Journal of Microscopy

Journal of Microscopy, 2012
Received 5 September 2012; accepted 25 October 2012
doi: 10.1111/j.1365-2818.2012.03686.x

Using FRET to analyse signals controlling cell adhesion and migration

K. KEMP-O'BRIEN & M. PARSONS
Randall Division of Cell and Molecular Biophysics, King's College London, London SE1 1UL, U.K.

Key words. Biosensor, cell adhesion, cell migration, cytoskeleton, FRET, GTPase cell signalling, microscopy.

Summary

Cell adhesion and migration are dynamic, complex processes that require tight temporal control of signalling cascades at defined subcellular sites to occur. Decades of research have used biochemical methods to identify numerous signalling pathways that are involved in the coordination of adhesion, membrane protrusion and contractility that all contribute to migration. However, understanding the way in which these signals are controlled within discrete sites in individual cells undergoing migration is essential to enable a clearer understanding of how these pathways are integrated during motility. Recent advances in the generation of fluorescent protein variants have enabled the development of probes to analyse localized changes in protein conformation, activation or interactions in single cells using microscopy. Many of these probes are based on the use of fluorescence resonance energy transfer that permits precise determination of interacting species based on the emission or lifetime properties of the fluorophores involved. Here, we provide an overview of some of the recent studies that have developed such probes and examples of how these biosensors have been used to further our understanding of cell migration signalling.

Introduction

Cell migration is a complex, integrated process that choreographs the morphogenesis of the embryo during development. In the adult, cell migration is central to homeostatic processes, such as mounting an effective immune response and the repair of injured tissues. The failure of cells to migrate or migration of cells to inappropriate locations can result in life-threatening consequences and pathologies, including vascular disease, chronic inflammatory diseases and tumour formation and metastasis. Understanding cell migration is therefore essential for developing new ways to target diseases as well as in emerging areas of biotechnology that focus on cellular transplantation and the manufacture of artificial tissues.

Most cells require adhesion to the extracellular matrix (ECM) and to adjacent cells for survival, differentiation and migration during both embryonic development and adult homeostasis. Adhesions are highly ordered, dynamic structures under tight spatial control at the subcellular level to enable localized responses to extracellular cues. The coordination of adhesion dynamics with intracellular signalling involves numerous signalling pathways that must be tightly controlled in time and space (Scales & Parsons, 2011). In simple terms, migration is characterized as a four-step cyclical process in which a cell extends a protrusion at its front, which in turn attaches to the substratum. This is followed by actomyosin contraction events to pull the cell body forward and disassembly of the rear attachment sites to permit forward movement (for recent review, see Wehrle-Haller, 2012). The cycle is initiated by external signals (such as chemotactic molecules), mechanical forces (such as from the ECM) or other cells, which are sensed and communicated initially via receptors at the plasma membrane. In response to these external cues, cells initiate a cascade of signalling events to result in the extension of F-actin-rich protrusions at the front that initiates the migration cycle. The orchestration of this complex process resides in a large cohort of signalling proteins that act as front/rear polarity indicators and dictate the discrete spatiotemporal dynamics of the adhesion machinery to control the cytoskeleton and ultimately guide migration (Rottner & Stradal, 2011).

Decades of research have revealed a considerable amount about the biochemical pathways that mediate different stages of the migration cycle. However, much of our current knowledge has been derived from biochemical analysis of cell lysates, as opposed to studies of signals that occur in living cells undergoing migration, largely due to the technical

Correspondence to: Maddy Parsons, Randall Division of Cell and Molecular Biophysics, King's College London, New Hunts House, Guys Campus, London SE1 1UL, UK. Tel: 0207 848 8164; fax: 0207 848 6435; e-mail: maddy.parsons@kcl.ac.uk

complexities of analysing such dynamic events in intact cells. However, recent advances in both fluorescence microscopy techniques and biological probe development have resulted in significant improvements in our understanding of signal propagation in live cells migrating in different contexts. Many of these probes make use of fluorescence resonance energy transfer (FRET) to monitor protein–protein interactions or changes in conformation (for a more in-depth FRET review, see Morton & Parsons, 2011a). Here, we summarize some examples of recent studies that use a variety of FRET-based probes that demonstrate the power of this method when analysing signalling events during cell adhesion or cell migration.

