Single-molecule localization microscopy - principles and applications,
Dr Sebastian Van De Linde
University of Strathclyde
A decade ago, single-molecule localization microscopy (SMLM) has been developed, which overcomes the limitations imposed by optical diffraction. During the first years the focus was on methodological advancements, such as multicolour and 3D imaging capabilities, but in recent years SMLM is increasingly applied to study biological questions. Importantly, SMLM does not only allow for studying cellular structures at high spatial resolution but also provides quantitative information. Quantitative molecular labeling and localization of cellular proteins have been verified by correlative SMLM and scanning electron microscopy. Further, computational methods such as particle averaging, which is originally applied in electron microscopy, can be used to generate accumulated super-resolved images from single-molecule coordinates.
I will provide a concise overview of the principles of SMLM with an emphasis on dSTORM, describe novel technical developments and conclude with applications in the fields of cell and neurobiology.

WDR35/IFT121 Regulates Entry of Membrane Proteins to Cilia
Tooba Quidwai (ESRIC student)
Cilia are highly organised microtubule-based organelles present on almost all mammalian cells that play key sensory and motile functions. Defects in cilia structure or function lead to a group of human diseases called ciliopathies. In order to function, cilia must maintain a distinct protein and membrane composition from the surrounding plasma membrane and cytosol, highly enriched in signalling receptors and effectors. How this compartmentalization occurs remains unclear.
Biogenesis of the cilium (ciliogenesis) requires the process of intraflagellar transport (IFT) which uses microtubule motor-based movement of cargos on highly-conserved multimeric protein complexes in (anterograde) and out (retrograde) of the nascent cilium. Components of these complexes can be biochemically and genetically characterised as belonging to either IFT-B (anterograde transport) or IFT-A (retrograde transport) particles. However, the individual functions of each IFT protein are not well understood. Null mutations in the IFT-A component WDR35/ IFT121 are embryonic lethal in both mouse models and human ciliopathies. Importantly, small, unstable WDR35 mutant cilia are formed but fail to become enriched in diverse classes of integral and membrane-associated proteins. To elucidate its role in the entry of membrane proteins to cilia, I present live and fixed cell imaging experiments to visualise the dynamics of membrane protein localisation at the periciliary base and interaction studies based on immunoprecipitation and mass spectrometry to define the molecular mechanism by which IFT proteins establish functional cilia.

Imaging toolbox for understanding single calcium channels.
Katarzyna Cialowicz (ESRIC student)
Calcium ions in the human body serve as second messengers and are responsible for cell homeostasis. Muscle contractions and neuronal communication are supported by electrical activity. Voltage-activated calcium channels are crucial for many cellular events. They play an important role in transducing the initial stimulus to the effector systems that modulate insulin secretion in pancreatic cells or neurotransmitter at nerve terminals. Dysfunction of these processes may cause many conditions, including diabetes or Alzheimer's disease. In recent years the amount of research on Ca2+ channels has markedly increased, but there are limitations related to physical restrictions in the spatial resolution of fluorescence microscopy. This phenomenon results in a loss of information with regard to the true location of a point source that is emitting light. Newly developed methods such as Stimulated Emission Depletion Microscopy (STED) or Photoactivated localization microscopy (PALM) allow imaging close to the molecular scale.
I will talk about my project which is focused on understanding the mechanism of action and distribution of N-type calcium channels by using novel microscopy tools I have helped to develop for calcium channel imaging.

Trapped between two beams – orienting living cells in a dual-beam laser trap using all in-fibre-based higher-order-mode manipulation
Kai Skodzek (ESRIC student)
Cell manipulation using a dual beam laser trap is a powerful tool for understanding cell mechanics and structures on a single to few-cell level. For example with increasing laser power a cell can be deformed and thus mechanical properties obtained. Holding a sample in between two laser beams controls it along the trapping axis while perpendicular to it rotation is possible. Rotating the sample in a controlled way tomographically enables increased three dimensional resolution especially for larger objects. In this talk I will present my current work on a novel dual beam laser trap that is capable of trapping multicellular objects furthermore, the trap uses a higher order mode laser signal to align the sample stronger perpendicular to the trapping axes. Shaping the higher order mode signal to rotate around the trapping axes will enable tomographic rotation of the sample.

