confocal zoom functionality. The repetitive, shorter exposures usually lead to brighter images, albeit with slightly worse signal-to-noise ratio but with much less bleaching per scan. The speed of the resonant scanner is fixed, usually at 8000 Hz, and it can be difficult to define regions of interest, except along horizontal lines. Leica, Nikon and now Olympus market resonant scanner units for their point-scanning confocal microscopes.

17.8 The Structured Illumination Approach to Optical Sectioning

Another means of being able to optically section the sample, to provide clear blur-free images, is implemented by using structured illumination microscopy (SIM). Currently there are three designs on the market capable of doing this: the original Optigrid from Qioptiq,

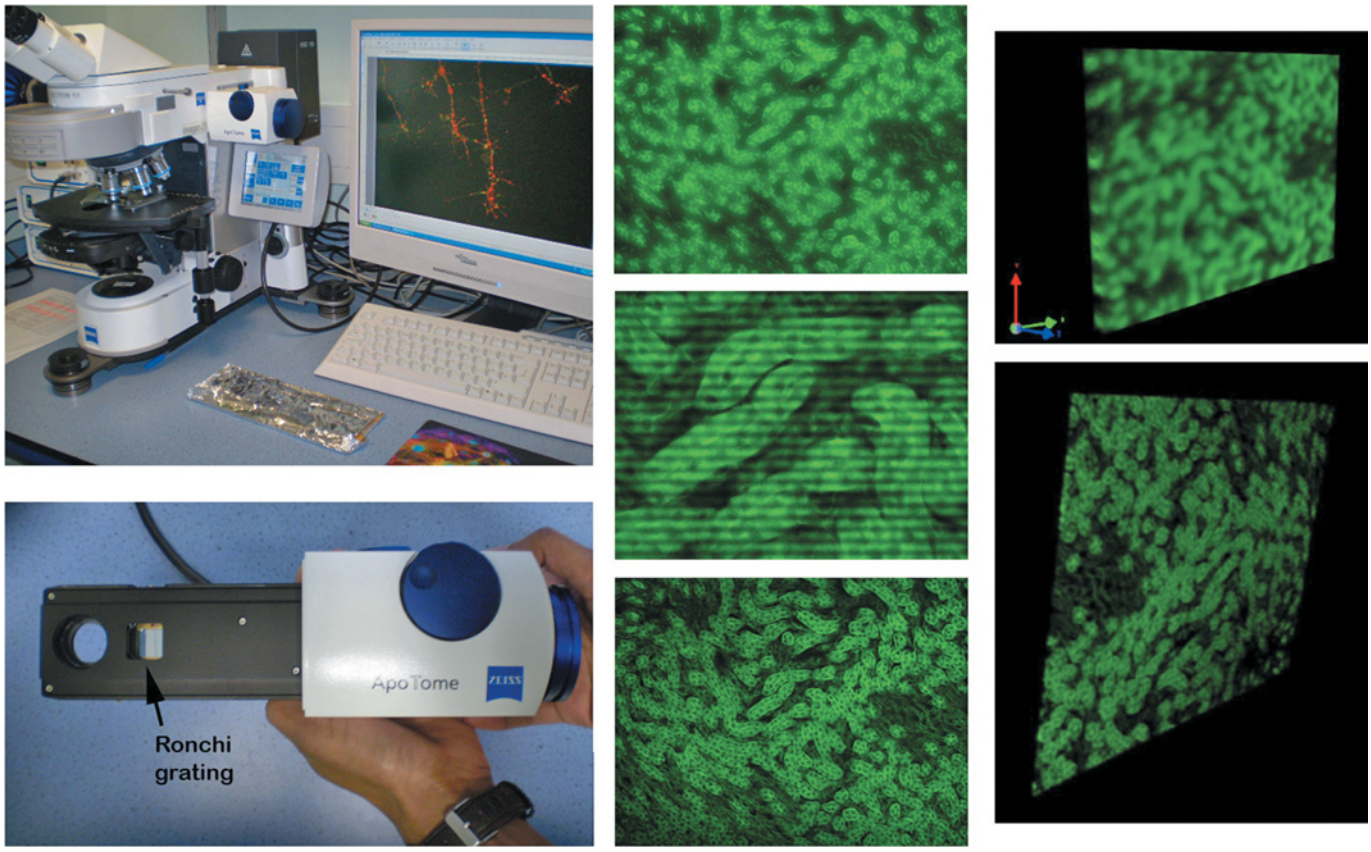


Figure 17.12 ***The ApoTome structured illumination optical sectioner*** *See the text for explanation of how this and similar structured illumination optical sectioning devices work. Source: Sanderson 2008. Reproduced with permission from The Quekett Microscopical Club.*

the Angstrom sold by Quorum and the Apotome 2 by Zeiss (Figure 17.12). All three structured illumination systems can be retrospectively fitted to modern widefield fluorescence microscopes. The ApoTome design fits only the manufacturer's own brand instruments, but the Angstrom and the Optigrid can be fitted to a range of microscope stands from all main manufacturers.

