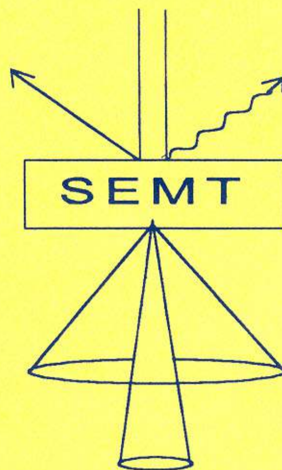
SEMT

ONE DAY MEETING

Wednesday 24 March 1999

London School of Pharmacy
Brunswick Square
London WC 1



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ACKNOWLEDGMENTS

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FUTURE PROGRAMME

Wed 26 May	2.00 p.m.	**Confocal and Beyond**	School of Pharmacy
Wed 13 Oct		**Visit to British Antarctic Survey**	Cambridge
Wed 8 Dec	2.00 p.m.	**Microscopy from the Natural World**	School of Pharmacy

WELCOME TO THE ONE-DAY MEETING

PROGRAMME

9.15 Coffee, Trade Exhibition

10.00 An introduction to the atomic force microscope and microthermal analysis
Paul Royall (London School of Pharmacy)

10.30 Ion beam microscopy - milling, imaging and analysis in one instrument
John Walker (FEI Europe Ltd. Cottenham, Cambridge)

11.10 High resolution quantitative immunocytochemical localisation of neurotransmitter receptors in the brain.
Prof Peter Somogyi (MRC Anatomical Neuropharmacology Unit, Oxford)

11.50 Some special applications of electron microscopy in plant cell research
Kim Findlay (Dept of Cell Biology, John Innes Centre, Norwich)

12.30 LUNCH Trade Exhibition - Posters - Micrograph Competition
Presenters to be with their posters from 1.30

2.00 Bioreactivity and fate of diesel exhaust particles
Prof. Roy Richards (Cardiff School of Biosciences, Cardiff, Wales)

2.40 Microscopy of things at home
Phil Robinson (Ceramic Technology, Staffordshire University))

3.20 Getting the most for your money - confocal imaging of multiple indicator dyes in living cells
Steve Bolsover (Dept of Physiology, University College, London)

4.00 TEA Presentation of micrograph competition prize

An introduction to AFM and microthermal analysis

Paul G. Royall, Duncan Q.M. Craig and David McCarthy.
Centre for Materials Science, School of Pharmacy, University of London,
29-39 Brunswick Square, London WC1N 1AX, UK.

Micro-thermal analysis, (*μTA*™), combines the principles of atomic force microscopy (AFM) with thermal analysis, [1,2,3]. For this application of AFM the tip is modified to allow the probe to act as a heat source with the ability to measure the response to the temperature program for a number of physical properties, which include thermal conductivity and the mechanical properties of the sample as a function of temperature. In addition to the thermal images, standard AFM topological images are gathered simultaneously in contact mode. Once an area has been scanned localised thermal experiments maybe conducted at points of thermal or topological contrast on the image. This facility allows the thermal or mechanical characterisation of specific domains of the samples surface identified in the topology or conductivity maps. Therefore *μTA*™ offers the ability to locate and characterise the microscopic regions of differing components or phases on the surface of complex systems. Such spatial resolution is not possible with standard thermal analytical techniques which measure the bulk properties of the material.

The ability to resolve spatially the thermal properties of different surface domains has allowed *μTA*™ to investigate the surface characteristics of various drug delivery systems. In this paper we report the novel use of *μTA*™ to characterise the surface of a number of compacts containing ibuprofen and HPMC, in addition to analysing the surface of progesterone-loaded poly(d,l-lactide) microspheres. These two examples present typical characterisation issues involved with tablets and drug delivery systems.

M. Reading, D. J. Hourston, M. Song, H. M. Pollock, A. Hammiche. Thermal analysis for the 21st Century. Am. Lab. 30: 13-17 (1998).
D. M. Price, M. Reading, A. Caswell, A. Hammiche, H. M. Pollock. Micro-thermal analysis: A new form of analytical microscopy. Mic. Anal. 65: 17-19 (1998).
D. Q. M. Craig, P. G. Royall, M. Reading, D. M. Price, T. J. Lever, J. Furry. Micro-thermal analysis for the characterisation of pharmaceutical materials. 26th NATAS Conference, Cleveland, Ohio. pp610. (1998).

