

Platform: Optical Microscopy and Super-resolution Imaging: Novel Approaches and Analysis II

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ROCS Microscopy: Super-Resolution Imaging of Cellular Structures at 100 Hz

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Many new, exciting imaging techniques have emerged during the last decade, providing significantly improved spatial resolution and contrast. However, this extra information comes at the cost of more photons required to illuminate the cell, which requires more time and energy and often damages biological structures. The smaller the structures to be investigated, the faster they usually move inside living cells, because of both Brownian motion and coordinated work of molecular motors. Therefore, alternative imaging approaches are necessary. In this talk I will present a novel technique called rotating coherent scattering (ROCS) microscopy. This imaging method is characterized by label-free, coherent imaging through scattering of a rotating laser beam, which reveals a variety of unexpectedly fast processes inside/of living cells. The technique operates at up to 100 Hz with a spatial resolution of currently 150nm, it can acquire thousands of images without loss in image quality and does not require postprocessing, such that the cells can be observed online.

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New Super-Oscillatory Technology for Unlabelled Super-Resolution Cellular Imaging with Polarisation Contrast

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Super-resolution microscopy is already showing huge benefits across the bio-sciences, but all widely-used techniques require the addition of fluorescent probes. We have demonstrated optical-super-resolution imaging in unlabelled living cells, using the phenomenon of super-oscillation.

Super-oscillation is a mathematical phenomenon, first described in quantum mechanics. It is widely accepted that any function that is band-limited (in frequency) oscillates no faster (in time) than its fastest Fourier component. However, a band-limited super-oscillatory function may oscillate arbitrarily fast in regions of relatively low intensity. In optics, this means that we can create an arbitrarily small hotspot at the focus of a lens using engineered interference of light. However, super-oscillatory hotspots are necessarily surrounded by sidebands that contain some fraction of the optical power - trading efficiency for resolution. We replace the objective in a confocal microscope with a super-oscillatory lens and use the confocal pinhole to reject the light scattered from the sidebands. The resolution of the image is determined by the size of the super-oscillatory hotspot.

We have developed a super-oscillatory system to image unlabelled cells at super-resolution and high speed. To do this we combine our super-oscillatory microscope with advanced polarisation-contrast imaging. The instrument is a modification of a standard confocal microscope, with two key components: spatial light modulators to shape the laser beam entering the microscope, and a liquid crystal panel to control the input polarisation. We capture four differently-polarised super-resolved images of the sample and then calculate the anisotropy and orientation angle of each pixel. This highlights those parts of a cell with significant molecular structuring, such as actin filaments, microtubules, and protein-enriched lipid bilayers such as vesicles and cell membranes.

We have applied this to a number of systems showing it is able to reveal new levels of information in living and moving biological samples.

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FLIM Phasor Fingerprint of Bacterial Metabolic State

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While a large number of bacteria are deemed beneficial, there is an increasing number of strains associated with pathology, including life-threatening diseases. Moreover, during the last decade, a large number of new infectious strains have been discovered. Treatment is often hindered by emergence multi-drug resistant bacterial strains. Bacteria adapt to adverse environmental conditions by changing their metabolic activity. Further, there also exists metabolic diversity within the same population. Non-invasive assessment of bacterial metabolism could shed light on their physiological status. In this work we employ two-photon fluorescence lifetime imaging microscopy (FLIM) of live, clinically relevant bacteria. FLIM of autofluorescent metabolic coenzyme reduced nicotinamide adenine dinucleotide (phosphate), NAD(P)H, has been extensively exploited for label-free metabolic imaging of mammalian cells to study cancer and other diseases. However, it has not been explored to similar extents in bacteria. We apply phasor analysis to FLIM and create FLIM-phasor fingerprints of bacteria species *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus epidermidis*. The FLIM-phasor method also allows metabolic fingerprinting of individual bacteria within a population. We demonstrate that the NAD(P)H FLIM-phasor fingerprint of bacteria is sensitive to bacteriostatic and bactericidal antibiotics treatment. Furthermore, it varied with cell growth phase of planktonic cultures, suggesting that characteristic shifts in the NAD(P)H FLIM-phasor are representative of modulation of metabolic state of the cells. The FLIM-phasor approach represents a powerful non-invasive imaging technique to study bacterial metabolism and characterize the phasor fingerprint of bacteria under various conditions. This method could prove important in understanding bacteria related pathology, drug response and therapy as well as emergence of newer drug-resistant bacterial strains, all in a label-free manner.

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An Efficient Multicolor Two-Photon Imaging of Endogenous Fluorophores in Living Tissues by Wavelength Mixing

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Two-photon imaging of endogenous fluorescence can provide important physiological and metabolic information from intact tissues in a label-free and non-invasive way. However, imaging of multiple intrinsic fluorophores, such as NADH, FAD, retinoids and porphyrins in living systems is generally hampered by sequential multi-wavelength excitation resulting in long acquisition times and motion artifacts. We report an efficient and simultaneous multicolor two-photon excitation of endogenous fluorophores with absorption spectra spanning the 700-1040nm range, using wavelength mixing. By using two synchronized pulse trains at two different wavelengths, an additional "virtual" two-photon excitation wavelength is generated, and simultaneous excitation of blue, green and red endogenous fluorophores is achieved. This method permits fast and reliable simultaneous imaging of the metabolic coenzymes NADH and FAD to being implemented, overcoming the difficulties associated with their difference in absorption spectra and disparity in concentration. We achieve efficient ratiometric redox imaging and simultaneous efficient two-photon fluorescence lifetime imaging (FLIM) of NADH and FAD in living tissues. Lifetime gradients of NADH and FAD associated with different cellular metabolic and differentiation states were measured in both reconstructed human skins and live *C. elegans* worms. Finally, we perform hyperspectral imaging of endogenous fluorophores during early zebrafish development.

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Coordinate-Targeted Fluorescence Nanoscopy with Multiple Off-States

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Far-field superresolution microscopy or nanoscopy techniques "superresolve" features residing closer than the diffraction-limit by transiently preparing fluorophores in distinguishable (typically on- and off-) states and reading them out sequentially. In coordinate-targeted superresolution modalities, such as stimulated emission depletion (STED) microscopy, this state difference is created by patterns of light, driving for instance all molecules to the off-state except for those residing at intensity minima. For high resolution, strong spatial confinement of the on-state is required. However, this also subjects fluorophores at