All designs work in the same way: they superimpose an image of a Ronchi ruling (placed in the excitation light path at the position of the epi-illumination field aperture) onto the sample. A Ronchi ruling is one composed of very high contrast black and clear rulings. When the grid is operating, these rulings can be seen moving up and down as stripes superimposed upon the image in the field of view of the microscope. Three images are acquired by moving the grid laterally (here within the plane of the paper) in three steps, each displaced by 1/3 of the ruling's period. These three grid movements produce one optical section. An algorithm recombines these three images to extinguish any portion of the images that contains unfocused light, from outside the depth of field of the objective (the out-of-focus signal is pretty much constant in all three images and subtracts out), while eliminating the 'grid-image' from the final resultant image. At the plane of focus, the differences between the illumination intensities are weak, whereas the differences in illumination intensity are strongest when the sample is out of focus.

Since it is necessary to collect three images at each z-position for each fluorochrome, this implies that the specimen must be stable and must not move during these three acquisitions. Therefore this technique is not very suitable for live cell imaging, where movement inevitably occurs. Where temporal resolution is required, the aperture correlation microscope (Section 17.6), using a spinning disc etched with a grid pattern, is the structured illumination equivalent of the spinning disc microsope.

These structural illumination microscopes have the same optical sectioning ability as the laser-scanning confocal microscope, but the algorithms won't easily handle *very* thick specimens (>30 μm) and can be prone to stripe artifacts. Nevertheless they can be easier to set up to collect an image and are cheaper to run, since they are fitted to a widefield fluorescence microscope and so use a mercury lamp, rather than a laser, as the fluorescence illuminator. For this reason also, the sample generally suffers less bleaching than would otherwise occur with laser illumination. In addition, the image formed by structured illumination in a widefield microscope is detected with a CCD camera, rather than a PMT with lower quantum efficiency. This makes the structured illumination method ideal for capturing images from very weak or sensitive and photo-labile samples. The structured illumination approach to optical sectioning is particularly suitable when using lower magnification objectives. It is easier to get an optical section from a large field of view at lower magnifications (e.g. less than 20x) with the structured illumination approach than by using point-scanning confocal microscopy.

This method is fundamentally the same as super-resolution SIM (Chapter 26, Section 26.6), but in the latter case, a much finer Ronchi grating is used.

17.9 The Airyscan Principle

The 'iron triangle' of imaging is discussed in Chapter 28 with particular regard to live-cell imaging. It is not possible simultaneously to image at high speed with a good signal-to-noise ratio and with good resolution. You can only have one of these three conditions (speed, SNR, resolving power) at the expense of the other two. With a smaller pinhole set below 1 AU, resolving power increases but primarily at the expense of SNR. Alternatively, SNR can be increased but primarily at the expense of acquisition speed. Fast scanning with the single-beam point-scanning confocal is possible at the expense of SNR, resolving power and the size of the image collected.

One approach to using a small pinhole for resolving power advantage yet simultaneously keeping the SNR high is to use a very sensitive multi-element area detector at the pinhole to collect all the light from an Airy disc simultaneously. This works because a displaced pinhole produces an image about equal to the resolving power of a pinhole aligned to the optical axis but of lower intensity. A high-sensitivity multi-channel 32-element gallium arsenide phosphide (GaAsP) detector constructed rather like an insect's compound eye is able to detect these offset signals and sum the signal shifted back on-axis. In this way much less signal is rejected by the 0.2 AU pinhole and may contribute usefully to image formation. Deconvolution of the image data ensures that resolving power is improved in all directions by a factor of 1.7x to give a lateral resolving power of 140 nm and an axial resolving power of 400 nm. This idea is not new, having been proposed by Pawley in 1996 and recently by Roth et al., 2013 and De Luca et al., 2013. Zeiss have very recently (Weisshart, 2014; Huff et al., 2015) made this idea commercially possible.