Ion beam microscopy - milling, imaging and analysis in one instrument

John F. Walker
FEI UK Ltd

While many forms of microscopy are regularly used to solve a vast array of problems, optical microscopy and SEM are ubiquitous. However, ion beam microscopy using gallium ions from a field-emission source can approach and in some cases exceed the resolution of SEM. In addition to the imaging capability is the ability to mill making the system an ideal in-situ preparation tool. Coupling this unique combination with the analytical capability of SIMS allows the user access to enormous problem solving power.

In the first part of the talk the basic principles are described. The layout of the column is shown and emphasis given to the liquid metal ion source (LMIS). Subsequently, various applications are described. The main techniques used are 1) viewing sub-surface structure by precise milling and virtually simultaneous imaging and 2) elemental mapping and other SIMS analysis to determine the composition of a specimen. The application of the above two techniques to several different systems is described.

High resolution quantitative immunocytochemical localisation of neurotransmitter receptors in the brain.

Peter Somogyi, MRC, Anatomical Neuropharmacology Unit, Oxford University, Mansfield Rd.
Oxford, OX1 3TH

Neurones in the brain receive synaptic input from several sources releasing the same neurotransmitter producing different effects depending on the location of the input on the neurone and/or the receptors mediating the physiological effect. The most common transmitters are the amino acids glutamate and GABA, each detected by a large number of functionally distinct receptors. Realistic explanations of neuronal operations require a definition of the receptor species in relation to the source of the synaptic input (1). Synaptic junctions are most easily identified by electron microscopy. Receptors are commonly localised by the immunoperoxidase method, which has low resolution because it relies on a diffusible endproduct that precipitates on all surrounding cellular elements making it difficult to allocate the source of the endproduct to a specific site in the plasma membrane. Because receptors activated by synaptically released transmitter are present in both the synaptic junction and in the extrasynaptic plasma membrane the presence of peroxidase endproduct on the synaptic specialisation or in the synaptic cleft cannot be taken as evidence that the given receptor is present in the synapse. Usually only particulate markers, such as colloidal gold, are suitable for the electron microscopic identification of synaptic receptors. My colleagues and I have been using post-embedding electron microscopic immunogold labelling on Lowicryl-embedded fixed brain tissue to assess the location of different classes of receptors in relation to transmitter release sites on the surface of neurones. Because not all antibodies provide a signal under post-embedding conditions sometimes it is necessary to employ pre-embedding immunogold methods using 1.4 nm gold-coupled secondary antibodies and silver intensification to reveal the gold. This method is particularly useful for visualising receptors outside the synaptic junction.

The results show that AMPA and NMDA type glutamate receptors, which are heteromultimeric ion channels, are highly concentrated in the *postsynaptic membrane specialisations,* but show only partial overlap in synapses of hippocampal pyramidal cells (1, 2). The largest spines have the most AMPA receptors and a significant proportion of spines lack detectable AMPA receptors. Calibration of the immuno-signal to functional receptors revealed that, on average, one immunogold particle represents ~2.3 functional receptors. Receptor content in individual synapses of the same connection can range from fewer than 3 up to 200 functional AMPA receptors. Synaptic AMPA receptor content is cell type specific and depends on the input pathway on a single cell, therefore both pre- and postsynaptic elements contribute to the quantitative pattern of receptor expression. Interestingly, the pathways which show NMDA receptor-dependent synaptic plasticity exhibit the largest variability in AMPA receptor content and contain a high proportion of synapses with few receptors. The NMDA receptor content of the same population of synapses show a different distribution.

