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Symposium 6: Functional imaging

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Chair: Mark Cannell and Paul Donaldson

Gaining new insights into physiological function from biophotonics

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Compared to conventional electronic based instrumentation, photonic methods are exquisitely sensitive. As an example, it is quite easy to measure photon fluxes of a few per second while equivalent electron flows are far below that realised even with sensitive patch-clamp amplifiers. However, the sensitivity of biophotonic methods extends to well below the level of the single cell by providing methods to detect and manipulate even single molecules.

Two decades ago, physiologists struggled to make measurements of intracellular calcium with ion-sensitive electrodes and bio-luminescent probes but the development of new fluorescent molecules for calcium measurement has made calcium measurement quite straightforward – if not always precise and easy. Cell calcium measurements with imaging systems have revealed new levels of complexity in signaling and as a result of the advances in instrumentation and methods it is now clear that the cell does not achieve function like a well stirred bucket of constituents. For example, the discovery of calcium sparks a decade ago clearly reinforced this idea – but we still don't understand how excitation-contraction coupling really works. This problem will be highlighted by some recent results from our laboratory where we have tried to develop and test ways of measuring the minute calcium fluxes underlying calcium sparks.

The microanatomy of the cell must be important for helping turn the cell from a large number of lipid and water soluble chemicals into life. Here new light imaging techniques are playing an important role and combination of computer image processing with high resolution imaging techniques reveals new levels of complexity in cell structure. Furthermore, with biophotonic methods, we can look inside the living working cell (an obvious advantage for those who are interested in how the living cell works). This has always been the classical province of the physiologist.

Promising new directions for physiological research include manipulation of proteins with molecular techniques and again, biophotonics provides powerful methodologies to study the results of such experiments. In this symposium we will look at new data and methods being applied to increased our understanding of physiology. We will see how new biophotonic methods offer ways to probe cell function with unprecedented fidelity and sensitivity. When one considers what may now be achieved with these methods, it would seem that the future of molecular-based physiology is very bright indeed.

Biosensors for investigating neuronal Ca^{2+} signalling

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Biosensors are genetically engineered protein sensors of a selected intracellular target (e.g. ions, metabolites) that can be directed to intracellular locations by targeting sequences that are encoded within the sequence of a protein. This presentation will focus on studies on designing and making new sensor molecules and addressing the utility of biosensors for making measurements, of Ca^{2+} (and ATP), in functionally important cell locations, such as pre-synaptic terminals. In particular, the coupling of these biosensors with fast imaging procedures will be highlighted by example studies.

New views of lens structure and function

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The ability of the ocular lens to focus light on the retina is the result of a unique cellular physiology and tissue architecture, which eliminates light scattering and improves the optical properties of the lens. The lens is a relatively simple avascular tissue. A single layer of cuboidal epithelial cells covers its anterior surface. At the lens equator these epithelial cells divide, elongate and differentiate into fibre cells, which form the bulk of the lens. Fibre cells adopt a flattened hexagonal profile that facilitates packing into an ordered cellular array, which minimises light scattering. During differentiation, the fibre cells lose their organelles, and undergo significant changes in the expression of cytoplasmic and membrane proteins. Since lens growth continues throughout life, younger fibre cells are laid down on top of existing fibre cells, internalising these older cells and thereby creating an inherent gradient of fibre cell age.

Maintenance of this lens tissue architecture requires special mechanisms, not only to supply the older anucleate fibre cells with nutrients, but also to control the volume of these cells. It has been proposed that the lens operates an active micro-circulation system that delivers nutrients to, and removes wastes, from the lens, thereby maintaining ionic homeostasis and the volume of the inner fibre cells (Donaldson *et al.*, 2001). It is believed that the ionic currents that drive this internal circulation are generated by spatial differences in the distributions of ion channels and transporters between the younger nucleated fibre cells in the periphery, and the older anucleate cells in the interior of the lens.

To systematically study this circulation system we have developed a series of imaging protocols that have allowed us to quantitatively assess how the distribution and function of key transport proteins vary during the course of fibre cell differentiation. These protocols allow us to map protein distribution over large distances with subcellular resolution (Jacobs *et al.*, 2001). Studies conducted on gap junctions (Jacobs *et al.*, 2001), glucose transporters (Merriman-Smith *et al.*, 2003) and an adhesion molecule, MP20 (Grey *et al.*, 2003), will be reviewed to illustrate how the adoption of our imaging protocols can yield new insights into the relationship between lens structure and function.