17.10 The Mesolens

Although they have been available for many years, the confocal microscope – like its widefield counterpart – can only image a large field of view (mm) at low resolution *or* a small field of view (µm) at high resolution. Some firms (see comments in the Introduction to this chapter) have introduced confocal macroscopes, which can image larger fields of view. However, at low magnification, where the numerical aperture of most objectives is low – and the depth of field large – it is impossible at low magnification to acquire good optical sections that are not excessively thick, because of poor axial resolution in depth.

Currently the only way around this is to use a high-NA objective and tile successive fields to provide a larger image overall. The problem with this approach is that very often the illumination is not perfectly even across the field of view, and so the edges of the tiles are very obvious. Furthermore, this method is time consuming, which can also lead to photobleaching.

A new giant lens is being developed by Amos, McConnell and Dempster, which has an unprecedented numerical aperture of 0.47 at a magnification of 4x, with a working distance of over 3 mm and a field size of 6 mm by 6 mm (McConnell et al., 2016). It is called the Mesolens because it lies between a conventional microscope objective and a close-up photographic lens. For example, the Mesolens can show an entire mouse embryo at 0.6 µm lateral resolution while observing cells and tissues at around 3 µm optical section thickness without losing a sense of depth. The Mesolens has a 3–5 times improvement in lateral resolving power and an improvement of 8–10 times in axial resolving power over a conventional 4x objective.

Such a 4x microscope objective has an NA of about 0.1, with a lateral resolution ($d_{x,y} = 0.61\lambda/NA$) of 3.4 µm and an axial resolution ($d_z = 2n\lambda/NA^2$) of about 100 µm. This means that it would not even be able to resolve detail within a 3 µm optical section. By comparison, a conventional objective of NA 0.47 will normally have a magnification of 20x or more, with a field of view of approximately 1 mm × 0.7 mm, rather than 36 mm^2. This 20x objective will resolve 0.7 µm laterally and about 5 µm axially. The Mesolens is capable of gathering approximately a hundred times[13] the amount of information in a focal series and can capture such a Z-stack in an hour that would take a day stitching and tiling with a 60x/NA 1.4 objective routinely used on a confocal microscope. Further information is available on the Mesolens website.

17.11 Concluding Remarks

At the conclusion of this chapter, it is worth reviewing the technical innovations that in the last forty years have made the laser-scanning confocal the predominant microscope platform for viewing fluorescent specimens, installed worldwide in most universities, hospitals and research institutes. In my view, each of these developments has been key to the evolution of the modern instruments now available as turn-key systems.

In the 1980s incorporating the **pinhole** to make a scanning microscope confocal was the key initial development (see Section 17.4, endnote 5 in Chapter 17 and Amos and White, 2003). The next significant development in the early 1990s was the **AOTF**, or Acousto-Optical Tuneable Filter, which replaced neutral-density filters for individually modulating the intensity of the lasers. The AOTF for the first time allowed blanking of the laser beam on the flyback during the raster scan. This helped significantly to reduce exposure of the specimen to light. The AOTF was first integrated into commercial instruments by Leica and became an essential requirement when tendering for a new instrument. The next development, around 2001, which became a convenient and essential tool to have, was **ROI**

scanning whereby the laser(s) could be controlled to raster within both defined and freehand regions rather than across the entire field of view. Not only did this allow for scanning only the area of interest, thus reducing exposure, but also saved significant time during image acquisition. The key advantage of ROI scanning, however, was to make FRAP a mainstream technique available to all. Just a year later Zeiss introduced their META detector, which permitted **spectral unmixing**, adding another dimension to image data sets. This advance allowed not only spectrally similar fluorophores and fluorescent proteins to be differentiated within the image for the first time but also brought FRET into mainstream imaging. By 2010 Zeiss again led the way by introducing high-sensitivity gallium arsenide phosphide (**GaAsP**) **detectors**, followed shortly after by similar PMT/silicon-based photodiode hybrid detectors. The latest significant development to impact the laser-scanning confocal market is the **Airyscan** introduced in autumn 2014. By incorporating a 32-element GaAsP detector into the confocal pinhole optical plane, it is now possible to secure the resolving power advantage of a reduced pinhole yet still maintain very high signal-to-noise levels. The only alternative is to acquire an image and deconvolve it – as the Airyscan also does – but using ten times the exposure. Like the previous technical advances, this newest technology significantly reduces the exposure of delicate specimens to light, whilst allowing lateral resolving power similar to super-resolution techniques.

17.12 Chapter Summary

This chapter explains how to take advantage of the specificity, sensitivity and potentially very high contrast of fluorescence imaging using confocal microscopes. The next chapter explains how to operate the most commonly used of these confocal microscopes, the point-scanning confocal.

