Posters

– 14. Correlative, multiscale and functional imaging –

P-521

3D tomographic imaging of biological objects using hard X-ray Bragg magnifier microscope

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We present an alternative method for 3D tomographic imaging of microscopic biological objects in hard X-ray regime, based on Bragg Magnifier (BM) principle. BM microscope uses asymmetrically cut Germanium crystals to magnify X-ray beam with advantages such as shorter propagation distances and increased dose efficiency, while achieving decent spatial resolution. This work focuses on the 3D reconstruction method to interpret the X-ray tomographic holograms using single-distance phase retrieval algorithm developed specifically for Bragg Magnifier, which is followed by filtered back-projection. We use a modification of contrast transfer function approaches developed for propagation based phase-contrast imaging and in combination with iterative constraint-based phase retrieval algorithm we obtained faster and more robust reconstruction method. Our algorithm was successfully applied to both synthetic and real-world experimentally measured holograms as demonstrated on 3D electron density reconstruction of model organism Tardigrade. We reached isotropic spatial resolution 300 nm approaching theoretical resolution limit for the given experimental setup.

P-522

Correlated cryo-fluorescence and cryo-electron microscopy can identify sites of membrane fusion

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Advances in cryo-electron microscopy have recently been coupled with correlated light and electron microscopy (CLEM), where fluorescence is used to locate regions of interest prior to data collection. While the goal of cryo-CLEM is typically localisation, the fluorescence can also probe function or dynamics if conditions are optimised for cryo-fluorescence microscopy. In this way, cryo-CLEM has the ability to assist not only in efficient high-resolution structural study, but also a description of the functional state of the molecule(s).

During membrane fusion, two bilayers enter into apposition, then progress to hemifusion and finally full fusion; this can be monitored by tracking dequenching of a concentrated lipid dye upon hemifusion with an unlabelled vesicle. We have identified conditions under which this assay can be performed on a cryo-CLEM grid, allowing localization based on lipid mixing, or unambiguous determination of lipid mixing in micrographs where this is not clear based on visualization alone. We have applied this method to a study of influenza virus-like particles fusing with synthetic lipid vesicles. We are adapting our cryo-CLEM system [1] to improve the signal-to-noise ratio and facilitate automation.

[1] Schorb, M. *et al.* (2016) *J. Struct. Biol.* 197(2): 83-93.

P-523

Pulse-shaped multiphoton excitation of single molecules

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Multiphoton excitation could have advantages over resonant excitation for single molecule spectroscopy. For example: deeper penetration of biological tissue due to reduction of out of focus excitation, access to different excited states, large spectral separation of excitation and emission and broad excitation spectra [1]. We seek to use the next generation of ultrafast lasers for single-molecule detection. However, dispersion from optical components can limit their use.

In this work we will describe a new home-built multiphoton fluorescence setup and present preliminary measurements. It deploys an ultra-broadband Ti:Sapphire laser (bandwidth 135 nm FWHM) and a pulse shaper for pre-compensation of broadening using the MIIPS-method [2, 3]. First experiments show that it is possible with this setup to achieve pulses of 9fs length in the focal plane of the microscope.

[1] He, G. S. *et al.,* Chem. Rev., **108**, 1245-1330 (2008)
[2] Coello, Y. *et al.,* JOSA B, **25** A140-A150 (2008)
[3] Lane, R. S. K. *et al.,* Opt. Express, **20** 25948-25959 (2012)

P-524

Image Mean Square Displacement analysis: a new method to study protein diffusion in cell membranes

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The Image Mean Square Displacement (iMSD) analysis is one of the newest techniques developed to investigate the diffusion of proteins within live cells. Based on the concepts of Fluorescence Correlation Spectroscopy and in particular on Spatiotemporal Image Correlation Spectroscopy method, iMSD analysis allows monitoring molecular dynamics obtaining protein diffusion laws. Starting from a stack of fast-acquired images, the new analysis technique gives the opportunity to map the diffusion of proteins in the entire cell, identifying the regions in which the protein is undergoing pure isotropic, confined, transiently confined or directed motion. As a result, maps of diffusion modes, of diffusion coefficients and of the size of fluorescent proteins diffusing inside the region are obtained, as well as local values for all the parameters characterizing the motion. The great potential of iMSD method is that diffusion laws can be obtained directly from imaging, in the form of an iMSD vs time-delay plot. Total Internal Reflection Fluorescence (TIRF) microscopy images have been analyzed to investigate the diffusion of Epidermal Growth Factor Receptor in live cell membranes, before and after stimulation with Epidermal Growth Factor.