Imaging protein–protein interactions in cell motility using fluorescence resonance energy transfer (FRET)

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Abstract

Protein–protein interactions and signal transduction pathways have traditionally been analysed using biochemical techniques or standard microscopy. Although invaluable in the delineation of protein hierarchy, these methods do not provide information on the true spatial and temporal nature of complex formation within the intact cell. Recent advances in microscopy have allowed the development of new methods to analyse protein–protein interactions at very high resolution in both fixed and live cells. The present paper provides a brief overview of using fluorescence resonance energy transfer to analyse directly molecular interactions and conformational changes in various proteins involved in the regulation of cell adhesion and motility.

Introduction

FRET (fluorescence resonance energy transfer) is a recently described approach that provides the means to visualize individual proteins and quantify biochemical reactions including phosphorylation, proteolysis and conformational changes. Labelling target proteins with specific fluorescent tags, either via genetic encoding [e.g. GFP (green fluorescent protein)/RFP (red fluorescent protein)] or chemical labelling (e.g. FITC/Cy3/Cy5) allows measurement of the 'fluorescence lifetime'. This is defined as the average amount of time a molecule spends in an excited state after absorption of a photon of light, and ranges from pico- to nanoseconds. FRET occurs when two different spectrally overlapping fluorophores are in close proximity to one another (typically <9 nm apart). This interaction causes energy to be transferred from the donor (e.g. GFP) to acceptor (e.g. Cy3) fluorophores, thus producing increased emission from the acceptor and a decrease in fluorescence lifetime of the donor. FRET can be determined by measuring either of these two phenomena: the former is known as 'acceptor-sensitized emission' and the latter 'donor-lifetime-based measurements' [1]. Although lifetime changes are the fundamental indicators of these interactions, intensity-based measurements are more commonly used in the literature recently due to the fact that this is a relatively easy and economical method of measuring FRET. However, there are many pitfalls associated with this method, namely the insensitivity, high background and the signal is entirely determined by the amount of fluorophores present. The alternative technique, routinely used in our own laboratory, is FLIM (fluorescence lifetime imaging microscopy), which measures changes in the fluorescence lifetime of the donor molecule. This technique is highly sensitive, demonstrates excellent resolution and does not depend on fluorophore intensity. There are two main methods for measuring fluorescence lifetime, namely time- and frequency-domain-based techniques. In the former, samples are excited using a short light pulse and the kinetics of the resultant fluorescent signal are measured over time. In frequency-domain measurements, the sample is excited by modulated light at a frequency equal to the reciprocal of the lifetime of the sample fluorophore (e.g. GFP). Changes in both phase shift and modulation can be used to calculate the fluorescence lifetime of the donor [2]. Until recently, the majority of FLIM experiments were performed using wide-field standard epi-fluorescence microscopes, which provide no spatial information regarding the site of protein interactions. Now, multi-photon or confocal laser scanning microscopes can be used to measure FRET [3,4]. This advance yields highly improved spatial resolution, controllable depth of field and the potential to generate a three-dimensional reconstruction of FRET signals within a cell by acquiring multiple optical sections. We are currently using this system in our laboratory to analyse interactions between molecules involved in cell migration and actin polymerization. Examples of some of the results obtained using both single- and multi-photon FLIM to analyse FRET interactions is discussed in the following section.

Examples of FRET interactions in cell motility

We have conducted experiments to investigate interactions between a number of proteins involved in actin-based cell motility and chemotaxis. The extracellular matrix receptor $\beta 1$ integrin has many important roles in cell motility, many

Key words: actin cytoskeleton, cell migration, fluorescence lifetime imaging microscopy (FLIM), fluorescence resonance energy transfer (FRET), signalling.

Abbreviations used: FLIM, fluorescence lifetime imaging microscopy; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; PAK1, p21-activated kinase-1; PKC, protein kinase C; RFP, red fluorescent protein.