1. There are many advantages to using fluorescence: specificity, sensitivity, high contrast.
2. The three disadvantages are blurring, bleaching and bleed-through.
3. Unless the sample is very thin, blurring is the most serious disadvantage. It occurs because fluorescent samples are self-luminous.
4. To restrict blurring, axially in the z-direction, confocal point-scanning is widely used.
5. The entire image is built up point-by-point in each frame, as the diffraction-limited spot of the illuminating beam rasters across the frame, like reading words in lines along a page of script in a book. Each frame corresponds to a plane in the z-axis.
6. The confocal microscope depends upon a pinhole to exclude out-of-focus light.
7. Altering the size of the pinhole will proportionally alter the thickness of the optical slice that is collected.
8. Photomultiplier tubes are used as detectors in point-scanning confocal microscopes. They very rapidly collect, and enhance, the single points of fluorescent light emitted as signal.
9. The confocal microscope is suited to imaging multiply-labelled specimens with minimal bleed-through. The narrow bandwidth laser illumination can be switched fast and efficiently.
10. Confocal microscopes are relatively noisy and slow at collecting an image compared to widefield microscopes.
11. The spinning disc microscope, containing thousands of pinholes, which are illuminated in parallel, is much faster.
12. The spinning disc confocal has a much lower light intensity per individual scanning spot, which reduces bleaching.
13. Cross-talk between pinholes in the spinning disc microscope occurs with refractive index mismatch and increases in proportion to the thickness of the sample.
14. Programmable array, slit or line-scanning microscopes and resonant scanning microscopes are alternative confocal designs that increase temporal resolution over the single point-scanning confocal microscope.
15. Structured illumination microscopes superimpose an image of a grating on the specimen. Computer algorithms subtract the out-of-focus light to give a confocal optical section. Aperture correlation microscopes are able to image dynamic processes at about video rate.
16. The Airyscan compound GaAsP detector improves resolving power and SNR.

Key Reading

Murray, J.M. (2011). 'Methods for imaging thick specimens: confocal microscopy, deconvolution, and structured illumination'. *Cold Spring Harbor Protocols 2011*/12: 1399–1437. doi: 10.1101/pdb.top066936.

- An excellent review on stratagems to avoid image blurring when collecting data sets from thick specimens. This paper is one I recommend to my students.

Chhetri, R.K. and Keller, P.J. (2016). 'Imaging far and wide'. *eLife 5*: e21072. Commentary on the Mesolens and acquiring large volume datasets.

- With several good references, this paper gives a useful insight into the future of image acquisition by light microscopy of very large 3D data sets.

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Yang, W. and Yuste, R. (2017). In vivo imaging of neural activity. *Nature Methods 14*/4: 349–359.

Internet Resources

Confocal Principles (Zeiss publication): bit.ly/confocal-principles.
Getting the best from PMTs: http://users.ox.ac.uk/~atdgroup/technicalnotes/Getting%20the%20best%20out%20of%20photomultiplier%20detectors.pdf.
HyD PMT detectors: http://www.formatex.info/microscopy5/book/818-825.pdf.
Spinning disc microscopy: http://cshprotocols.cshlp.org/content/2010/11/pdb.top88.full and http://zeiss-campus.magnet.fsu.edu/articles/spinningdisk/introduction.html.
Resonant scanning microscopy: https://www.microscopyu.com/techniques/confocal/resonant-scanning-in-laser-confocal-microscopy.
Aperture correlation microscopy: http://zeiss-campus.magnet.fsu.edu/articles/opticalsectioning/aperturecorrelation/introduction.html.
High-resolution episcopic imaging (Indigo Scientific): http://www.indigo-scientific.co.uk/Optical-HREM-High-Resolution-Episcopic-Microscopy.
ImageJ split fields of view display plugin: https://imagej.nih.gov/ij/plugins/cairn-splitter.html.
The Mesolens: http://www.strathclydemesolab.com and https://www2.mrc-lmb.cam.ac.uk/group-leaders/emeritus/brad-amos/.

