

Coherent X-ray Science (CXS) Super-Resolution Optical Microscopy Workshop

Report by Leann Tilley and Trevor Smith

“It is very easy to answer many fundamental biological questions; you just look at the thing”. This is quote from Richard Feynman (Nobel Prize in Physics) in a talk to the American Physical Society in 1959. He went on to suggest that what Physicists should do to help biologists is to make better microscopes. And indeed in the last few years optical microscopy methods have undergone breathtaking developments. In particular, cell biologists are very excited about new light microscopy techniques that use ingenious approaches to overcome the “diffraction limit” of light. These new techniques enable imaging of cellular processes at a level of resolution up to an order of magnitude beyond the current limits.

The impact of the new Super-Resolution microscopy methods is being recognised around the world. To inform Australian scientists about the new methods, the ARC Centre of Excellence for Coherent X-Ray Science (CXS) organised a Super-Resolution Optical Microscopy Workshop at the Convention Centre, in Melbourne, on 1st October 2010. The workshop formed part of the highly successful international OzBio2010 Conference which was held in the same venue over the course of the week. The CXS workshop aimed to bring Physicists and Biologists together at a very exciting time for optical microscopy. There were a total of 185 registrants and it was a fantastic opportunity to meet within and across disciplines and countries.



Professor Keith Nugent opened the meeting explaining the goals of the workshop. He gave an overview of some of the activities within CXS and explained how Physicists, Chemists and Biologists work together to achieve the aims of the Centre. He explained that CXS, which was established to develop X-ray imaging, has seen the exciting opportunities offered by the new Super-Resolution visible light methods and is keen to provide leadership and scientific contributions in this area.



The workshop featured a talk by an invited overseas speaker who is an inventor of one of the new Super-Resolution Microscopy techniques as well as excellent presentations by leading Australian proponents. The plan of the workshop was to provide an overview of the new techniques as well as providing high level insights into the

development and application of particular techniques. Some highlights from the excellent program of speakers are described below.

Professor Guy Cox, Sydney University, served as Deputy Director of the Electron Microscope Unit, University of Sydney (now the Australian Centre for Microscopy and Microanalysis) from 2001 to 2005. He explained the laws described by Raleigh and Abbé dictating that the resolution of conventional light microscopes restricted to about half the wavelength of the exciting light. Thus conventional microscopes can resolve 200 - 250 nm in the lateral plane and ~600 nm in the vertical direction. This is about the size of a mitochondrion or a bacterium and is a major limitation on the ability of cell biologists to study sub-cellular events.



Guy gave an overview of the new imaging techniques that use different strategies to overcome the diffraction limit. He provided a more in-depth analysis of Stimulated Emission Depletion (STED) Microscopy. He explained that STED microscopy uses the non-linear de-excitation of fluorescent dyes to increase resolution. It employs a conventional excitation laser in combination with a STED laser to produce a toroidal or doughnut-shaped illumination pattern. The STED laser deactivates the fluorescence outside a small central zone, reducing the effective excitation area from which fluorescence emission can occur. A resolution of ~80 nm in xy can be readily achieved, with ~20 nm the best reported (2, 8).

Associate Professor Sam Hess, University of Maine, USA, invented a technique known as fluorescence photoactivation localization microscopy (FPALM). FPALM and the related technique Stochastic Optical Reconstruction Microscopy (STORM) separate fluorophores in time that are inseparable in space and are sometimes called “pointillist” techniques (1, 4). They rely on photoactivation processes to turn fluorescent molecules on and off (1, 3). With only a few well separated molecules “turned on” each emitter is individually captured in a given image without overlap. 2-D Gaussian functions are fit to accurately determine the location of each emitter in that image. These molecules are then turned off and a new set

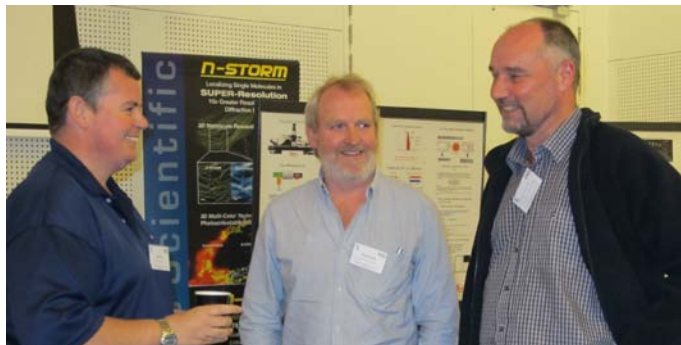


turned on which are reimaged and located. This process is repeated thousands of times until the entire image is reconstructed. The technique enables imaging of subcellular structures in living cells with a “resolution” of 20-30 nm which is well below the diffraction limit (where “resolution” is defined as the distance between objects that can be reliably measured rather than true optical resolution).

Sam described the application of FPALM to the question of how the influenza virus exploits cell membrane organization for infection and assembly. His work is providing unprecedented views of the clustering and dynamics of hemagglutinin, the fusion protein from influenza.