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Quantitative phase microscopy - a new way to interrogate the structure and function of unstained viable cells

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The optical transparency of unstained viable cell specimens limits the extent to which information can be recovered from bright field microscopic view as these specimens generally lack visible, amplitude modulating components. However, if the phase-shift component of the cellular material is utilised, an information-rich image can be obtained. Optical phase microscopy, and derivatives of this technique such as Differential Interference Contrast (DIC) and Hoffman Modulation Contrast (HMC) have been widely applied in the visualisation of cellular specimens to enhance contrast. Whilst providing significantly enhanced contrast, useful in viewing specimens, the capacity to extract quantitative information from the phase content available in these optical techniques has not previously been explored.

Quantitative Phase Microscopy (QPM) is a new method for visualising transparent objects. This recently developed computational approach extracts quantitative phase measurements from images captured using a bright-field microscope without phase or interference contrast optics. QPM works via an algorithm which is applied to an in-focus image and a pair of equidistant de-focus images (one positive and one negative de-focus). From these images a phase map is generated which can be used to quantitatively emulate other contrast image modes such as DIC, dark field or HMC. The generation of these analogue images using phase mapping obviates the inherent problems associated with optical phase imaging, including cell edge distortion and edge halo effects. As it is implemented on an optically simple bright field microscope, the QPM methodology is also an economical alternative for cellular imaging applications.

Of particular importance is the capacity to quantitatively analyse the recovered phase images using QPM. The phase map generated from the bright field images contains information about cell thickness and refractive index and can allow quantitation of cellular morphology under 'real-time' conditions. For instance, the proliferative properties of human airway smooth muscle cells have been evaluated using QPM techniques to track cell culture confluency and growth (Curl *et al.*, 2002). In addition, cell volume measurement techniques have been applied to investigate the responses of erythrocytes to different osmotic challenges using QPM (Curl *et al.*, 2003).

QPM is a valuable new imaging tool which extends the capacity to interrogate viable cells to obtain structural and functional information in a rapid and non-destructive manner.

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High throughput imaging of extended tissue volumes

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In many fields, three-dimensional (3D) information is necessary to understand the organisation and spatial relationships within biological samples. Our group has previously used a confocal microscope and microtome to assemble large, high-resolution volume images from resin-embedded heart tissue (Young *et al*., 1998). Digital reslicing, segmentation and volume rendering methods can be applied to the resulting volumes to provide quantitative structural data about the 3D organisation of myocytes, extracellular collagen matrix and blood vessel network of the heart not previously available. Information such as this is necessary to quantify the heart wall remodelling associated with various types of cardiac disease. It is also required for computer models, which are necessary to examine the effects of myocardial structure on the function of the heart. For example, we have used structural data extracted from an extended volume image of rat left ventricular myocardium (3.8mm $\times$ 0.8mm $\times$ 0.8mm at 1.5µm pixel size, 0.72×10^9 voxels) to model the influence of structural discontinuities on the propagation of electrical activation in the heart (Hooks *et al*., 2002). However, acquisition of volume images of this scale requires weeks of painstaking work using conventional techniques.

We have developed a novel high throughput imaging system that enables extended volume images to be collected flexibly and efficiently. The system consists of a confocal microscope (Leica TCS 4D) with a Kr/Ar laser, a variable speed Ultramill (Leica) which cuts to 1µm over a 75mm path using diamond or tungsten carbide tips, and a three-axis translation stage (Aerotech) with XYZ movement of 1000, 200 and 75mm, respectively at 100nm step size. This stage controls the positioning of specimens for imaging and milling. The microscope and mill are supported above the translation stage using rigid mounting systems designed to facilitate alignment of imaging and cutting planes. The system is mounted on an anti-vibration table. Z-stack volume images are acquired for overlapping x-y areas that cover the region of interest. The imaged volume is then milled off and the process is repeated. The images acquired may then be combined to reconstruct the volume in 3D. A major advantage of this method is that alignment of the sample elements is maintained throughout the imaging and milling operations, thereby preserving spatial registration and making reconstruction of the complete volume image easier and faster.

The system is controlled using a dedicated computer (Dell P4, 1.8GHz, 1GB RAM, Windows 2000) using custom software written using the LabVIEW™ programming language. A single user interface has been developed that enables image acquisition and milling to be controlled interactively or automatically and allows the operator to process, reconstruct and visualise the image volumes. The flexible user interface provides the ability to image chosen sub-volumes at high resolution, but placing them within the context of a large volume imaged at lower resolution.

Preliminary studies carried out with cardiac tissue specimens demonstrates that the system has the capacity to acquire a 62.5 million voxels per hour, each averaged over 8 scans. This translates to acquisition of a fully registered image volume $1mm^3$ at 1µm pixel size (10^9 voxels), with 8× averaging, in 16 hours, representing greater than an order of magnitude speedup from the manual technique. The volumes imaged to date have been limited to heart tissue perfusion stained with picrosirius red. We are currently working on techniques to extend the range of tissues and fluorescent markers suitable for imaging with the system.

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