Metabotropic (G protein coupled) glutamate receptors (mGluRs) are excluded from the postsynaptic density (3), instead mGluRs I and 5 are concentrated in a *perisynaptic annulus* surrounding the synaptic specialisation and they also occur in decreasing density in the *extrasynaptic membrane,* or, as mGluR2, are not associated with synapses at all. Therefore, synaptic G protein-coupled receptors are segregated from ionotropic receptors in the synapse. *Presynaptic* mGluRs also show synapse specific patterns of distribution (4); group III receptors (e.g. mGluR7) are strongly concentrated in the synaptic active zone, the site of transmitter release, whereas group II receptors are along preterminal axons and in the non-synaptic terminal membrane.

Receptors for GABA show the same principles of distribution (1,5). The heteromultimeric A-type receptor, which forms an anionic channel, is highly concentrated in the synaptic specialisation, and it also occurs in the extrasynaptic plasma membrane in a subtype and cell type specific manner. Surprisingly, at least one receptor subtype is excluded from the synaptic junction where GABA is released. Up to three sizes of gold particles have been used on the same section to reveal the location of different receptor subunits on the same cell (5).
The highly constrained locations of receptors underlie localised molecular interactions in defined membrane domains, leading to selective effects depending on the receptor subtypes and their location. The location and composition of receptors as well as their numbers in the plasma membrane undergo dynamic changes governed by pre- and postsynaptic neuronal activity (6).

(1) Somogyi P, Tamas G, Lujan R, Buhl EH (1998) Brain Res Rev *26:13-135*. (2) Nusser Z, Lujan R, Laube G, Roberts JDB, Molnar E, Somogyi P (1998) Neuron *21:545-559*. (3) Baude A, Nusser Z, Roberts JDB, Mulvihill E, McIlhinney RAJ, Somogyi P (1993) Neuron 11:771-787. (4) Shigemoto R, Kulik A, Roberts JDB, Ohishi H, Nusser Z, Kaneko T, Somogyi P (1996) Nature 381:523-525. (5) Nusser Z, Sieghart W, Somogyi P (1998) J Neurosci 18:1693-1703. (6) Nusser Z, Hajos N, Somogyi P, Mody I (1998) Nature 395:172-177

Some special applications of electron microscopy in plant cell research.

Kim Findlay (Dept of Cell Biology, John Innes Centre, Norwich)

The outward appearance of a plant is essentially very different from an animal, yet the same basic structures and organising principles apply equally well to cells in plants as they do to cells in humans. At the cellular level, there are only three real differences between animals and plants as far as preparation for the EM is concerned; plants have cell walls, vacuoles and plastids. Insignificant as they may seem, these differences can offer us serious problems during fixation, embedding and sectioning, and have been the main stumbling-block in advancing the study of plant cell biology as compared to that in animal research. Botanical microscopy has concentrated on the study of meristematic tissues (e.g. root tips) in order to avoid these problems as much as possible, but when we need to sample the great majority of plant cell types, walls, vacuoles and plastids become the microscopists' nightmare. This talk describes some of the difficulties involved with preparation for EM imaging of plants and uses several examples of the work done at the John Innes Centre to illustrate this, concentrating on the research into cell walls themselves using both TEM and ambient and low temperature FEG-SEM in particular. Plants offer us many challenges as electron microscopists; the resultant images can demonstrate both the beautiful and bizarre.

Bloreactivity and fate of diesel exhaust particles

Roy Richards, Cardiff School of Biosciences
Cardiff University

Carbon and diesel exhaust particles (DEP) form part of the aerial pollution mixture found in the urban environment in the UK. There is some concern that these tiny individual (ultrafine) particles (20-30nm), with potentially complex surface chemistry, may he detrimental to

health. Unfortunately, there are few human or animal studies with DEP or other ultrafine particles by which we can explain their mechanisms of action or bioreactivity.

