SOCIETY OF
ELECTRON MICROSCOPE
TECHNOLOGY

Hon Sec:
Dr G M Lewis
19 Bellfield Avenue, Harrow Weald, Middx. HA3 6ST
0181-428 4264 (Phone, Answerphone & Fax)
e-mail: cglewis@surfaid.org

CONFOCAL AND BEYOND

Wednesday 26 May - 2.00 p.m.

1999

LONDON SCHOOL OF PHARMACY
Brunswick Square

2.00 **Simplifying confocal microscopy: a real objective**
Dr Alan Entwhistle (Ludwig Institute for Cancer Research, London)

2.30 **Real time white light confocal microscopy**
Prof Tony Wilson (Dept. of Engineering Science, Oxford University)

3.00 **TEA** and **Software Demonstrations** by **Leica** and **Zeiss**

3.30 **Future trends in confocal microscopy**
Hugh Thomas (Leica Microsystems UK Ltd., Milton Keynes)

4.00 **Use of confocal microscopy to study gap junctions in communication in the retina.**
David Becker (Dept of Anatomy, University College, London)

4.30 Close

Members and others interested are very welcome to attend this meeting

RSVP to the Secretary by Monday 24th May **PLEASE**

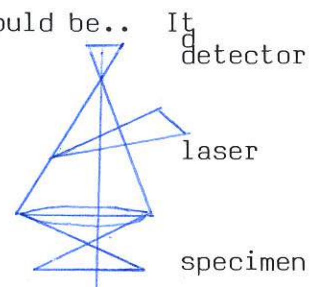
S E M T   Confocal and Beyond   26 May 1999

Simplifying confocal microscopy: a real objective

Dr. Alan Entwhistle

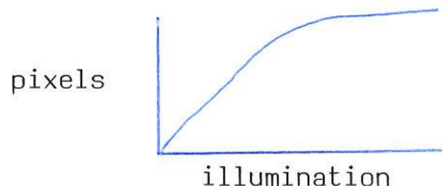
Ludwig Institute for Cancer Research, London

The confocal microscope is not easy to use, but it should be.. It requires a collimated light source, & a lens. A pinhole aperture is placed where the beam is focussed before the detector, & its position is critical; the position varies with use, the laser, drift, etc.



The beam scan is confocal; so much information is produced that a computer is necessary to process it. We need high spatial information and a high signal/noise ratio.

Increasing the amount of light gives maximum fluorescence up to saturation; but also increases the fading. Beyond optimum illumination, the pixels are saturated.



It is best to turn down the photomultiplier tube until you can only just see the image; this can then be enhanced on the computer. It is not necessary to adjust the photomultiplier tube.

It is necessary to take numerous views rapidly, because the fluorophores fade within microseconds. When pumped into the triplet state,there is no further movement. The ideal dwell time is about a microsecond, so the scan speed should be about 1 second.
So there are numerous frames to integrate. As we increase the number of frames, we also increase the sharpness and clarity. More information is obtained over 64 seconds than over 16 seconds.
Several lasers can be used simultaneously. Opto-acoustic modulators change the beam to fit the pinhole. Choose the fluophors and time; align the microscope at the beginning of the session. It is essential to have good software.

It is essential to be familiar with the light microscope, and <u>know</u> how to use it well.

Real-time white light confocal microscopy
Prof. Tony Wilson
Dept. of Engineering Science, Oxford

The pinhole produces optical sectioning, in sub-micron optical sections. Lasers are used for brightness, but allow only a limited choice of wavelengths. The results are usually not real-time; there are problems with alignment of the pinhole; it is necessary to scan.
We want easy alignment; no scanning; real-time results; no laser. This is basically the ordinary light microscope, but with the latter there is no possibility of optical sectioning.
The correlation approach leads to a better light budget.
We define optical sectioning in terms of spatial frequencies.
We require all spatial frequencies to attenuate with defocus:
- illuminate with one spatial frequency, to produce a fringe;
- obtain the optical-section image, with fringe superimposed;
- remove the fringe

to produce the optical-section image.