Associate Professor Katharina Gaus is an NHMRC Senior Research Fellow and leads the Cell Membrane Biology group at the Centre for Vascular Research, University of New South Wales. Kat uses PALM and STORM to study the dynamic mechanisms that underlie membrane signalling. These single molecule imaging techniques can be used to quantify the number of proteins participating in signalling clusters, the number of clusters and the ratio of proteins within clusters. She described the thresholding parameters used to distinguish fluorescent signals from background and noted that labeling density is an important determinant of the performance of these pointillistic methods. Kat's work has provided important insights into the principles that control the organization of signalling proteins in the T cell membrane.

Associate Professor Trevor Smith, University of Melbourne, described the more established techniques of Scanning Near-field Optical Microscopy (SNOM) and Total Internal Reflection Fluorescence microscopy (TIRFM), both of which can be classified as “near-field” techniques. SNOM was the first optical method capable of overcoming the diffraction limit and is based on squeezing the excitation light through the very small aperture, some tens of nanometres in diameter, of a drawn optical fibre, restricting the region of the sample that generates emission to these dimensions. TIRFM can provide imaging with axial resolution on the tens of nanometres, and some new approaches based on this method such as Harmonic Excitation Laser Microscopy (HELM) or Standing Wave Illumination, are reported to achieve ~90 nm lateral resolution (5) or better. Trevor discussed the combination of some Super-Resolution techniques in addition to the extension of Super-Resolution microscopy through the use of time-resolved emission detection, providing picosecond temporal resolution in addition to the sub 100 nm spatial resolution. He applies these techniques to biological as well as non-biological samples, including luminescent polymer films.



Associate Professor Cynthia Whitchurch is a NHRMC Senior Research Fellow in the ithree Institute and is Director of the UTS Microbial Imaging Facility at the University of Technology, Sydney. Cynthia described her work using 3-D Structured Illumination Microscopy (3D-SIM) to study bacterial cell biology. In 3D-SIM applications the sample is illuminated with a periodic pattern that interacts with the fluorescence from small features in the sample to generate Moiré images with resolvable spatial features (5-7). An algorithm is used to reconstruct an image with a 2-fold increase in resolution over conventional optical microscopy (xy ~100 nm, z ~250 nm).

Bacteria are very small with rod-shaped species having a typical width of 0.5 - 1.5 microns and a length of 1 - 10 microns. As a result studying microbial cell biology using conventional light microscopy is a major challenge. Using the Delta Vision OMX microscope Cynthia has been able to ask questions about cell division, cellular differentiation and protein trafficking in bacteria.

Professor Leann Tilley is Director of Research at the La Trobe Institute for Molecular Science. Her laboratory is using a range of high resolution microscopy techniques to image

malaria parasite-infected erythrocytes. Leann was an early adopter of 3D-SIM. She travelled to UCSF to use the developmental instrument of Prof. John Sedat to study sub-structure within the exomembrane system of malaria parasite-infected erythrocytes. More recently she has worked with Cynthia Whitchurch to apply 3D-SIM to study 90 nm knob structures that are important *P. falciparum* virulence determinants. In other examples she showed that 3D-SIM is able to resolve apical pores (~150 nm diameter) in GFP-labelled merozoites and ~100 nm striations in the sub-pellicular membrane complex of *P. falciparum* gametocytes. She discussed some of the challenges associated with obtaining good 3D-SIM images and stressed the potential for artefacts with any imaging technology. She commended the use of multi-modal or correlative imaging methods, which greatly increase confidence in the data.

The attendees of the workshop enjoyed lively discussions during the question times and in the afternoon tea and drinks sessions. The advantages and disadvantages of the different Super-



Resolution techniques and conventional imaging techniques were discussed at length. Issues to be weighed up include the desired optical resolution, whether existing fluorophores and labelling protocols are compatible with particular imaging methods, the time required to acquire the data and reconstruct the image, and the need for live cell

imaging. It became apparent that scientists need access to a range of instruments and techniques because different techniques are best suited to different applications and each modality has advantages and disadvantages.

The CXS has sponsored the development of the Cellular Nano-Imaging Consortium (CNIC) as an affiliation of scientists with interests in Super-Resolution Optical Microscopy. CNIC brings together institutions and research leaders with cross-disciplinary expertise and an interest in using and/or developing nano-imaging optical methods. It provides on-line access to information about conventional and Super-Resolution optical imaging techniques and details the resources that are currently (and potentially) available to interested parties. Through CNIC additional workshops and conference sessions will be organised to inform Australian scientists about new high-resolution imaging modalities. The CNIC site can be accessed at www.coecxs.org/cnic.

The Organizing Committee for the workshop (Leann Tilley, Trevor Smith, Fabienne Perani, Keith Nugent and Tania Smith) thank the session chairs, Sarah Russell (Swinburne University / Peter MacCallum Cancer Centre) and Mark Prescott (Monash University) and all of the participants for contributing to such an excellent program. The Committee would also like to thank a number of commercial companies whose generous sponsorship made the workshop possible. These are: Coherent Scientific (Nikon), Zeiss, Leica Microsystems, Berthold Australia Applied Precision), FABLS and La Trobe Institute for Molecular Science (LIMS). Photos are available at www.coecxs.org.



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