Light and electron microscopy, together with biochemical assessment of lung tissue has provided evidence for a sequential series of changes which are notably similar with a wide ranging group of bioreactive particles or chemicals. These changes after insult, including those to the lining fluid, type 1 epithelium, endothelium, alveolar permeability, free cells, type 2 epithelium and interstitium, their correction or non-reversibility will be described. A comparative toxicological study in rats with small masses of instilled particles (1) a non-ultrafine quartz mineral (pure crystalline silicon dioxide) which causes progressive lung damage (2) an ultrafine amorphous silica (Cabosil-pure silicon dioxide) which causes transient damage and (3) an ultrafine carbon black thought to be relatively inert was carried out to assess the potency of DEP. DEP were found to be weakly bioreactive but had the ability to induce a transient lung permeability change (1 week post-instillation) and possibly a mild inflammatory response. Further light and electron microscope studies showed that DEP induced some swelling in alveolar septa in which particles had passed the epithelial barrier and were located in the interstitial cells (6 weeks post-instillation). The actual cells containing DEP, which are often present in large aggregations, are difficult to identify as are some of the other interstitial components. However, many of the particles make a slow journey to the lymph nodes such that at 30 weeks post-instillation there are large aggregations of DEP in the hilar regions. This DEP aggregation seems tolerated at this site, unlike the effects observed with bioreactive quartz which induces a three fold increase in lymph node size.

Microscopy of Things at Home

Phil Robinson Ceramic Technology, Staffordshire University

This paper serves as a reminder of the strengths, as well as the weaknesses, of light microscopes, using the domestic scene as a context in which to illustrate the principal points made.

Everyone is familiar with the beam-matter interactions diagrams which serve to remind us of the possibilities of information gathering of electron microscopes. They may be less familiar with the "checklist" for light-matter interactions, which condense to information on colour, boundaries with refractive index change, and anisotropy. Additionally, unless it is decided to make modifications of temperature and/or pressure, all light microscopes are "environmental", and may be employed in examinations of watery materials, of preparations using other liquids, such as refractive index liquids, and even of live specimens. There are not the perennial problems of desiccation, charging and beam damage. When preparations are transparent to light, we may collect information from depths of hundreds of micrometres. While resolving powers in the specimen plane may be one micrometre, comparable with the spatial resolution of X-ray microanalysis in many investigations, the z axis resolving power may be measured in nanometres.

These points will be reviewed briefly, and illustrated with examples selected from the materials which are likely to be found "in the home", although, perhaps, not in every home at any time!

Getting the most for your money: confocal imaging of multiple indicator dyes in living cells.

Stephen Bolsover and Michael Duchen, Physiology Imaging Consortium, University College London.

Most confocal microscopes can acquire data from a number of channels simultaneously, each channel corresponding to a different emission wavelength. This gives users the opportunity to combine dyes to get more information from each experiment.

In one such approach, one dye that reports a varying intracellular parameter is combined with a dye whose function is only to report its own spatial location. For instance, the Bolsover lab. is studying individual nerve cells within developing zebrafish embryos; each cell contains both rhodamine dextran to indicate cell position and Oregon Green BAPTA dextran to indicate cytosolic calcium concentration. We will give a number of similar examples.

The most useful aspect of multi-dye recording is that one can get information about how different physiological parameters are changing in the same living cell and therefore look for correlations between them. The first step in complexity is to use two dyes, each reporting an intracellular parameter. For instance, the Duchen lab. is combining Fluo-3 to report cytosolic calcium concentration with TMRM, which reports the mitochondrial membrane voltage. Other examples will be given.

Ratiometric dyes have a number of advantages over dyes that simply get brighter or dimmer as they report the parameter of interest. Confocals offer a limited choice of excitation wavelengths but many emission channels, so ratiometric emission dyes such as Indo-1 for calcium are most suitable. The Bolsover lab., in collaboration with John Greenwood at the Institute of Ophthalmology, is following the calcium concentration in brain endothelial cells with Indo-1 (using two emission channels) as T cells, marked with the vital dye PKH26 (Sigma) and visualized on a third channel, crawl over them.

If the emission wavelengths are far enough apart, signals from three dyes can be reliably separated. The Bolsover lab. is studying calcium-induced translocation of calmodulin in patch clamped nerve cells using three fluorophores: Fura-Red (Mol. Probes) to measure calcium, fluorescein_calmodulin to show calmodulin's location in the cell, and TA-calmodulin (Katalin Török, QM+WC) to detect calmodulin activation.