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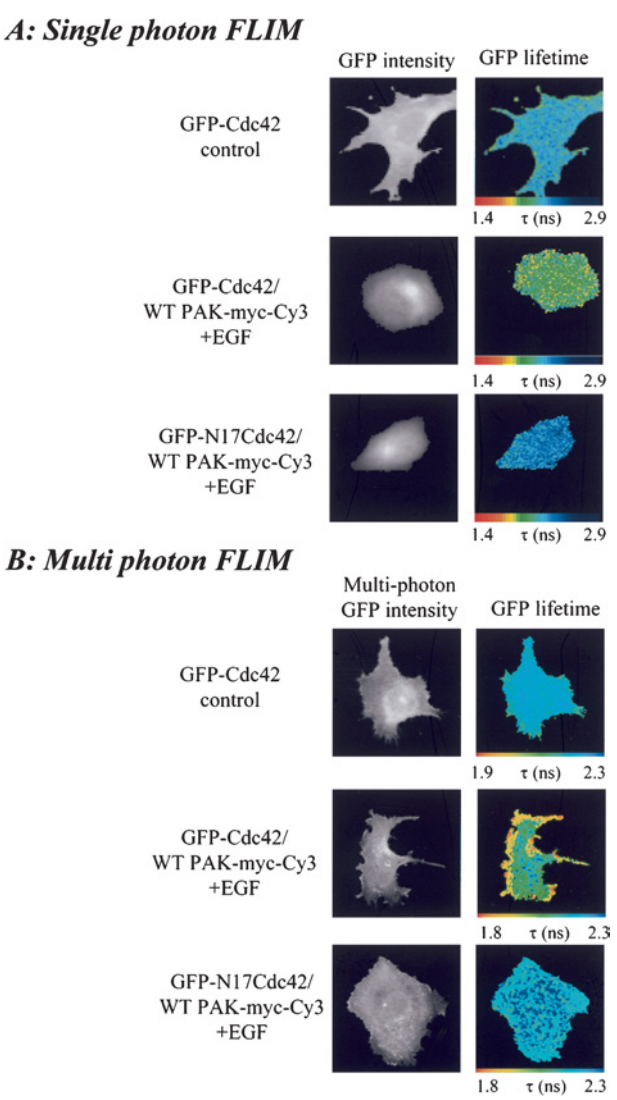
of which are as yet poorly understood. We were interested in the intracellular signalling molecules involved in the control of β1-dependent cell motility. Although many studies have used standard confocal fluorescence microscopy in an attempt to visualize integrin interactions with signalling partners, this has been hampered by poor resolution and clumping of proteins; hence true interactions are impossible to detect using this method alone. Consequent to biochemical evidence of an interaction between the enzyme PKCα (protein kinase Cα) and β1 in breast carcinoma cells, we overexpressed the GFP-tagged form of PKCα, fixed the cells and co-stained using a Cy3-labelled antibody to recognize the active ligand-bound form of the β1 integrin (mAb12G10). We then performed single-photon frequency-domain FLIM on these cells and demonstrated a significant interaction between the PKCα regulatory domain and an active β1 integrin [5]. This was evident by a decrease in the lifetime of the GFP–PKCα from a control GFP lifetime of 2.2 to approx. 1.8 ns, indicating transfer of energy between the two molecules. We further demonstrated that inhibition of this interaction using a peptide mimicking the 21-amino-acid tail of β1 integrin also inhibited β1-dependent cell motility.

We have also used single-photon FLIM to analyse interactions between PKCα and the actin-bundling protein fascin [6]. Fascin is a substrate for PKCα, and is phosphorylated at Ser^{39} after the activation of the enzyme by phorbol ester treatment. Single-photon analysis of FRET populations was performed, using GFP-fascin as a donor and PKCα-myc-Cy3 as an acceptor. The result demonstrates that fascin interacts with PKCα only in the presence of phorbol ester. Further studies revealed that cells expressing a mutant of the fascin Ser^{39} site to prevent phosphorylation by PKC demonstrated no change in the fascin-GFP lifetime indicating no FRET was taking place.