Towards in vivo bacterial detection in human alveolar lung tissue using Smart Probes and fluorescence lifetime imaging microscopy (FLIM),
Dr Tushar Choudhary
Antibiotic resistance is a serious global concern. One way to tackle this problem is to develop new and sensitive approaches to diagnose bacterial infections and prevent unnecessary antibiotic use. With recent developments in optical molecular imaging, we are one step closer to in situ rapid detection of bacterial infections. The Proteus Interdisciplinary Research Collaboration (IRC) is funded by EPSRC and is a consortium of three universities, The University of Edinburgh, The University of Bath and Heriot Watt University. The aim of Proteus is to deliver a healthcare technology which will sit next to the bedside of a critically ill patient in the Intensive Care Unit. The fiber optic based equipment will have the capability of in vivo imaging of distal lung

to see infection (bacteria, fungus etc) and inflammation (immune cells). It will also sense clinically relevant parameters such as pH, glucose and oxygen in the distal lung.

We present here bespoke fluorescent probes for bacterial detection in ex vivo human lung tissue using fluorescence lifetime imaging microscopy (FLIM). Our in-house custom probes can selectively attach to either Gram positive or Gram negative bacteria based on type and provide distinguisability based on fluorescence lifetime. Two in-house synthesised bespoke probes were used in this study to detect and differentiate between Gram positive and Gram negative bacterial strain using their fluorescence lifetime in the ex vivo human lung tissue. The average fluorescence lifetime of Gram positive probe (n=12) was 2.40 ± 0.25 ns and Gram negative (n=12) was 6.73 ± 0.49 ns. The human lung tissue (n=12) average fluorescence lifetime value was found to be 3.43 ± 0.19 ns. We demonstrated here use of novel bacterial probe for detection and discrimination of Gram positive and Gram negative bacteria using FLIM. Furthermore we were also able to distinguish between dead or alive bacteria in ex vivo lung tissue based on difference in their lifetime. We have developed Fibre-FLIM methods to enable clinical translation within the Proteus Project (www.proteus.ac.uk).

Title New ways to look at how roots interact with soils, Dr Lionel Dupuy
The James Hutton Institute

Roots and their interactions with soil and microorganisms contributes to nutrient bioavailability, crop growth, and soil biodiversity and fertility, but techniques for observing soil biological organisms *in situ* are lacking. In this talk, I will present new approaches developed in my lab to observe, image and characterize roots and microorganisms live an *in situ*. For example, we have developed a new substrate termed transparent soil that can combines complex soil-like environment and the capabilities of imaging biological processes at different scales, resolutions and throughput rates using modern microscopy techniques. I will also present new approaches to understand root and soil physical interactions, including new micro-mechanical sensors, 3D imaging and image analysis.

Organelle Changes in a Huntington's Disease Model using Cryogenic Soft X-ray Tomography, Dr Michele Darrow
Diamond facility, Oxford
Huntington's Disease (HD), a neurodegenerative disorder characterized by movement and executive function disruption, is linked to an expanded and unstable CAG trinucleotide repeat which translates as a polyglutamine (Q) repeat in the protein product. Healthy controls demonstrate a fairly broad range of trinucleotide repeats and 40+ repeats have been described as pathological. Due to this expansion, exon 1 of the Huntingtin protein (HTT) is aberrantly processed by cellular machinery leading to an unintended cleavage product (mHTT). Aggregates of mHTT exon 1 have been found in mouse models and in patient brain.

PC-12 cells were used as an HD model to study cell morphology changes due to the presence of mHTT. Cells expressing mHTT-GFP exon 1 were grown on gold finder grids, cryo-immobilised by plunge freezing, and imaged. GFP fluorescence was used to identify cells of interest prior to imaging in cryogenic soft x-ray tomography (SXT). SXT makes use of x-rays in the "water window" (2.4 nm wavelength, 500 eV) where common biological elements such as carbon and nitrogen absorb x-rays and are therefore more visible, whereas water (vitreous ice) is relatively transparent. This allows whole, fully-hydrated cells to be imaged at approximately 40 nm resolution without requiring sectioning or staining techniques.

Tilt series were collected and reconstructed, and segmented using a new software tool called SuRVoS (Super Region Volume Segmentation). Cellular features were annotated based on their inherent properties. Cytoplasmic organelles were classified using specific characteristics of the data, such as the average intensity of the organelle, the size of the organelle, the variance of the voxels that make up the organelle and the location of the organelle in the 3D volume. Next, population statistics for each organelle class, such as the average and standard deviation of the class's size, shape, intensity and variance will be used to describe the morphology of a non-perturbed cell, allowing the identification of changes due to perturbations, in this case, the presence of mHTT aggregates.