POSTERS

THE USE OF CONFOCAL LASER SCANNING MICROSCOPY TO STUDY HYDRATED ORAL BIOFILM MICROSTRUCTURE.

CS Andrews 1, J Pratten 2, DQM Craig 1, M Wilson 2,
1 Centre for Materials Science, School of Pharmacy, University of London; 2 Department of Microbiology, Eastman Dental Institute for Oral Health Care Sciences, University of London.

Purpose. Confocal laser scanning microscopy (CLSM) is emerging as a powerful technique for the study of biofilms. An investigation of the distribution of viable and nonviable cells within oral biofilms is described as proof of concept for more detailed investigation of the chemical environment using fluorescent probes. Methods. Enamel disks (5 mm) were cut from bovine incisors, polished and conditioned for 1 minute in sterile artificial saliva The disks were mounted in a constant depth film fermentor (CDFF) (University of Wales, Cardiff) which was maintained at 37°C. To provide a multispecies inoculum of organisms found in the oral cavity, saliva was collected from 10 healthy individuals and pooled. The disks were exposed to a recirculating inoculum (500 mL artificial saliva and 1 mL of pooled saliva) for 8 hours. The inoculum flask was then disconnected and the CDFF fed from a reservoir of sterile artificial saliva at a flow rate of 0.72 L/day, the waste being collected in an effluent bottle. Vital staining of unfixed biofilms was performed using LIVE/DEAD Bac light™ Bacterial Viability Kit (Molecular Probes Inc.). Stained and unstained biofilms were examined using a Wild-Leitz CLSM, images were captured straight to an IBM PC. Results. Using CLSM in reflectance mode (A), which requires no staining, the microstructure of mixed species oral biofilms was revealed to be complex, with stacks of bacteria, separated by channels. In fluorescence mode (B), live bacteria fluoresced in the green wavelength with Bac Light™, and could be distinguished from dead cells which fluoresced in the red wavelength (not shown).
Conclusions. The distribution of viable and nonviable cells and the structure of hydrated oral biofilms can be directly studied using CLSM in either reflectance mode (A) or fluorescence mode (B). This suggests that planned further investigations of biofilm structure and chemistry are feasible.
The authors would like to acknowledge the financial support of Unilever Research.

S.E.E.C. Romania

Anne Drewe (Dept of Microbiology, ICSTM, Charing Cross Hospital)

An update of our work and contacts with Romania.

Periodontopathogenic Bacterial Biofilms and Planktonic Cells - An Ultrastructural Comparison.

N MORDAN* D.JENSEN, M WILSON, and H NEWMAN.
Eastman Dental Institute, London, UK.

Dental plaque, the aetiologic agent of both dental caries and chronic inflammatory periodontal disease, is a bacterial biofilm formed by adherence of planktonic free-living bacteria to a surface and their subsequent proliferation. Much past research has involved the assessment of bacterial death in planktonic culture with a view to arresting the advance of plaque *in vivo*. However it is now apparent that the behaviour of members of a biofilm, in particular their resilience to adverse conditions differs from that of planktonic cells. In this study we cultured *Actinobacillus actinomycetemcomitans* in Wilkins Chalgren broth with 10% defibrinated horse blood and as a biofilm (on cellulose nitrate membrane filters on Wilkins Chalgren agar plates) for 24, 48 and 72 hours. Resulting cultures were fixationed with glutaraldehyde, post-fixed in osmium tetroxide, dehydrated and embedded in LR White resin. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed in a JEOL 100CX transmission electron microscope. Although there was progressive alteration in the appearance of both protoplasm and cell wall with time, the rate of change was faster for cells in broth as opposed to biofilm. Planktonic bacteria showed intact cells at 24hrs but by 72hrs there was advanced lysis and protoplasmic condensation. However biofilm cells displayed only slight lysis and little protoplasmic condensation at 72hrs. Planktonic cells displayed surface-associated fibrils at all time periods though those in biofilm only did so at 72hrs. These differences in ultrastructure between planktonic and biofilm bacteria are evidence of the slower growth rate of cells in biofilm which in turn may account for the refractory nature of biofilms.