It is also possible to introduce an ID grating into the illumination path, a piezo-driven 25µm pitch grating.

It is possible to have the conventional image and the optical section on the screen at the same time.

The grid works well to produce the fringe pattern, with widefield lifetime imaging. If there is no physical grid, it is possible to use a laser at an angle - this uses no lens, but only mirrors.

We cna now get a real-time TV-rate 3-D image, with a simple light source, & novel fluorescence geometry.
This will lead to exotic wavelengths, deep UV, auto-fluorescence; widefield imaging.

Future Trends in confocal microscopy
Hugh Thomas
Leica Microsystems UK Ltd, Milton Keynes

Confocal microscopy eliminates flare from out-of-focus images; the Z resolution is good, and optical sections can be obtained.

TCS-SP is a spectral imaging confocal system, PC-based, developed in 1966.
In 1967 the multi-band detector and confocal spectrophotometry were developed.
There are no filters in the detector; transmission is 85%. Four integrated spectral photometers. λ = 400 - 750 nm.
The system is flexible for dye selection. It has maximum emission sensitivity; minimum emission cross-talk; it maps the spectral characterisitics of emitted light.
There is optimal spectral detection, separation, and detection of emission.

2-photon laser scan only excites the label at the focus of the beam; there is no out-of-focus fluorescence, so no need for a confocal aperture.
But:
- bleaching is higher than with the single-photon mode at the focal plane, the point of excitation;
- XYZ resolution is less than with single-photon;
- 3-photon effects damage DNA
- expensive, Clas IV, lasers are necessary, with special precautions.

Bleaching only occurs at the focal plane.
UV excitation without UV light - causes less damage to live cells.
There is less scatter, & better penetration.

The system can be used for live-cell imaging; ion concentration measurements; thick tissue. A short pulse length increases the efficiency of excitation, but high peak power leads to more specimen damage.
The system for live cells and deep penetration is expensive

Use of confocal microscopy to study gap junctions in communication in the retina

David Becker
Dept. of Anatomy, University College London

Gap junctions allow ions (? & dyes) to enter the cells. They consist of rings of proteins, which can open & close rapidly, e.g. between all the cell types of the retina, & vary the properties of the channels.
There is connexin expression in the developing chick retina.
Calcium is active during cell division.

ABSTRACTS

Simplifying confocal microscopy: a real objective

Dr Alan Entwhistle
Ludwig Institute for Cancer Research, London

A period of thirty years elapsed between the first conception of the confocal microscope by Minsky and instruments being manufactured and sold in significant numbers. During this interval, the theory which underpinned the confocal beam scanning microscope was elucidated, much of it by Brakenhoff, Sheppard and Wilson but the construction of a commercial product had to await the elaboration of a number of technologies. In instruments illuminated by a single diffraction limited spot, like the confocal microscope, the dispersion of the illumination must be very small. Suitable light sources are exemplified by the continuous wave laser and mass production of the confocal microscope only followed after their development. Beam scanning instruments also produce prodigious amounts of data which must be stored. When the confocal microscope is employed for its most common use, examining fluorescent emissions, very large numbers of small electrical signals from photoelectric devices have to be recorded. Computer based or digital technologies are ideal for this job and it is no coincidence that the commercial exploitation of the confocal principle only followed after the production of small, relatively inexpensive digital chips.