Notes

1. There are several thousand active professional microscopists who read, and contribute to, the Confocal Listserver. Amongst the inevitable chatterboxes, which any forum attracts, a few contributors offer consistently good advice: experience will tell you who these are.
2. Prior to optical sectioning of fluorescent samples, developmental biologists studying embryogenesis resorted to reconstructing analogue 2D sections into 3D models. This was useful and informative but cumbersome, time consuming and prone to distortion. Digital imaging has assisted the re-emergence of this approach called high-resolution episcopic imaging (HREM). Tissues are embedded in plastic resin containing the fluorescent dye eosin and successively sliced or milled. Successive digital images of the block surface are taken in register to create a 3D data set. See Geyer et al. (2009) for further details. This equipment is available through Indigo Scientific, whose details are given in the Internet Resources, at the end of the chapter, and in the list of suppliers in Appendix 5.
3. The point spread function has a complex shape, which at first may be difficult to visualise. In the plane of focus, the PSF is simply the two-dimensional Airy pattern – a bright circular Airy disc in the centre surrounded by a pattern of annular rings of decreasing intensity. The Gaussian plot associated with the Airy pattern is an intensity plot of the PSF in the focal plane. In three dimensions the central part of the PSF assumes the shape of a rugby football, and the concentric rings of the larger Airy pattern spread out away from the central plane as a complex set of conical structures (see Figure 17.5, and also Figure 10.15, Figure 21.2 and Figure 21.9). These side-lobes are greatly reduced in the confocal microscope and virtually non-existent in the multi-photon microscope. Each point in the image is replaced by its corresponding PSF, the intensity of each PSF being directly proportional to that of the point from which it arose.
4. Many people assume that a laser is mandatory as an illuminant for a confocal microscope. It is not. The single-beam scanning confocal fluorescence microscope in the transmitted-light configuration normally does require a laser for the reasons explained above, but Professor Tony Wilson has demonstrated a reflected-light confocal microscope, similar in design to Petráň's tandem-scanning microscope and the aperture correlation microscope, which captured an optical section by the illumination of a single candle. The author has witnessed this demonstration in the old lecture theatre of the Department of Human Anatomy, Oxford.
5. In a delightfully personal memoir, Marvin Minsky emphasises the problem of blur due to the scattering of light and describes how he first developed the principle of the confocal microscope into a working prototype. Carbon arc lamps were used before the advent of the laser; see Minsky (1988). For a fascinating report by Amos and White on how they developed the confocal microscope into a practical instrument for biological research, see Amos and White (2003). These references are given at the end of this chapter.
6. The central maximum intensity (corresponding to the Airy disc) of the PSF is reinforced, while the lower intensity maxima from the surrounding rings, the 'side-lobes' of the Airy pattern, are attenuated. Since these side-lobes give rise to much of the out-of-focus light, the confocal microscope is therefore a good optical sectioner.
7. In a line scan with averaging set to 8, if – due to Poisson noise variation – there is a single noisy pixel value of 30, and remaining pixels are valued at 0 (to make the calculation easy and obvious) in the remaining seven scans, then the average value assigned to that pixel will be ≈ 4. The noise is therefore integrated out of the image. Some people prefer line scanning to the more usually employed frame scanning, because if the noisy pixel occurs in a later frame, the average function is computed from that frame onwards, not the entire eight frames.
8. Zeiss have recently increased scan speed to a notable extent by cooling both the galvanometer mirrors and the electronic circuitry (a significant source of image noise in the single-beam laser-scanning confocal) in the LSM 800 series.
9. The earliest spinning disc designs, developed by Petráň and Hadravsky, were called 'tandem scanners' because they used opposing pinholes on two matched discs. This design has been superseded by the Yokogawa Nipkow disc design with microlenses incorporated, because it is a much more light-efficient design. Modern spinning discs have an array of 20 000 very fine (30–50 μm diameter) pinholes.
10. If the camera exposure is very short (or the rotational velocity of the disc falls, which is unlikely), streaking will occur in the image. If the alignment of the pinholes is not perfect or the timing synchronisation between the camera and the spinning disc is offset, then streaking and scan lines may degrade the image.
11. Just as a pixel (the *picture element*) is the smallest irreducible part of a digital image which represents the sampling intensity of the analogue photon stream emitted from the sample, so a voxel is a 3D volume element, which includes the step size between optical sections along the z-axis. Most voxels are anisotropic voxels because of the relatively poorer resolution along the z-axis, but a few image analysis packages require voxels having equal dimensions. These are called isotropic voxels; effectively the sample is significantly over-sampled along the z-axis to create these isotropic voxels.
12. Note this is the fixed illumination pinhole in front of the laser, not the variable aperture emission pinhole in front of the detector that controls the thickness of the optical section and the signal collected.
13. The Optical Index (see Chapter 31, Section 31.7) of a 60x/NA 1.4 plan-apochromat objective lens is about 23. Some of the best-designed objectives for confocal microscopy have an NA of 1, and an OI of 50. By comparison, the Mesolens has an OI of 117. It packs an unprecedented amount of resolved detail into the primary image plane.