Basic principles of FRET and ways to measure it

FRET is a technique that allows detection of fluorescent molecules that are in very close proximity, thus indicating protein–protein interactions. For FRET to occur, the emission spectrum of one fluorophore (donor) must overlap with the excitation spectrum of the second (acceptor). When the donor protein, tagged with the fluorophore having the shorter wavelength, comes into close proximity with the acceptor protein (typically <9 nm), a nonradiative resonance energy transfer takes place from the donor to the acceptor fluorophore leading to three measurable outputs: (1) the fluorescence intensity of the acceptor increases; (2) fluorescence intensity of the donor decreases and (3) a reduction in donor fluorescence lifetime occurs. Depending upon the experimental context, one or more of these outputs can be used to obtain information regarding interactions between proteins of interest (Day & Davidson, 2012).

Many methods to perform and measure FRET exist and the most commonly used involve the transfection of proteins that are fluorescently tagged with, for example, cyan fluorescent protein (CFP) as the donor and yellow fluorescent protein (YFP) as the acceptor. These fluorophores are commonly used as they can be imaged using standard filters and lasers on a widefield or confocal microscope. FRET can be measured in these samples provided that the levels at which each protein is expressed are the same, thereby resulting in equal intensities of each fluorophore in one cell. The ratio of intensity of both fluorophores in each pixel across each image can in this case be used to calculate FRET; an approach known commonly as ratiometric or sensitized emission FRET. Essential to this technique is the exact 1:1 expression level of the donor:acceptor probe pair – analysis of two different labelled proteins expressed from two different plasmids is thus not possible using this technique. The best, and most common way to ensure equal expression of the fluorophores is the expression of a single 'biosensor' plasmid that encodes the protein or domain(s) of interest flanked on either side with the donor and acceptor fluorophores, such as CFP and YFP. Such intramolecular biosensors are generally reliant upon conformational changes within the protein, such as induced by interactions with effector binding partners, that lead to induction or loss of FRET between the donor and acceptor fluorescent proteins (see Fig. 1).

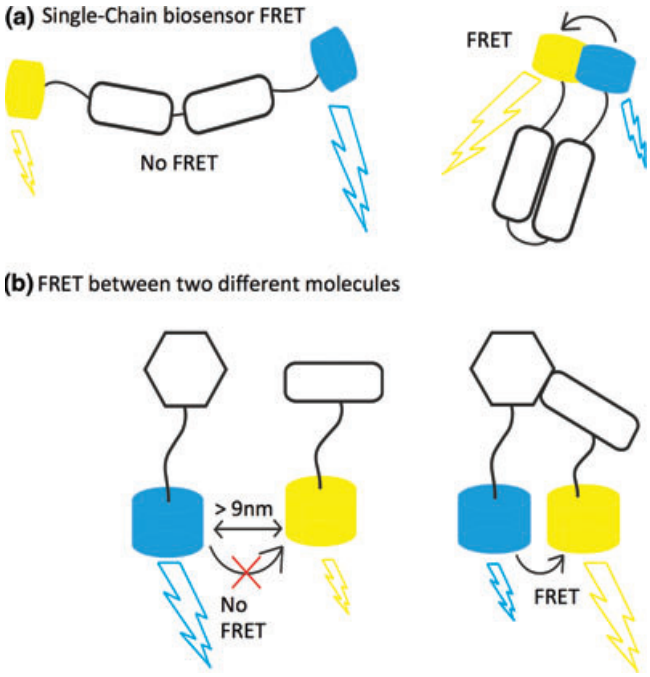

Fig. 1. Basic principles of FRET. Many proteins undergo a conformational change upon activation or binding to other proteins, and this can be studied through use of single-chain FRET probes or 'biosensors'. When single-chain FRET probes (A) are in their extended state, FRET efficiency is low as the two fluorophores (CFP/YFP) are more than 9 nm apart, too far for FRET to occur. This results in an even balance between acceptor and donor emission intensity. However, as the protein changes conformation (such as through activation or binding to effector molecules encoded within a biosensor) the two fluorophores are brought together resulting in FRET between the donor and acceptor. This is seen as an increase in acceptor fluorescence emission or a decrease in donor fluorescence emission. This principle can be utilized to answer a wide range of biological questions about single proteins or specific subdomains of proteins undergoing interactions. (B) FRET between two different molecules can be analysed by coexpressing different fluorescently tagged proteins of interest on separate plasmids. When there is no interaction, the fluorescent proteins are too far apart to undergo FRET, however upon protein interaction the fluorophores are brought close enough together to undergo FRET and an increase in FRET efficiency is seen. This can be measured by a decrease in donor fluorescence lifetime, or increase in acceptor fluorescence emission (using acceptor photobleaching).

