

COMBINING FLUORESCENCE ANISOTROPY WITH TIR EXCITATION TO IMAGE PROTEIN-PROTEIN INTERACTIONS BY FRET

Daniel R. Matthews^a, Leo Carlin^a, Paul Barber^c,
Boris Vojnovic^c, Malcolm Irving^b, Tony Ng^a, Simon M. Ameer-Beg^a
daniel.matthews@kcl.ac.uk

^aRandall Division, Dimpleby Department of Cancer Studies, King's College London, New Hunt's House, Guy's Medical School Campus, London, SE1 1UL.

^bRandall Division, King's College London, New Hunt's House, Guy's Medical School Campus, London, SE1 1UL.

^cAdvanced Technology Division, University of Oxford, Gray Cancer Institute, PO Box 100, Mount Vernon Hospital, Northwood, Middlesex, HA6 2JR.

From simple phenotyping to high-throughput screening, automated microscopy is having a wide-ranging impact in the biological sciences. The advent of automated, high-speed fluorescence imaging is due to a general realization that a more biological, as opposed to biochemical, approach to screening applications is required. There are currently a number of instruments available commercially which allow the researcher to take this approach. There are, however, only two reports of high-throughput screening instruments based on the measurement of fluorescence lifetime^{1,2} rather than intensity, which remains the standard approach.

For many years total internal reflection (TIR) excitation has been applied in microscopy applications as a method of optical sectioning. Beyond the critical angle an evanescent wave exists at the sample surface with a decay length of the order of 100nm. In cell-based fluorescence microscopy this provides excitation in a range which covers the membrane of the biological sample. This has been further developed by adding polarised excitation allowing information about the orientation of bound molecules to be extracted, in addition to the spatial location³. Of course for unbound molecules an ensemble measurement loses this orientational information. Polarised TIR excitation implemented in single molecule imaging can however maintain the information about orientation of unbound species and this has recently been investigated⁴.

Here we show that polarised excitation can be used with TIR to monitor energy transfer processes. In previous work we have shown that the measurement of fluorescence anisotropy of sensitised emission in Forster resonance energy transfer (FRET) assays can be used as a metric for the rapid determination of the activity of biosensor molecules based upon the interaction of green and red fluorescence proteins⁵. A RAC1 variant of these biosensors, termed a "Ras and interacting protein chimeric unit" (Raichu) probe, was constructed in our laboratory. Expression in Jurkat T-cells has allowed us to demonstrate the sensitivity and dynamic range of the acceptor anisotropy technique to monitor the population of the biosensors in live cell assays. We have furthered this approach by using TIR excitation to provide high-spatial resolution information about our protein of interest. The variants of biosensors all report on the activity of proteins responsible for cell motility and in a broad sense are localised to the cell membrane. The ability to measure FRET in the spatial region of interest (the membrane) without the interference of fluorescence signals from the body of the cell, and at high-speed, is a major advance for automated microscopy and particularly for high-throughput and high-content screening. Here we describe our work on the implementation of TIR excitation in the measurement of acceptor anisotropy in FRET assays.

1. A. Esposito et. al., *Molecular and Cellular Proteomics*, **6(8)**, 1446-1454 (2007).
2. C. B. Talbot et al., *Journal of Biophotonics*, **1(6)**, 514-521 (2008).
3. D. Axelrod, *Biophysical Journal*, **26**, 557-574 (1979).
4. S. E. D. Webb, *Optics Express*, **16(25)**, 20258-20265 (2008).
5. D. R. Matthews, *Progress in Biomedical Optics and Imaging*, **9(18)**, (6859-19-1)-(6859-19-12) (2008).