The present study employed both single- and multi-photon FLIM to analyse changes in protein phosphorylation by protein–protein interactions or extracellular stimuli [7]. PKCα, for example, is autophosphorylated after the activation at Thr^{250}. The population of active PKCα in both cells and tissues can be monitored by analysing intra-molecular FRET between the fluorescently labelled full-length molecule (e.g. GFP) and a labelled phospho-specific antibody to recognize Thr^{250}-phosphorylated site. The use of antibody: antibody FRET, as in this case, allows endogenous proteins to be analysed, and in this case breast cancer tissue samples were used to demonstrate that a higher population of active PKCα is present in invasive versus solid tumours [7]. We are also currently using multi-photon FLIM to determine the phosphorylation of proteins directly involved in actin polymerization such as PAK1 (p21-activated kinase-1) and N-WASP in cells using intramolecular FRET (M. Parsons, B. Vojnovic and S. Ameer-Beg, unpublished work). We have analysed the interactions between GFP-Cdc42, a member of the small GTPase family of proteins and PAK1-myc-Cy3, a downstream effector. Results from both single- and multi-photon lifetime imaging demonstrate a significant decrease in GFP-Cdc42 lifetime when the molecule is active (in the GTP-bound state) and can therefore bind to PAK1 (Figure 1).

Figure 1 | FRET between GFP-Cdc42 and PAK1-myc-Cy3 in single- versus multi-photon FLIM

MDA MB 231 breast carcinoma cells were micro-injected with plasmids encoding GFP-Cdc42 (WT or N17 dominant-negative variants) and PAK1-myc. Cells were then fixed and stained using an anti-myc-Cy3 conjugated antibody. Single- and multi-photon FLIM was performed as described previously [2,4]. (**A**) Images acquired using single-photon FLIM. The top panels show the lifetime of GFP alone (typically 2.2 ns) in the absence of Cy-3 antibody. The middle panels show the decrease in GFP lifetime in the presence of WT Cdc42-myc-Cy3 plus epidermal growth factor (EGF) to stimulate Cdc42 activation. The cell shows a relatively uniform decrease in lifetime across the cell. The efficiency of FRET is also shown again as a uniform change across the cell. The bottom panels show that the N17-Cdc42 dominant-negative control has no interaction with PAK1, and therefore no change in the GFP lifetime as compared with control values. (**B**) Similar images acquired using the same constructs but on a multi-photon FLIM. This time, the localization of interaction between WT GFP-Cdc42 and PAK-myc-Cy3 in the presence of an EGF can be seen clearly at the cell periphery and in membrane protrusions. These results demonstrate that higher resolution imaging is possible on multi-photon versus single-photon lifetime imaging microscopes.



The Figure demonstrates the difference in resolution of the FRET signal between single- (Figure A) and multi-photon (Figure B) microscopy. Multi-photon imaging allows the detection of specific structures such as filopodia and vesicles (Figure B), which is not possible using epifluorescence single-photon images (Figure A). Improved resolution through the use of multi-photon microscopy is crucial to the future of imaging to allow clear spatial and temporal data acquisition, which will shed light on the true localization and kinetics of protein interactions and modifications during cell motility.

Future applications

The application of FRET to the study of protein interactions during cell motility and adhesion has already greatly enhanced the understanding of the behaviour of proteins in a motile cell. The ongoing development of new fluorescent probes, microscopy and image quantification software for more efficient and in-depth analysis will widen the scope for applications of this technology. The combination of FRET with other imaging techniques, such as total internal reflection microscopy, will allow better resolution of interactions at the single cell and tissue level [8]. Similarly, the use of multi-photon technology combined with living colour fluorescent proteins or probes permits the visualization of whole organisms, thus enabling FRET analysis to be performed on proteins in live animals. High throughput FRET measurements may provide a more sensitive alternative to existing methods (such as yeast two-hybrid) for screening potential new protein–protein interactions [9]. It would also be possible to use such systems for screening pharmacological agents aimed directly at disrupting protein interactions of modifications in the treatment of disease. Certainly, technologies such as these will provide unique insight into the way in which proteins behave during cell adhesion and motility both *in vitro* and *in vivo*.

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Received 3 December 2003