An alternative to ratio FRET measurements is photobleaching of either the donor or acceptor within a specific region of interest, followed by measuring the change in intensity of the other fluorophore within the same region. This can be performed on intra- or intermolecular FRET sensors, with the latter containing proteins or protein domains coupled to fluorophores on separate plasmids (Fig. 1).

Acceptor photobleaching is generally preferred as it results in an increase in donor intensity when FRET occurs, which is an easier read-out to measure than the loss of acceptor intensity upon donor photobleaching. The acceptor photobleaching approach is an inherently irreversible process, and thus suited only to analysing either a single event in live cells or fixed specimens. Despite the fact that acquisition of FRET data is relatively easy, the analysis of resulting data can be quite difficult and several issues need to be considered, such as the correct controls (both biological and from a technical perspective), calibrations for spectral crosstalk between fluorescent channels and corrections for photobleaching in order to avoid false positives. Additional problems that might arise include bleed-through from one fluorophore to another (often due to the use of incorrect filters) and a poor signal-to-noise ratio, both of which give rise to erroneous results (Miyawaki, 2011).

An alternative way to measure FRET is by fluorescence lifetime imaging microscopy (FLIM). FLIM uses specific detectors to measure the inherent fluorescence lifetime of the donor molecule and can be performed using either one- or two-photon illumination sources, depending upon the type of FLIM. There are two types of FLIM: frequency domain and time domain, and each offers different advantages and caveats. Frequency domain FLIM uses high frequency intensity-modulated light to excite the sample resulting in emission at the same modulation frequency. If the emission is delayed, this can be measured as a phase shift and used to calculate the fluorescence decay time. Time-domain measurements however are made using pulsed light sources whereby the pulse width is much shorter (typically ps) than the lifetime of the fluorophore (typically ns). The time-dependent intensity decay is measured after each pulse and the lifetime calculated from this. In both cases, a reduction in the normal fluorescence lifetime of the donor (typically green fluorescent protein (GFP) with a lifetime of around 2.3 ns) indicates FRET, and can be presented in a pixel-by-pixel lifetime map across an individual cell, or as cumulative FRET efficiency histograms of averaged lifetime over the entire cell or region of interest for statistical analysis. FLIM has a number of benefits over intensity-based approaches as it is independent of fluorophore concentration and highly sensitive thus able to offer significantly better spatial resolution and improved signal-to-noise ratios (Morton & Parsons, 2011b). However, FLIM requires more specialized (often expensive) acquisition equipment such as pulsed laser sources or specialized detectors, and often requires highly complex data analysis and as a result is less widely used for FRET analysis compared to intensity-based approaches.

A limiting feature of FRET/FLIM is the choice of fluorophore pairs that can be analysed. GFP remains the best and most widely used donor molecule for FLIM. This is due to the monoexponential fluorescence decay kinetics exhibited by GFP that make it ideal for lifetime analysis. Multiexponential decay curves in the uncomplexed state (such as is seen with CFP, for example) make subsequent calculations of interacting populations very complex and prone to artefacts. Hence, GFP-mCherry or GFP-mRFP is the most widely used fluorophore pair for FRET/FLIM analysis in studies to date. However, very recently, two new fluorescent proteins have been developed that exhibit monoexponential decay kinetics: mTFP (or Teal) and mTurquoise. These variants of CFP offer alternative fluorescent protein donors for FLIM and can be used in combination with YFP or brighter, more photostable variants of YFP (e.g. Venus) as suitable FRET/FLIM protein pairs (Grashoff *et al.*, 2010; Klarenbeek *et al.*, 2011).

Use of single-chain FRET biosensors to study signalling during cell adhesion and migration

Single-chain FRET probes encode both donor and acceptor fluorophores expressed as part of a single protein. Most are based on induction of a conformational change following activation of protein effector binding that will result in an increase or decrease in distance between the donor and acceptor fluorophores and in turn the occurrence of FRET. Single-molecule FRET biosensors have major advantages over two-protein FRET (i.e. encoded on separate plasmids; Fig. 1) as additional complications of donor and acceptor expression do not need to be taken into account (see above).

