Confocal Microscopy

The Development of a Modern Microscopy

"We are now able to take a series of photographs at slightly different focal planes or levels through a single cell or group of cells. These photographs result in a consecutive record of the internal architecture of the cell or cells. This accomplishment we have called "optical sectioning". ... By means of this new development a transparent specimen such as a group of cells may be sectioned optically. ... Detail above or below the focal plane does not interfere." [1]

Imaging in the Microscope

It could be claimed that the development of the modern microscope started with Abbe [2], who made many important contributions to different aspects of microscope design. But perhaps most importantly, he developed the basic principles of Fourier optics, that a lens performs a Fourier transformation, and that the microscope acts as a low-pass filter for spatial frequencies in the object. These ideas paved the way for the invention of Zernike's phase contrast microscope and the Schlieren method, and are a popular concept in modern optics. Abbe developed theories for coherent imaging, as is appropriate if the object is illuminated with a plane wave, but also for -incoherent imaging, as from a self-luminous object. The mathematical details of Abbe's theory were published later by Lummer and Reiche [3]. However, Abbe's ideas were not fully appreciated, and several publications argued that coherent illumination was unimportant and impractical [4]. This disagreement has even been presented as a dispute between Abbe and Rayleigh [5], who of course had published his own theory of resolution of optical instruments, in particular applied to the telescope [6]. There was a view that so-called critical illumination, where the source is focused on to the object, would give the best image formation. But it was found that Köhler illumination, in which the source is placed in the front focal plane of the condenser lens, gives equally good resolution. These controversies were eventually settled upon the development of the theory of partially-coherent image formation by Hopkins, in 1953 [7], who showed that critical and Köhler illumination are in principle equivalent, and, furthermore, that the aberrations of the condenser lens are unimportant: the condenser aperture
plays only a minor role in determining the resolution of the optical system.

Fourier theory can be regarded as more fundamental than Rayleigh's two-point resolution criterion, which is rather arbitrary. Rayleigh's criterion is based on the assumption of an unobstructed circular pupil function, which is special in the sense that it is the pupil that for the paraxial case maximises the intensity at the focus for a given power input [8]. Now it is known that pupils can be designed that can give an arbitrarily small central spot, at the expense of reduced power in this central lobe [9]. Abbe showed that the spatial frequency cut-off for an incoherent system is $2 \frac{NA}{\lambda}$, where the numerical aperture is $NA = \eta \sin \alpha$, which is twice that for a coherent system, with a cut-off frequency of $\frac{NA}{\lambda}$. Hopkins showed that, if the condenser numerical aperture is greater than or equal to that of the objective, for weak transilluminated objects the cut-off frequency is also $2 \frac{NA}{\lambda}$.

**The Ultra Microscope and Angular -Gating**

The ultramicroscope uses angular separation of the illuminating and detection beam paths to eliminate the strong undiffracted light component and thus allowing observation of weakly-scattering objects such as nanoparticles [10]. The deep field photographic microscope (66-8-DFM, KEM Equipment Company, Elk Grove Village, Illinois) used illumination "only within the focal depth of the objective while the specimen is being scanned. Thus out of focus parts of the object are always in darkness, and the final photographs show high resolution throughout the depth of scan." A similar instrument was manufactured by the Irvine Optical Corporation (Dynaphot II-A Burbank, California), which could produce images with greatly increased depth of field [11]. These instruments were fore-runners of the selected plane illumination microscope (SPIM) [12], also known as orthogonal-plane fluorescence optical sectioning, OPFOS [13]. This principle is now recognised as angular gating, one of several methods to give rise to optical sectioning, and to
eliminate multiply scattered light.

**Scanning Microscopes**

Zvorykin and Ramberg, in 1949 [14], p.383 described the flying spot microscope developed by RC Webb of RCA. This generates a scanning light spot using a scanned cathode ray tube. Young and Roberts [15] described the flying spot microscope system in detail, and pointed out the many advantages it has over ordinary microscopes. A microscope with a Nipkow disc scanning was constructed by Mellors and Silver [16]. Freed and Engle [17] reported a -vibrating mirror scanning system. Caspersson [18] described microscopes employing mechanical movement of the specimen or of a weak lens in the optical path. It is interesting to recall that there was strong interest in flying spot microscopy during the 1950s, culminating in a special meeting on the subject, reported in volume 97 of the Annals of the New York Academy of Sciences. Adam [19] used a rotating mirror for scanning. Phelan and DeMeo [20] used a single mirror for two-dimensional scanning. Olympus Optical offered a commercial laser scanning microscope (LSCM) in 1976. Lincoln Laser developed a commercial system using a polygon mirror scanner, while Newport Electro-Optics Systems developed a laser scanning microscope using an acousto-optical scanner, both in the 1980s.

**Confocal Microscopes**

It is widely accepted that the confocal microscope was invented by Marvin Minsky, who filed a patent in 1957 [21]. It should be appreciated that Minsky's patent was a long time ago, and preceded the invention of the laser, the development of sensitive photomultipliers, or the widespread availability of computers for image storage. Confocal imaging has the advantages that it exhibits an optical sectioning property, allowing the formation of 3D images and the rejection of scattered light when imaging into scattering media (confocal gating). It also exhibits a doubling of the spatial frequency cut-off, equal to 2 NA/λ for a coherent system and 4 NA/λ for an incoherent system.