As a whole, SXT is a powerful technique that can describe the effects of various cellular perturbations such as disease, infection or treatment, in a whole cell context.

From the Dark Ocean Comes Light: Hannah Imlach, Artist in residence within The Institute of Biological Chemistry, Biophysics & Bioengineering and the Centre for Marine Biodiversity and Biotechnology,
Hannah Imlach is a visual artist working predominantly in sculpture and photography. Her research-led practice explores how we relate to our immediate environment and the threats caused by changing climate. Hannah's fully immersive residency at Heriot-Watt has focussed on the ecology of Scotland's cold water coral reefs, deep sea fluorescent organisms and how fluorescent proteins have revolutionised biological understanding through super high resolution imaging. This research informs a new body of sculptural work which will be exhibited in early 2017.

Tracing autophagosome biogenesis using three-dimensional electron microscopy, Eeva-Liisa Eskelinen
University of Helsinki

Autophagy is a fundamental housekeeping and survival pathway that recycles organelles and aggregate-prone proteins in eukaryotic cells. Nascent autophagosomes, also called phagophores, nucleate from a subdomain of the endoplasmic reticulum (ER). Several other organelles such as mitochondria, Golgi complex, plasma membrane and recycling endosomes have also been linked with phagophore biogenesis. We have previously shown using electron tomography (ET) of aldehyde-

fixed cells, that phagophores form membrane contacts with several other organelles, including ER, ER exit sites, Golgi cisternae, endosomes/lysosomes, and mitochondria (Biazik et al. 2015a). We currently focus on improving the ultrastructural preservation of our electron microscopy samples. To achieve this, we are using electron tomography (ET) of high-pressure frozen, freeze substituted cells to visualize phagophores and their surrounding organelles in near-native state (Biazik et al. 2016b). ET is performed to visualize the three-dimensional ultrastructure of phagophores. The preliminary results have revealed that high-pressure freezing and freeze substitution preserve small vesicles and microtubules better than conventional aldehyde fixation. We aim to use this approach to further dissect the relationship of nascent autophagosomes with other organelles and the cytoskeleton. Future membrane flux experiments are however needed to determine whether membrane contacts also signify lipid translocation. Further, we are currently setting up correlative light-electron microscopy workflow in order to trace phagophore biogenesis at earlier stages that are not possible to identify by morphology alone.

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Jonathan Taylor
Multi-timescale heart imaging: from milliseconds to hours
Light sheet microscopy allows us to probe the development of the zebrafish heart over timescales spanning seven orders of magnitude. These begin with the fluid-dynamic processes taking place on scales of milliseconds inside the beating heart, extending to the heartbeat itself at a rate of several Hz, through cell migration and the immune response to injury on timescales of seconds to minutes, all the way up to the early development of the heart itself over a period of hours. We have developed realtime realtime optical gating technology for our custom-built multi-channel light sheet microscopes, enabling us to computationally “freeze” the motion of the heart where necessary, with no unnecessary exposure of the fish to potentially harmful laser light. This leaves us free to observe biological processes and response to injury in a minimally-invasive manner. I will show how this has enabled us to perform high-resolution mapping of blood flow profiles inside the heart, and to track in 3D the infiltration of macrophage cells in and around the heart. Through this we can observe the immune response to injury in unprecedented detail in vivo. We can even observe the unperturbed development of the heart in continuous timelapse imaging for 18 hours or more.

Optimising fluorophore performance in single molecule localisation microscopy using novel SPAD imagers

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Single molecule localisation microscopy (SMLM) requires sequential switching on and off of a small subset of fluorophores to enable the localisation of individual fluorophores and subsequent generation of a super-resolution image. The choice of fluorescent probe and buffer conditions can impact on the quality of super-resolution images. Quantitative characterisation of the switching properties of a variety of fluorescent probes has been investigated to enable the selection of best probes for SMLM[1-3]. Additionally, attainment of good quality images is hindered by the uncertainty in the image acquisition rate, in order to capture the millisecond fluorescence event for each fluorophore, with suitable signal-to-noise ratio, to enable their position to be determined with the highest accuracy and precision.

CMOS SPAD (single photon avalanche diode) imagers allow for fast frame rates and single photon resolution, which potentially allow for higher accuracy and precision of fluorphore position than traditional EMCCD or sCMOS imagers [4, 5]. However, these imagers are not widely available, limiting their use in SMLM studies.