GTPases

The small GTPases were one of the first families of molecules to be analysed using single-chain FRET biosensors. GTPases become active when bound to guanosine triphosphate (GTP), inducing a conformational change, which allows for the binding of the GTPase to its effector protein. Tagging an effector itself alone (such as Ras) was deemed unsuitable for FRET due to the absence of a large conformational change. Therefore, a chimeric construct was instead generated encoding the Ras binding domain of Raf linked to H-Ras with N and C terminal CFP and YFP fluorophores. Upon activation and GTP loading, the Ras binding domain of Raf binds to H-Ras inducing a large conformational change in the chimeric protein that is sufficient to alter the FRET efficiency between the CFP/YFP fluorophores (Mochizuki *et al.*, 2001). This probe was called Ras and interacting protein chimeric unit, or 'Raichu' and was the first in a range of similarly designed probes for different GTPase family members. Indeed, in the same publication, the authors also replaced the H-Ras part of the chimeric protein with Rap1A and this was shown to function as a Rap1 biosensor. The authors used these probes to show that upon epidermal growth factor (EGF) stimulation, Ras is activated at the periphery of the cell whereas Rap1 is activated at the perinuclear region.

Rac and Cdc42 GTPase single-molecule FRET probes were subsequently made in a similar fashion to the Ras/Rap1 Raichu probes. These consisted of the CRIB domain of Pak1 and Rac1 or Cdc42 between CFP and YFP with the

membrane-targeting CAAX box of K-Ras at the N-terminus to locate the probe to the cell membrane (Itoh *et al.*, 2002). GTP binding to Rac1 or Cdc42 induces binding of the GTP-binding domain (CRIB) domain of Pak1 and causes a conformational change in the chimeric protein, which brings together the CFP and YFP components. This results in increased FRET and allows for visualization of localized active GTPase species in response to changes to the GTPase exchange factor/activating protein equilibrium at the membrane. Active Raichu-Rac and Raichu-Cdc42 were both seen at the leading edge of the cell even when the cell changed direction. At higher magnification, active Raichu-Cdc42 was more concentrated at the very tip of the leading edge as denoted by higher FRET (Itoh *et al.*, 2002). A variety of RhoA-binding domains from known RhoA effectors were subsequently screened in order to find the RhoA-binding domain with the optimal change in FRET within the chimeric protein and as a result, the RhoA-binding domain of Protein Kinase N was used in a new Raichu-RhoA probe (Yoshizaki *et al.*, 2003). Further modifications have been made to insert other GTPases and effector proteins that now span a large number of different family members (Pertz, 2010). Subsequently, the GTPase FRET biosensors are now used in many labs to study various aspects of GTPase signalling during cell adhesion and migration and have proven to be powerful tools to dissect upstream regulators of these pathways (see Fig. 2).

Recently, development of novel postacquisition analysis tools for these probes has been further improved to enable highly localized spatiotemporal activation patterns to be determined in living cells. Complex computational analysis and modelling approaches enabled the spatiotemporal analysis of RhoA, Rac1 and Cdc42 activation profiles in different cells to be correlated, and thus identify the time and location of the activation of the three GTPases during membrane protrusion in fibroblasts (Machacek *et al.*, 2009). This analysis revealed a characteristic pattern of GTPase activation in highly confined subcellular domains, on the order of micrometres, and occurring with precise time shifts, on the order of tens of seconds. RhoA was activated directly at the leading edge at the onset of membrane protrusion, followed by inactivation during membrane retraction. However, Rac1 and Cdc42 were activated later during the protrusion phase, and continued to persist longer during retraction. The areas of active Rac1 and Cdc42 lagged behind and were mutually exclusive with the pool of active RhoA directly at the leading edge (Machacek *et al.*, 2009). This study demonstrated the complex feedback networks that exist between GTPases in a living cell, and highlights the importance of studying these events at high temporal and spatial resolution.

