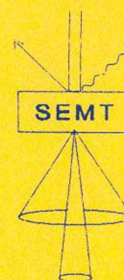
SOCIETY OF ELECTRON MICROSCOPE TECHNOLOGY



CONFOCAL IMAGING

2.00 p.m. Wednesday 15 October 1997

EASTMAN DENTAL INSTITUTE
Grays Inn Road
London WC1X 8LD

1.00 **Trade Exhibition**

2.00 **Imaging ion concentrations in living cells with the confocal microscope**

(Steven Bolsover (Dept. Physiology, University College London)

2.30 **Optical Studies of hippocampal synaptic plasticity**

Nigel Emptage (Divn. Neurophysiology, Natl. Inst. of Medical Research, Mill Hill)

3.00 **Tea and Trade**

3.30 **3-D fluorescence microscopy; a quantitative tool ?**

Nick White (Dept. Plant Sciences, Univ of Oxford)

4.00 **Confocal microscopy in a study of nuclear organization and activity**

Peter Shaw (John Innes Centre, Norwich)

4.30 **Closing discussion and Trade**

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Please reply to the Secretary - Dr Jill Lewis
19 Bellfield Avenue, Harrow, Middx. HA3 6ST
0181 428 4264 (Telephone, Answerphone, Fax)

I hope to be present at the meeting on 15 October 1997

Name ....

Address ....

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Phone......................................

ADVANCES IN CONFOCAL MICROSCOPY

October 15th 1997

Abstracts

Imaging ion concentrations in living cells with the confocal microscope.

Steven Bolsover (Dept Physiology, University College, London)

The availability of fluorescent indicator dyes developed by Tsien and others has led to an explosion in the use of fluorescence imaging for the measurement of ion concentrations in living cells, giving us a far more precise view of the spatial complexities of ion concentrations within cells. However, there are two types of measurement for which standard fluorescent microscopy is insufficient. Firstly, confocal microscopes can be used to measure ion concentrations within cells in blocks of tissue, even though cells above and below the cell of interest contain the indicator dye. My laboratory has used this approach to follow [Ca++]i in developing motoneurones within whole zebrafish embryos. Secondly, confocal microscopes can be used to resolve ion concentrations in regions of single cells that are completely enclosed within other fluorescent volumes of the same cell. My lab has used this approach to follow [Ca++]i within the nuclei of stimulated nerve cells.

As with any technique, confocal imaging can give rise to signals that do not truly represent the pattern of the phenomenon one is studying. In particular, when dye fluorescence is different in two cell regions, such as the nucleus and cytosol, four independent factors can be responsible:

1. The degree of loading of the dye with ion ([ion:dye]/[free dye]) may be the same in both locations, but the fluorescent properties of the dye may be different in the two locations.
2. The free ion concentration may be the same in the two locations, but the degree of loading of the dye with ion may be higher in one location due to an effect of the environment on the affinity of the dye for the measured ion.
3. Dye may be present in cell volumes that one does not expect it to be in, for example, many measurements of cytosolic [Ca++]i are in fact severely contaminated by signal from dye that is located in subcellular organelles.
4. Lastly, the measured gradient may be real.

I will discuss problems 1 to 3 and (a) the degree to which one needs to worry about them and (b) what procedures can be carried out to evaluate and reduce them.

3-D fluorescence microscopy: a quantitative tool?

Nick White (Dept of Plant Sciences, University of Oxford)

I will talk about our recent work on 3-D and 4-D microscopy of intact plant and animal tissues. I will also cover some new developments in laser scanning microscopy and digital microscopy. The aim will be to show, in practical terms, how to make multidimensional microscopy a quantitative tool and to describe the current state of progress in these areas.

Optical studies of hippocampal synaptic plasticity

Nigel Emptage (Divn of Neurophysiology, Natl Inst for Med Res, Mill Hill, London)

Long-term potentiation, an activity dependent form of synaptic plasticity, has become the dominant model for the study of learning and memory in the mammalian brain. However, despite more than 20 years of investigation, fundamental mechanistic features of this form of plasticity have remained illusive. One specific example is whether the locus of the increase in synaptic efficacy occurs within the pre-or post-synaptic neurone. Attempts to address this issue using electrophysiological methods have produced conflicting results creating a need for an alternative approach. One alternative that offers considerable promise, is *living tissue imaging,* specifically taking advantage of the ever increasing array of biologically relevant fluorescent probes. In this talk, I shall discuss one and two-photon microscopy as used to investigate activity at a single synapse in organised neural tissue. I will argue that the use of ion-sensitive fluorescent probes, such as those detecting calcium conmcentrations, can provide reliable assays of activity at the synapse, and that this information can be used to address fundamental questions such as the locus of long-term potentiation.

Confocal microscopy in the study of nuclear organization and activity.