Alternatively, further improvement in the quality of the super-resolution images could be achieved by optimising the exposure time of the conventional EMCCD to complement the photoswitching properties of a fluorophore, such that photons emitted are captured at a frame rate appropriate for fluorophore on time.

To achieve this, CMOS SPAD imagers are used to characterise switching properties some of the most commonly used SMLM fluorophores in a range of buffer conditions. This information allows for optimisation of imaging parameters for acquiring SMLM images using the traditional EMCCD.

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Light-induced localisation in nuclear bodies: a signalling hub regulating gene expression in plants

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Light is essential not only for energy production but also as an informational signal that directs and optimises plant growth and development. Specialised photoreceptors, light signaling components and transcriptional regulators have been observed to translocate into the nucleus and form highly dynamic subnuclear structures also known as photobodies. The exact composition, mode of formation and molecular function of photobodies remains elusive. Here, we investigate the light-induced nuclear body formation and function of Tandem-Zinc-Finger Plus3 (TZP), a transcriptional regulator that induces gene expression during major developmental re-programming in the model plant Arabidopsis.

Accepting from the Best Donor; Analysis of Fluorescent Pairs to Optimise Dynamic FLIM-based FRET Experiments

Kirsty J Martin, Ewan J McGhee, Juliana P Schwarz, Karen Pickering, Andrew Campbell, Joel Johannson, Bryan Miller, Martin Drysdale, Douglas Strathdee, Saskia Brachmann, Volker Stucke, Gisbert Weckbecker, Francesco Hofmann, Owen J Sansom and Kurt I Anderson

Abstract

FRET biosensors have proven very useful tools for studying the activation of specific signalling pathways in living cells. Most biosensors designed to date have been predicated on fluorescent protein pairs that were identified by, and for use in, intensity based measurements, however fluorescence lifetime provides a more reliable measurement of FRET. Both the technology and fluorescent proteins available for FRET have moved on dramatically in the last decade. Lifetime imaging systems have become increasingly accessible and user-friendly, and there is an entire field of biology dedicated to refining and adapting different characteristics of existing and novel fluorescent proteins. This growing pool of fluorescent proteins includes the long-lifetime green and cyan fluorescent proteins Clover and mTurquoise2, the red variant mRuby2, and the dark acceptor sREACh. Here, we have tested these donors and acceptors in appropriate combinations against the standard or recommended norms (EGFP and mTFP as donors, mCherry and either Ypet or Venus as acceptors) to determine if they could provide more reliable, reproducible and quantifiable FLIM-FRET data to improve on the dynamic range and breadth of application of biosensor technologies. These tests were performed for comparison on both a wide-field, frequency domain system and a multiphoton, TCSPC time domain FLIM system. Clover proved to be an excellent donor with extended dynamic range in combination with mCherry on both platforms, while mRuby2 showed a high degree of variability and poor FRET efficiencies in all cases. mTFP-Venus was the most consistent cyan-yellow pair between the two FLIM methodologies, but mTurquoise2 has better dynamic range and transfers energy consistently over time to the dark acceptor sRCh. Combination of mTFP-sRCh with Clover-mCherry would allow the simultaneous use of two FLIM-FRET biosensors within one sample by eliminating the crosstalk between the yellow acceptor and green donor emissions.

Multifocal microscopy applications to cell studies

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Abstract
Widefield microscopes are 2D instruments where imaging is limited to single planes. In a scenario where particles move in 3D and time (4D problem), this technology becomes insufficient, with many applications requiring real time access to the axial dimension. Point spread function (PSF) engineering techniques provide a solution, where they encode the axial information into the PSF, but they can only do that over a limited axial range of 2 μm [1].
An increasingly established technique to achieve 4D imaging is multifocal microscopy (MUM), which allows the simultaneous acquisition of several equispaced planes, to obtain 3D data over a longer axial range (around 8 μm) in real time [2]. A distorted diffraction grating [2], which associates different focal lengths to each different diffraction order, offers a simple and practical solution to imaging multiple axial planes, without cumbersome optical setups. With MUM the axial positions of particles can be recovered by extrapolating the image data as particles progress through adjacent imaging planes, meaning even heavily undersampling axial plane separations can be used for axial localisations.
Here a diffraction multifocal relay is presented. It has been applied to track 1 μm yellow-green beads flowing on top of cells to observe the local shear stress generated. This study can help to improve the understanding of cell tissues behaviour when mechanically stimulated and shows the versatility of this instrument to tackle biological problems.