Kinases

FRET-based probes have also allowed for spatiotemporal visualization of kinase activity in intact migrating cells. One of the first to be made was for cAMP-dependent protein kinase (PKA), a key kinase in many signalling pathways during adhesion and migration (Hu *et al.*, 2001). PKA is anchored to specific sites by different anchor proteins (AKAPS), which allows for control of specific functions within discrete subcellular locations (Kritzer *et al.*, 2012). Zhang and colleagues created the AKAR construct which encodes a CFP/YFP FRET pair with a phosphoamino acid-binding domain from 14-3-3 protein and a phosphorylatable sequence specific to PKA in between, each separated by a flexible linker sequence (Zhang *et al.*, 2005). Upon phosphorylation, a change in conformation occurs leading to the phosphoamino acid domain binding to the newly phosphorylated residue to bring the two fluorophores together to undergo FRET. This intramolecular binding is also reversible by dephosphorylation enabling dynamic changes in FRET to be measured. A range of chimeras were made and tested for emission ratio changes over time before the AKAR1 chimera was selected and further tested, illustrating the extensive optimization procedures that must be carried out prior to using a new biosensor for biological measurements. The optimized AKAR1 construct was distributed evenly throughout the cytoplasm of the cell but not the nucleus and acceptor photobleaching demonstrated increased FRET efficiency after stimulation with the cAMP activator Forskolin (Zhang *et al.*, 2005). A new variant of this probe, termed AKAR2, has subsequently been used to show different levels of PKA activities in the cytoplasm and nucleus in live cells over time (Zhang *et al.*, 2005). A targeted mitochondrial variant, AKAR3, was also used to analyse differences between PKA activity in the cytoplasm, nucleus and mitochondria by ratiometric FRET with improved constructs for AKAR1 and AKAR2 providing better sensitivity (Allen & Zhang, 2006). These biosensors have recently been used in different cell types to show that PKA activity is increased at the leading edge during migration and invasion in an RII-AKAP binding-dependent manner (Lim *et al.*, 2008; McKenzie *et al.*, 2011).

A different approach was taken by another group to design a FRET probe for the analysis of the kinase Akt (Yoshizaki *et al.*, 2006; Yoshizaki *et al.*, 2007). The Akt indicator, or Akind, has YFP inserted between the PH domain and the catalytic domain of Akt and the CFP fluorophore was placed at the C-terminus. When inactive, Akt has a closed conformation. This keeps the fluorophores apart, but upon activation a conformational change occurs exposing the PH domain and recruitment of the kinase to the plasma membrane. This also induces the two fluorphores to come into closer proximity and undergo FRET. Akind was shown to become transiently active and move to the cell periphery following EGF stimulation in live cells (Yoshizaki *et al.*, 2007).

Another recent example of the use of both ratiometric and acceptor photobleaching of FRET in adhesion biology was in the development of a FRET-based sensor to study conformational changes in the four point 1 ezrin/