Peter Shaw (John Innes Centre, Colney, Norwich)

The major advantage of the confocal microscope over conventional fluorescence microscopy is that the optical arrangement excludes most of the out-of-focus light, giving clear focal sections, and allowing 3-D reconstructions to be made as a series of images at successive focal planes. In contrast, with conventional optics the focal plane is degraded by blurred contributions from the other planes of the specimen. Thus the advantage of confocal microscopy is greatest for thick and brightly labelled specimens. Confocal microscopy has been particularly important in studies of plant cells and tissues, since the cells are often large and deep, and cells of interest are usually found deep within the plant. In order to image cells and tissues faithfully in three dimensions, it is important to use specimen preparation techniques which preserve the 3-dimensional structure as much as possible. We have developed labelling techniques for thick tissue sections cut using a vibratome. Typically these sections are 2-3 cells thick and permit confocal microscopy to be used to obtain 3-D reconstructions of entire cells or groups of cells.

I shall describe some of the ways fluorescence *in situ* and antibody labelling techniques have been coupled with confocal microscopy in my laboratory to study the organization of nuclei in plants. We have demonstrated that the organization of sites of transcription of ribosomal RNA within the nucleolus, the sub-nuclear compartment where ribosomes are synthesised, comprises many (100 or more) small foci. There are many copies of the ribosomal genes (thousands in some plants). Correlatioin of transcription labelling with *in situ* labelling of the genes suggests that each transcription site represents a single gene. We have also analysed the nuclear organization of the chromosomes during interphase in wheat root cells. The chromosomes are arranged in a very regular parallel array. The centromeres, specialised regions at the centre of the chromosomes, are clustered in one region of the nuclear periphery, while the telomeres - the ends of the chromosomes - are arranged on the opposite side of the nuclear periphery. Thus the two arms of the chromosomes lie next to each other, in the so-called Rabl configuration. Correlation of chromosome labelling with sites of transcription within the nucleus shows that the nuclear transcription sites are also widely dispersed foci, and that they are dispersed uniformly throughout the nucleus, rather than being concentrated at the centromere or telomere regions. The sites are also distributed throughout the chromosomal domains, rather than being concentrated at the domain boundaries.

S.E.M.T. Confocal Imaging 15.10.97

Imaging ion concentrations in living cells with the confocal microscope

Steven Bolsover
Physiosology, University College London

The first slide was of salamander retina on a flat surface; confocal imaging was not nedded because the surface was flat. Confocal is most useful for volumes within cells, or cells within a solid tissue. All the tissue receives light, but <u>only</u> that part in the plane of focus passes through the aperture to the image plane.
Fluo-3 dye is often used. It is possible to colour-code the fluoresent images, by dividing the fluorescent image by the control image; but care is needed with the interpretation.
The dye Indo-1 is ratio-metric, the calcium-free part of the image appearing green, and the calcium-containing part blue.
Depolarisation allows calcium into the cell through the membrane, giving a warmer colour. The nuclear envelope is not a serious barrier to calcium ions. It is possible to make an XY picture.

The zebrafish embryo takes only 3 days from fertilisation to a free-swimming fish. Calcium is needed for the development of the motor neurones, going from the spinal cord to the muscles. There is a 4-minute oscillation in the developing axon, which is very important for the correct development of the nervous system to make the right connections.

Assumptions:

1. That we know what fraction of the signal comes from the intracellular dye. (that the error from autofluorescence would be the same everywhere). But e.g. the mitochondria may have a lot of autofluorescnece, the nuclei only a little.

2. That we can calculate the ion concentration from the autofluorescent signal. But the error is not the same everywhere; the affinity of the dye for the ion may be different.

3. That the dye is in the cytosol and nucleoplasm, not in the organelles. But it may still load the organelles.

Dye may enter the organelles by (1) transport of free dye, (2) endocytosis, (3) hydrolysis of ester within the organelles. This can only be avoided with certainty by injecting dextran-loaded dye into the cytosol, to avoid the organelles.

4. That the presence of the dye and the process of measuring it are not affecting physiological cellular phenomena.
But putting the tissues in buffer and blasting them with light is liable to affect the working of the cell.

He is not sure whether deconvolution can be trusted for the reliability of absolute numbers.

Confocal microscopyin a study of nuclear organisation and activity

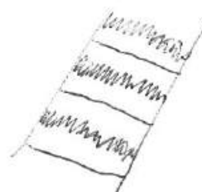
Peter Shaw
John Innes Centre, Norwich

A lot of genetic engineering is being done at the Centre, so there is a lot of interest in nuclear organisation.
Apertures are placed in the beam for the laser and for the photomultiplier.
Usually we just want one phase; occasionally 3-D.

The cells are first permeabilised with non-ionic detergent; then stained with bromo-UTP for transcription. Much more fluorescence is found in the nucleolus than in the nucleoplasm.

Ribosomal RNA genes transcribe in the nucleolus; the spacer and coding regions do not completely overlap, but the silent regions do. If labelling is with different dyes for the active regions, numerous small foci can be seen, one for each gene.
The ETS anti-sense labels the same centres as does bromo -UTP, but more diffusely.
The first and second cleavages occur in different regions of the nuceleolus.
The metaphase chromosome is in the resting stage; there are techniques to stop it unravelling. Treatment with detergent leads to the formation of nuclear halos because of loops in the chromsome; this indicates that the interphase chromosome has loops in it.
Each chromosome has its own discete domain.