It seems the confocal principle was appreciated before Minsky's patent was filed. Goldman [22] described a confocal slit system with line illumination in 1940, and demonstrated 3D imaging of the eye. Koana, in 1943, used the confocal pinhole to remove stray light, thus increasing the accuracy of microphotometry [23]. Although Koana's paper was written in Japanese, his collaborator Naora described the method in English, in the widely-read journal Science, among other papers [24, 25]. Zvorykin and Ramberg [14], p.298 describe a photoelectric microphotometer using line illumination and a slit in front of the detector.
Lukosz, in 1963, proposed that structured illumination can be used to increase the spatial frequency cut-off [26]. The object is illuminated by the image of a mask, which can be regarded as producing modulation, which transforms high spatial frequencies into the pass band of the optical system. In Lukosz's system, demodulation was achieved optically using a second mask, but digital methods are now frequently used. The method can be used with coherent or incoherent illumination, giving cut-off frequencies of 2 NA/λ or 4 NA/λ, respectively. Lukosz also proposed a near-field version using a grating moved close to the object plane. McCutchen, in 1967 [27] proposed a similar argument to explain the resolution improvement of confocal imaging, in which the focused image of a small stop is used to modulate the object spatial frequencies. He also described fluorescence microscopy, and near field microscopy using a small aperture moved close to the object.

A form of confocal microscope, using an array of apertures on a spinning Nipkow disc, was patented by Pêtrán in 1966 [28-30]. Pêtrán called this the tandem scanning microscope. This type of microscope was constructed commercially in small quantities by Pêtrán, and later in the 1980s by Tracor Northern. Goldman's confocal slit system [22] also incorporated angular gating, using a divided aperture system, in which the illumination and the image light pass through complementary semicircular apertures. Maurice [31] described the concept of the specular, or divided aperture, microscope. Svishchev [32-34], Baer [35], Maurice [36], and Koester [37] described combinations of the specular method with different patterns of illumination. Davidovitz and Egger [38, 39] used laser illumination in a confocal microscope. They acknowledge Baer for the suggestion of the "application of a laser to Minsky's microscope".

Sawatari [40] performed confocal detection using a heterodyne method. This is based upon the directional properties of heterodyne detection [41, 42]. Fujii showed that heterodyning can be used to construct a lens-less microscope [43]. Slomba, in 1972, described a laser flying spot scanner for use in automated fluorescence antibody instrumentation [44]. "A pinhole aperture ... is arranged to pass only the energy contained in that spot to the photomultiplier tube." This system was clearly confocal. Koppel et al., in 1976 [45] described a microscope with an "image plane field diaphragm", which is clearly equivalent to a confocal pinhole. A laser scanning microscope using mechanical object scanning was developed in Oxford in 1975, and a review of this work later published [2]. Early confocal fluorescence images were published by Cox of the Oxford group in 1984 [47]. The Oxford microscope was commercialised (the first commercial confocal microscope) by Oxford Optoelectronics, and later assigned to Lasersharp. Another important contributor to this era of the development of confocal microscopy was Brakenhoff.
Present day confocal microscopes rely on a dedicated computer for control and image storage. Here we mention some early applications of computers to microscopes. The TICAS system, in 1968 [49] digitised microscope images using a scanning stage. "The achromatic condenser ... focuses the field diaphragm as a ... spot of light in the plane of the cell to be scanned. The specimen is imaged onto a measuring diaphragm just in front of the detector ..." Thus it seems the TICAS system was confocal.

Spectre II was a computer-controlled microscope system in which the image was scanned optically (also in 1968) [50]. "The computer-controlled fine focus ... is presently unique among microscope scanners. It permits us to use the technique of 'optical serial sectioning ...'". Jarvis (also in 1968) [51] described a system with combined flying spot/moving table/video microscope scanner. A confocal microscope with computer image storage and processing was described by Cox in 1983 [52].

Partially Coherent Image Formation Theory

Hopkins [7] analysed the case of an incoherent source with a condenser lens, which together gives rise to a partially-coherent effective source. But now we know that this treatment does not hold for all imaging systems, for example confocal imaging. Further generalisations have been made to the cases of -coherent sources [53], confocal imaging [3], confocal systems with a finite-sized incoherent source [55], arrays of coherent or incoherent sources [56], and -coherent or incoherent structured illumination [57]. The paper by Sheppard and Choudhury [3] was perhaps the first paper to use the term "confocal -microscope". Interestingly, the authors had great difficulty in getting this paper published, as it was seen to contradict the accepted model of Hopkins.

The Future

Since its early days, confocal microscopy has become a common in many laboratories. But it has some limitations, and for many applications we may expect it to be replaced in the future by other techniques, structured illumination, multiphoton microscopy, or digital holographic microscopy. Of course, the exact details are difficult to predict.

References:

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