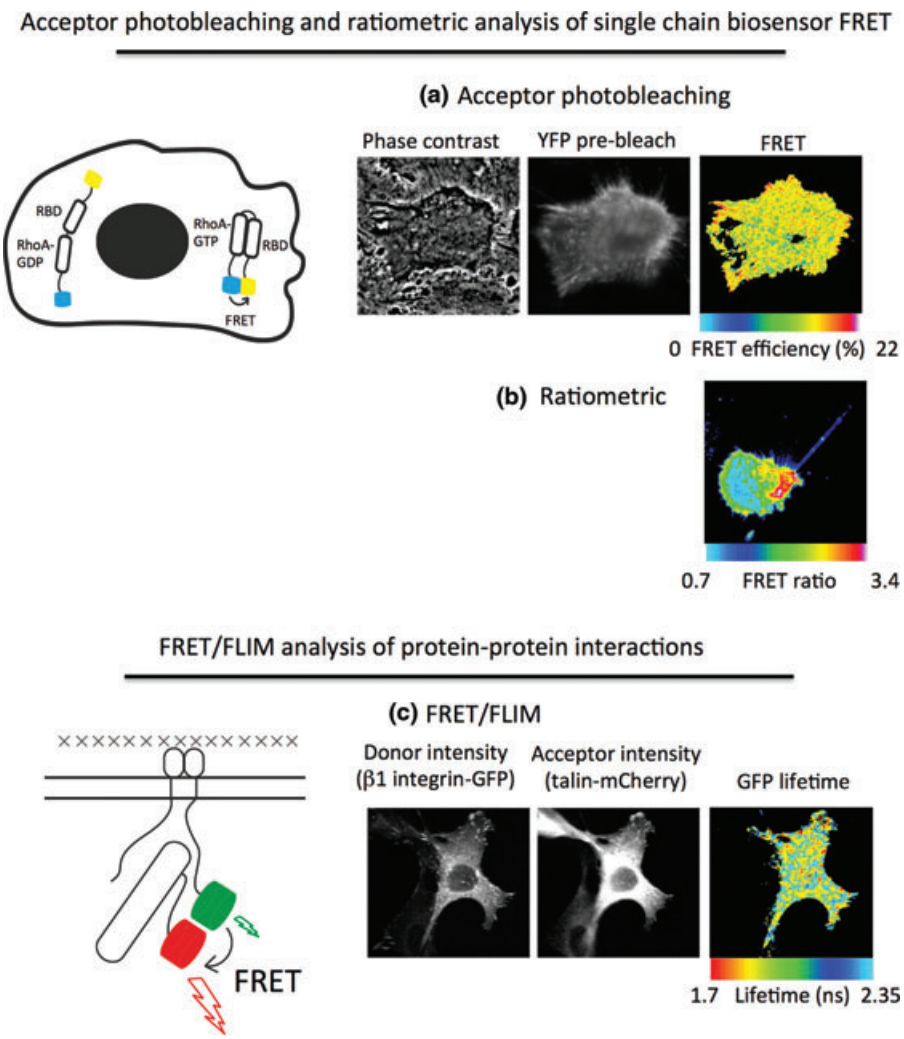

Fig. 2. Examples of data arising from different FRET analysis techniques. (A) Acceptor photobleaching FRET: occurs following a change in intensity of one fluorophores following photobleaching of the other. Cartoon on the left shows potential changes in the RhoA Raichu biosensor FRET in different locations within a single cell. The example data images in (A) show acceptor photobleaching of the CFP/YFP single-chain RhoA FRET biosensor (see text for details) in fixed epithelial cells acquired on a confocal laser-scanning microscope. Images show the cell by phase contrast (in contact with neighbouring epithelial cells), the YFP prebleach image (central panel) and the FRET channel (right-hand panel). The YFP acceptor was photobleached and the resulting increase in the intensity of the donor fluorophore (CFP) measured and FRET efficiency calculated. In the example image increased FRET is seen in the red/orange coloured regions. Acceptor photobleaching FRET can be used to calculate FRET efficiency and therefore can be used to quantify interacting species between different cells or conditions. (B) Ratiometric FRET: image of the same RhoA Raichu biosensor expressed in human epithelial cells (as in A), but analysed by measuring the intensity of ratios of both fluorophores in each pixel of the image and expressed as a ratio allowing for visualization of when and where FRET is highest. Areas showing pink/white pixels denote highest ratio between donor/acceptor fluorescence intensities and therefore highest FRET. This technique provides spatial information regarding localization of interacting species, but is not quantitative. (C) Fluorescence lifetime imaging microscopy (FLIM): whereby the changes in total donor lifetime can be measured by using specific detectors that enable the fluorescence decay kinetics to be quantified. Cartoon on the left shows the example protein pairs being analysed in the images on the right: $\beta 1$ integrin-GFP and mCherry-talin expressing fibroblasts. Donor and acceptor intensity images are shown alongside the lifetime map of the donor (far right), where red areas indicate high FRET (low lifetime). In this instance, a decrease in donor lifetime indicates FRET, which can be mapped on a pixel-by-pixel basis. In addition, data can be pooled from multiple cells or experiments to permit quantification of interacting species between conditions.

radixin/moesin domain of focal adhesion kinase (FAK). As outlined in the previous section, traditionally for the purpose of intramolecular FRET analysis proteins are tagged on their N and C termini. However as FAK is a large and relatively bulky protein, this strategy was deemed inappropriate. To increase the likelihood of energy transfer between fluorophores, the authors of this study instead inserted a CFP tag within the coding region of FAK, i.e. between the four point 1 ezrin/radixin/mosein and kinase domains, and the YFP tag on the N terminus (Papusheva *et al.*, 2009). The sensor

was shown to behave as conventionally tagged FAK protein and was then used to show that FAK exists in different conformational states at different stages of cell migration and spreading. At the same time another group published the use of another FAK biosensor that instead relies upon the molecule being in an autoinhibited state to drive FRET (Cai *et al.*, 2008). In this case, the loss of autoinhibition, such as during FAK activation, leads to loss of FRET between N- and C-terminal fluorophores. The authors' demonstrated activation of this biosensor occurs in live cells and that this was dependent upon binding to the phospholipid Phosphatidylinositol 4,5-bisphosphate.