The development of lasers and computers, whilst essential for the production of beam scanning confocal microscopes, fails to address another important limitation on their performance: the diffraction limited spot illuminated by the laser must exactly coincide with the equally tiny volume in space that is probed by the detector There is nothing in the design of these instruments that says this must automatically be so, the only reason that these two minuscule volumes coincide is because someone has very carefully aligned the detector arm of the confocal microscope with the illuminator arm. Moreover, this careful alignment can soon be undone by thermal, mechanical or electrical drift A third development, cheap, fast opto-acoustical scanners, devices that can change the direction of propagation of a beam of light in the microsecond time scale using ultrasonic deflection, means that confocal microscopes can now be designed which can be both largely self-aligning and then correct their own alignment dynamically. Further exploitation of this technology and its integration with computer software that is designed to address the limitations of the average user and the fluorophores they use, finally makes possible the design of a confocal microscope that is simple to use. Sadly, despite all that the manufacturers might tell you about the 'user friendliness' of their instruments, this is still a dream and more sadly, unless the potential users of these instruments actually start to take the trouble to learn to use a light microscope properly, it will remain a dream for ever.

Real time white light microscopy

Prof Tony Wilson
Department of Engineering Science
University of Oxford
Parks Road, Oxford Ox1 3PJ

The key elements in the optical system of a confocal microscope are the point source and point detector. The use of these has the effect that only light which has arisen from the focal region is detected efficiently since the point pinhole detector serves to attenuate greatly light from out of focus regions of the object. However the use of a single pinhole source and detector means that only one object point is imaged and so it is necessary to introduce scanning to image a finite region of the object. Further drawbacks to this single point source/point detector approach are that the image is not usually obtained in real time and also incandescent light sources are not usually sufficiently bright and hence lasers are usually used. We will describe a new form of confocal microscope which has been developed in our laboratory together with Rimas Juškaitis and Mark Neil in which there is no need to scan and traditional white light sources may be used and the image is formed in real time at T.V. rate.

In order to be able to replace the laser source with an incandescent white light source it is necessary to increase the light budget of the system. One approach would be to build many confocal systems in parallel. This is the approach adopted in the tandem scanning microscope which employs an aperture disc containing many pinholes. However since it is necessary to avoid cross-talk between neighbouring 'confocal systems' it is necessary to space the pinhole apertures far apart. In practice this means that only about 1% of the available light is used for imaging and that the aperture disc must be rotated (scanned) in order to 'fill in' gaps between the points in the object sampled by the neighbouring confocal systems. However these systems produce real time confocal images with non laser sources.

Our approach is to increase the light budget further and to obviate the need for scanning by placing the pinhole apertures as close together as possible. This will result in efficient use of the available light but a method of preventing cross-talk between neighbouring confocal systems must be found. We achieve this by using an aperture mask which is programmable in the sense that the transmissivity of the individual pixels may be changed in time and then use aperture correlation techniques to eliminate cross talk. We apply independent sequences to vary the transmission of each pixel such that the sequences presented to any one pixel is uncorrelated with that presented to any other pixel in the aperture. In this way we are able to build an instrument which produces focal images in real time. We will present two designs of such a system together with images obtained in both reflected light and fluorescence. Finally, a novel approach using no moving parts will be described.

Future Trends in Confocal Microscopy

Hugh Thomas
Leica Microsystems UK Ltd

This presentation covers three main topics:

1. What is confocal microscopy?
2. Introduction of a new "Spectral" Confocal microscope design concept.
3. An overview of 2-photon microscopy - advantages and disadvantages.

This talk covers a brief overview of classical confocal microscopy outlining the main advantages of the technique which has been commercially available for just over a decade. This is developed into a discussion on a new "spectral" confocal scanhead design where the emission filters are replaced by a series of spectrophotometers; the main benefits being enhanced flexibility for dye selection, reduction in cross talk and "lamda" scanning allowing the real emission spectra of the dye to be mapped.

Finally, a brief resumé of a new technique called 2-photon excitation microscopy outlining the basic technique and demonstrating the main advantages, such as much better specimen penetration and reduction in photobleaching which is very appropriate for live cell applications.

Use of confocal microscopy to study gap junctions in communication in the retina

Dr David Becker
Dept of Anatomy, UCL