Life & Science Imaging

Fast confocal fluorescence lifetime imaging

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Fluorescence lifetime imaging microscopy (FLIM) is a powerful and widely applied advanced microscopy technique, particularly in the biological sciences. In a standard single beam laser scanning microscope arrangement, acquisition times are typically of the order of 3 - 5 minutes, which represents a significant limitation when dynamic information is sought in a biological experiment. We have recently shown that acquisition times can be significantly reduced by the use of a scanning multiphoton multifocal arrangement, in which an array of beamlets is scanned across the sample and the fluorescence is imaged onto a single photon avalanche diode (SPAD) array detector.

In this report we demonstrate one-photon excitation multibeam confocal laser scanning microscopy as a method for rapid FLIM, with low laser powers and acquisition times two orders of magnitude shorter than conventional time-correlated single photon counting (TCSPC) based laser scanning FLIM. This reduction in acquisition time will enable real-time monitoring of intracellular processes, including protein-protein interactions.

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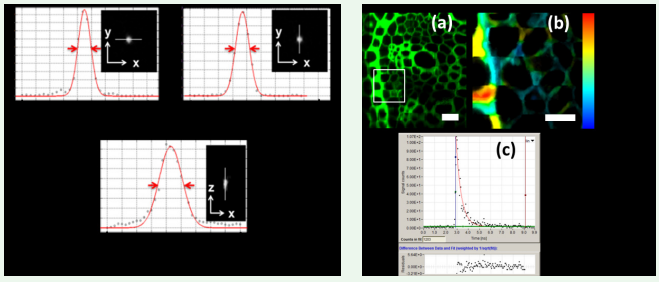

(Left): Optical resolution measurements of a 175 nm green microsphere.
(Right): (a) Fluorescence intensity image of convallaria section recorded using an array of 15x13 beamlets (b) 8x8 beamlet TCSPC FLIM image measured in 2 s corresponding to the ROI shown by the white box in (a). Scale bar 10 µm. (c) Representative decay curve from 3x3 pixels in the time-resolved image.

Multiplexed label-free detection of single proteins

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Optical detection of single molecules usually relies on the fluorescent properties of the molecule or on the introduction of a fluorescent label. Recently, alternative approaches eliminating the need for labels were introduced, using the local refractive index sensitivity of surface plasmon resonances for sensing. Protein sensing down to the single molecule level was shown. These breakthroughs were achieved either by total internal reflection type dark field microscopy, or with photothermal microscopy using photoinduced heat dissipation to generate a refractive index contrast.

These methods exclusively relied on the readout of one gold nanoparticle sensor at a time. However, it would be desirable to extend these techniques to allow for multiple-particle read out and thus simultaneous observation of multiple events for implementing next-generation clinical diagnostic assays. Schemes based on plasmon resonance shift detection of multiple particles were presented only recently, using either time gated events like a spatial light modulator in the conjugate image plane as a form of complex slit, sample scanning, or pure intensity readout to reach their multiplexing capabilities.

Here, we present a novel wide-field detection scheme, which allows simultaneous monitoring of the surface plasmon resonance of multiple gold nanorods. It directly records 2D spectrally dispersed images of gold nanorod samples. The spectra are corrected via an overlapped particle image that correlates the particle positions to the reference slit position, allowing us to use the full 2D space of the sensor. We present an unprecedented low noise fluor, allowing us to observe single protein particle binding events with high signal-to-noise ratios.

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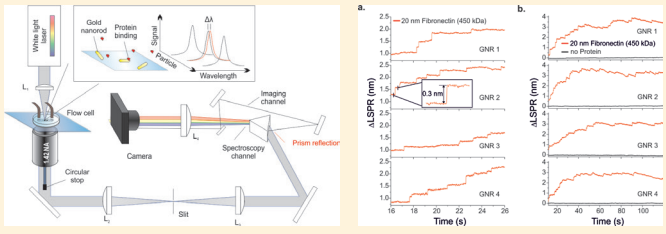

(Left): Experimental setup of the dark field microspectroscope.
(Right): Time-dependent surface plasmon resonance shift for protein free (black) and 20 nM Fibronectin solution (red).
A magnified region shows clear single protein binding steps, the inset emphasizes the unprecedented single to noise ratio (a). The full trace shows the saturation behavior after 1 minute (b).

FRET-FLIM as an approach to validate proteomics datasets

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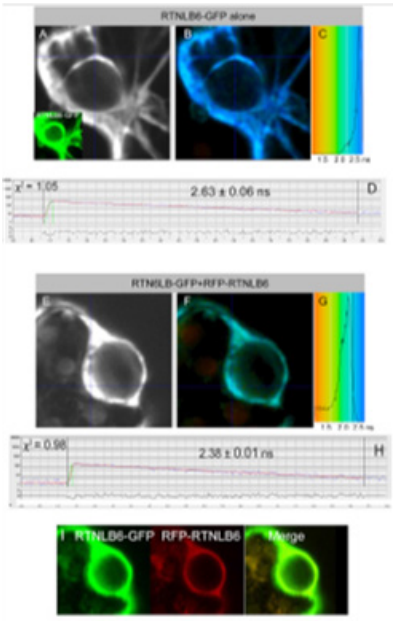
The cortical endoplasmic reticulum (ER) in plants is pleomorphic and structured as a tubular network capable of morphing into flat cisternae, mainly at three-way junctions, and back to tubules. Plant reticulon (RTNLB) proteins tubulate the ER by dimer- and oligomerization, creating localised ER membrane tensions that result in membrane curvature. Some RTNLB ER-shaping proteins are present in the plasmodesmal (PD) proteome [1] and may contribute to the formation of the desmotubule, the axial ER-derived structure that traverses primary PD [2]. Here we investigate the binding partners of two PD-resident reticulon proteins, RTNLB3 and RTNLB6, which are located in primary PD at cytokinesis [2].

We used a dual approach to identify interacting partners of RTNLB3 and RTNLB6 [1,2]. First, GFP-immunoprecipitation assays coupled to mass spectrometry, to identify proteins potentially binding to RTNLB3 and RTNLB6 was performed. Second, a detailed FRET-FLIM (Förster Resonance Energy Transfer by Fluorescence Life Time Imaging Microscopy) analysis was conducted with the identified proteins, to confirm prey-bait interactions in vivo.

Our mass spectrometry interaction data identified a large percentage (40%) of ER proteins, including other RTNLB family members. However, we also found a relatively large number (25%) of proteins present in the published PD proteome [1], and a surprisingly high proportion (35%) of plasma membrane proteins. Of the PD-resident proteins we identified, a significant number were shown previously to be targets of viral movement proteins (MPs). Additional proteins identified suggested roles for RTNLBs in transport and pathogen defence. We suggest that RTNLBs may play key roles in anchoring and/or signalling between the cortical ER and PM. This proteomics data was subsequently validated through in vivo assessment of interactions by FRET-FLIM analysis.

References: [1] Fernandez-Calvinoet al, 2011
[2] Knox et al, 2015



FRET-FLIM analysis of RTNLB6 without an interaction partner (A-D) or RTNLB6 dimerization (E-I).

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