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Analysis of the potential impact of ocean acidification on the pelagic gastropod community in the North East of Scotland

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The potential impact of ocean acidification (OA) on plankton calcifiers is a focus of interest for the marine science community. Most planktonic studies have focused on coccolithophores with mixed results, while other calcareous groups of great ecological significance have received less attention.

This study will present the first investigation of the impacts of OA on pelagic gastropods at the Scottish Coastal Observatory monitoring site at Stonehaven (56º 57.8´ N, 02 º 06.2´ W). Temperature, salinity, nutrients, phytoplankton and zooplankton have been monitored at the site weekly since 1997. Carbonate chemistry parameters; total alkalinity (TA) and dissolved inorganic carbon (DIC) were measured between 2009 to 2015 but analysis has only been completed to end 2013. During this study the dissolution and elemental composition of archived pelagic gastropods shells from 2011-2013 were examined using Scanning Electron Microscopy (SEM) and X-ray microanalysis.

TA and DIC showed a seasonal pattern with considerable interannual variability. The seasonality of calculated pH values was influenced by the phytoplankton biomass in the water. Evidence of shell dissolution in pelagic gastropods was observed and the relationship with OA and environmental parameters examined. SEM images and molecular analysis reveal a diverse pelagic gastropod community at the Stonehaven monitoring site and provides the first record of the polar pteropod *Limacina helicina* in Scottish waters.

Cryo-EM of Virus Infected Cells: Towards *in situ* structural biology

Swetha Vijayakrishnan, Frazer Rixon, David Bhella

Cryo-EM imaging and reconstruction of frozen vitrified samples has become the method of choice for analysing the structure of medium to large complexes such as viruses and macromolecular assemblies. Until recently, most such analyses have been confined to purified materials. However, there has been increasing interest in extending studies into cells where the majority of biologically interesting structures and processes are located. Limited penetration by the illuminating electrons prevents imaging of specimens thicker than ~500 Å, restricting this form of analysis to thinner samples, such as intact bacterial cells and peripheral regions of some eukaryotic cells. To overcome this limitation, sectioning methods have been employed including cryo-sectioning of (CEMOVIS) and focussed ion beam milling of (cryo-FIB) vitrified specimens. However, these methods are technically challenging, subject to artefacts (CEMOVIS), or involve specialised equipment of limited availability (cryo-FIB). Here we describe the application of the well-established Tokuyasu sectioning method for preparing material for imaging as vitrified sections by cryo-EM tomography. In this procedure, fixed samples are infiltrated with sucrose, which acts as a cryoprotectant, allowing easy sectioning. The cut sections are thawed, washed and then vitrified and imaged in the cryo-EM. Using this approach, we have carried out sub-tomogram averaging to generate low-resolution icosahedral structures of intranuclear herpesvirus capsids. To our knowledge, this represents the first direct determination of virus structure from within the nucleus of a cell using cryo-EM techniques. We strongly believe that this work in general has great implications for structural determination of viruses and/or viral proteins as well as viral processes during infection within the context of their native environment, the cell.

Intracellular Drug Imaging by Stimulated Raman Scattering Microscopy

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The first "laser guided" surgery on a brain tumour (reliant on real-time, *in vivo* Raman imaging techniques) to be performed in the UK was reported in August 2015.[1,2] Rapid advances in the field of Raman imaging, particularly stimulated Raman scattering (SRS) microscopy are opening up many new avenues for imaging and quantification of drugs and small molecules in living systems.[3-5] These advances mean that for the first time, images of small molecules within cells might be acquired without the use of "bulky" fluorescent labels or nanoparticle sensors (which might perturb cellular biology). Here, we present label-free visualisation of tyrosine kinase inhibitors within colon cancer cells using SRS microscopy. Three-dimensional mapping enables the direct assessment of drug localisation within the cells using biocompatible detection.

The detection of drugs with low intracellular concentrations still remains a challenge. In these instances, Raman labelling approaches may facilitate detection.[6] However, a modular workflow for rational label design is yet to be established. Such approaches would allow SRS microscopy to be fully exploited in the drug development process. Here, we present a modular screening approach for Raman label design, with subsequent labelling of the drug, anisomycin. Detection of the Raman-labelled anisomycin analogues using SRS microscopy enables the real-time rate of uptake to be assessed. Furthermore, combining multi-colour SRS microscopy and dual-modality imaging allows drug accumulation to be mapped across intracellular structures, and to be correlated with markers of cell cycle status. These studies indicate the potential for SRS microscopy in the drug development process.

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