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Confocal laser scanning microscopy in connective tissue research

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The confocal laser scanning microscope (CLSM) is a key tool in many areas of research where light microscope imaging, predominantly of fluorescent subjects, is important. Although there are low magnification applications, it is mostly used as a high magnification, high resolution light microscope and as such, in keeping with the interests of Dr Iolo ap Gwyn in whose honour this part of the of the meeting has been designed, could be regarded as being at the interface of light and electron microscopy. Fundamentally there are two forms of confocal microscope – one specialised for high speed "real time" imaging for viewing of rapid, dynamic events, such as calcium transients in cells, and another associated with high quality three dimensional imaging. Whilst the boundaries between these instruments have become blurred in recent years, it its the latter type and its applications that I shall deal with here.

The principle of confocal microscopy was developed in the 1950's, but its development was dependent on relatively recent advances in laser, detector and computer technology to take advantage of the technique. Fundamentally, CLSM is based on the fact that, especially at high magnifications, the plane of focus of the microscope is much smaller than the thickness of the specimen being examined. In conventional microscopy, this means that at any one focus position a small amount of specimen is in focus, but the overwhelming majority is out of focus. Since the image is made of both, the out of focus light severely degrades the image from the focal plane – a particular problem with fluorescence microscopy. The key to the confocal principle is that the optical arrangement of the illumination and image forming parts of the microscope. These result in only in-focus light being accepted by the microscope from the specimen – the so-called "optical sectioning" effect. The amount of light coming from the focal plane is small, and thus requires sensitive detection and amplification by photomultipliers, but the image is undegraded and very sharp. Also, the focal plane can be moved accurately through the depth of the specimen, taking an image at user-set intervals to give a stack of images of the specimen in perfect register. These images can be combined to give a sharp view of the whole specimen with no out of focus fluorescence. In addition, we can use the image stack to gain depth information: microscopy always gives the x-y plane; confocal imaging gives the z-plane as well, allowing 3-dimensional modelling of structures.

Applications of the CLSM in our laboratories have included cytoskeletal analysis of chondrocytes in tissue sections and explant cultures placed under mechanical loading, and 3 dimensional modelling of tendon cell shape, cytoskeletal and cell-cell junctional analysis in tissue sections and intact tendons - it is possible to use the optical sectioning properties of the instrument to gain structural information from underneath the surface of intact specimens in favourable circumstances. Recent and ongoing studies include cellular and cytoskeletal control of early matrix deposition in developing intervertebral disc, cornea and bone. All of these studies involve high magnifications, the production of optical section series and 3 dimensional modelling, sometimes of 3 fluorogens simultaneously. Current and future work will be directed towards understanding the finer details of what we can observe with our labelling, microscopical and computer modelling techniques in relation to ultrastructural studies of cell and matrix deposition and interaction.

The CLSM can be used as an analytical as well as structural tool, with appropriate use of fluorescent reagents and appropriate software packages. There are numerous reagents that allow concentration dependent imaging of ions, of intracellular pH and of particular organelles. In addition further dynamic aspects of cell and matrix and even protein interactions behaviour can be examined using techniques such as FRAP (fluorescence recovery after photobleaching: fluorescent signals can be bleached by high intensity laser irradiation; fluorescent molecules then diffuse in from the surroundings allowing fluorescence recovery) and FRET (flourescence resonance energy transfer: different fluorogens transfer energy when very close together, thus allowing stimulation of one with the wavelengths of light appropriate to the other; can be used for measuring distances between fluorescent tagged molecules). Indeed, applications are limited only by availability of fluorescent reagents and the imagination of the microscopist!