In wheat, the centromeres and teleomeres line up; this brings the transcription sites uniformly through the chromsome.



3-D fluorescence microscopy: a quantitative tool ?

Nick White
Dept. Plant Sciences, Oxford

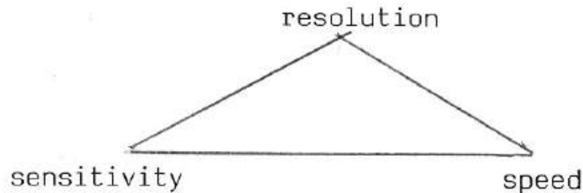
If sufficient high-power pulsed laser is put in (but not enough to fry the sample!), there is a good probability of obtaining 2 photons per molecule, and thus exciting the whole specimen. Multi-photon confocal microscopy does not need apertures, because there is only one plane with enough excitation.

If chloroplasts are present in the specimen, they will absorb some red light; so we must be careful about the wavelength used.
Deconvolution improves resolution.

It is necessary to tell the software what immersion medium and mountant are being used; the sample is an optical component of the system. If the microscope <u>or</u> the specimen bend light, this compromises confocality.

With the 1-photon system, the light may not come back at the same wavelength because of attenuation; with the 2-photon system, only 800 λ comes back.
It is possible to use colour to show relative volumes of cells.

Imaging with isotropic response:
- direct measurement
- reliable visulaisation
- FFT-based deconvolution



the confocal triangle

Handbook of biological confocal microscopy
ed. J.B. Pawley, 2nd. ed.

Objectives are still not well correected for the far red; but this is not very important, because in the 2-photon phase, the pulse is lengthened.

Optical studies of hippocampal synaptic plasticity

Nigel Emptage
Divn.Neurophysiology, NIMR Mill Hill

He uses the 2-photon technique, which is simple but needs more instrumentation.
The active site can be identified:

- calcium-sensitive dyes, e.g. calcium green
- fluorescent probes, eg GFP
- lipophiolic dyes, eg FM 1 - 43 (problematic with nerve tissue)
- sodium-sensitive dyes eg SBF1 (not for resolving small changes)
- voltage sensitive dyes eg TMRE (cannot yet resolve small changes)

The intensity decreases with the depth within the specimen; by 140μ (eg in brain slice), 50% of the intensity has been lost.
Red light penetrates much better than green of blue. But the dyes are not excited by red and infra-red light, which has less energy; so fluorochromes are used with 2-photon microscopy - $E = h\nu$
Total volume excitation over numerous cycles will bleach above and below the area of interest with one continuous photon; 2-photon system only bleaches the level being examined.

The 2-photon system uses red light, which causes less biological damage, can be used on living cells, and used conventional visible-light optics.

SEMT Meeting October 15 1997

List of Registrants

Paul Ansell	Hitachi Scientific Instruments
Michael Bayliss	BVS, Royal Vet Coll, London
Douglas Banning	School of Pharmacy, London
Andrea Boyd	Dept Oral Medicine, Guy's Hospital
John Bredl	Royal Veterinary College
Terry Cooper	Taab Laboratories, Aldermaston, Berks.
Heather Davies	EMU, Biol. Sci., Open University, Milton Keynes
Mala Dhir	Biorad, Hemel Hempstead
Anne Drewe	Microbiology, Charing Cross & Westminster Med School
Barry Dowsett	CAMR, Porton Down, Wilts.
Jaydesh Dudha	BVS, Royal Vet Coll, London
John Gilbert	Jeol (UK) Ltd., Welwyn Garden City
Alan Gray	London Hospital Medical College
Takesh Hamura	School of Pharmacy. London
Elaine Harrison	Biol Dept, Open University
Gisele Hodges	Queens University, Belfast
Tania Hopcroft	Royal Veterinary College
As'ad Abu Khalil	School of Pharmacy, London
Mike Kelly	Dept Oral Pathol., London Hospital
Andrew Kent	Neuroscience, UMDS, Guy's Campus
Gill Lewis	EMU, Eastman Dental Inst
Iben Larsson	Fellowship House, WC1 2AB
Joanne Loaman	Dept Pharmacol, UCL
David McCarthy	EMU, School of Pharmacy
Hilary McPhail	Dept Physiol., St Mary's Hospital Medical College
Steve Marsh	Dept Pharmacol, UCL
John Millar	VBS, Royal Veterinary College
Nicky Mordan	EMU, Eastman Dental Hospital
Angie Poole	Royal Vet College
Phil Salmon	Royal VeterinaryCollege, London
Padmini Sarathchandra	EM, Surgical Research, NPIMR, Harrow
A.Selyanko	Dept Pharmacol, UCL
Elaine Shervil	Royal Vet College
Alain Stewart	Biorad, Hemel Hempstead
Rosemary Suswillo	Royal Veterinary College
Verina Waights	Dept Biol, Open University
Amanda Wilson	EM, St George's Hospital
Etsuo Yonemochi	School of Pharmacy, London