Another essential adhesion kinase, the tyrosine kinase Src, has also been studied using a FRET-based biosensor approach. The Src biosensor encoded the src homology domain (SH2) of Src coupled to a synthetic substrate with CFP and YFP at either end. Binding of the SH2 domain to the substrate induced high FRET that could be measured in intact cells (Wang *et al.*, 2005). The authors demonstrated that Src was activated upon integrin ligation using fibronectin-coated beads and that this depended upon intact actin and microtubule cytoskeletons. This probe has subsequently been used in other studies to analyse activation of Src during cell adhesion and migration in a number of contexts (reviewed in Seong *et al.*, 2011).

Lipids

Lipids play a vital role in adhesion and migration signalling. Lipids are highly dynamic molecules that undergo modifications to distinguish different intracellular environments for the localization of membrane-bound proteins. As the lipid composition of membranes is highly dynamic, it has been very difficult to clearly identify when and where specific phospholipid species are being generated in live cells. The first phospholipid to be successfully analysed using a FRET probe was Phosphatidylinositol (PI) (3,4,5) triphosphate (PIP3) (Sato *et al.*, 2003). Local PIP3 production is increased in response to activated PI3-Kinase and is important for the recruitment and activity of many proteins, such as Akt and Grp1 (Falasca & Maffucci, 2012). Previously, GFP-tagged versions of PIP3-binding PH domains have been used to localize PIP3 in cells, but these approaches suffer from artefacts introduced by fluorescence intensity changes at membrane ruffles and lack of differentiation between probe localization at separate membranes within a cell (Venkateswarlu *et al.*, 1998). The FRET-construct used to overcome this consisted of a specific PIP3-binding PH domain from GRP1, termed the lipid-binding domain (LBD), sandwiched between a CFP and YFP FRET pair which was then tethered at the acceptor end to the plasma membrane via a CAAX-box motif from K-Ras. The linkers between each domain of the probe were rigid α-helical repeated protein sequences, which flank an individual diglycine motif operated as a hinge. Thus, in the non-PIP3 bound state the two fluorophores show low FRET, but in the presence of PIP3 the lipid-binding domain causes a change in molecular conformation to bring the two fluorophores together and allow intramolecular FRET. The probe was validated through microinjection of synthetic Phosphatidylinositol 4,5-bisphosphate or PIP3 and the CAAX box was modified to change the localization from the plasma membrane to the endoplasmic membrane and these two variants were termed fllip-pm and fllip-em, respectively (Sato *et al.*, 2003). This probe was further validated using known activators of PI3K that induced PIP3 production and resulted in increased FRET in a dose-dependent manner. The two FRET probes, fllip-pm and fllip-em, were shown to respond equally to similar microinjected concentrations of PIP3. The authors demonstrated that PIP3 levels increased at both the plasma and endoplasmic membranes following platelet derived growth factor (PDGF) stimulation, but increased more at the latter due to *in situ* production at these membranes in response to endocytosis of activated receptor tyrosine kinases.

The fllip-pm construct was further modified to use as a biosensor for another lipid, phosphoinositol, 3,4 bisphosphate (PI(3,4)P2) using the lipid-binding domain of GRP1 replaced with the PH-domain of TAPP1 which binds to PI(3,4)P2 (Yoshizaki *et al.*, 2006). The probe functions in the same way as previously described for the PIP3 FRET biosensor but the probe recognizes and had higher FRET in the presence of PI(3,4)P2 in the plasma membrane. The authors used the PI(3,4)P2 FRET probe in conjunction with two other previously discussed probes, the GTPase FRET probe Raichu-RalA which functions the same as other Raichu probes, and the Akind FRET probe for active Akt described in the previous section. With the Akind probe, the authors found that it was translocated to the cell periphery where higher FRET observed following treatment with EGF, and most prominently at lamellipodia. Following EGF stimulation, PIP3 was found to be increased diffusely along the plasma membrane as shown by increased FRET, PI(3,4)P2 however was seen to increase more at the plasma membrane at lamellipodial protrusions, similar to that seen with the Akind Akt FRET probe. Taken together, these probes have provided powerful evidence of the activation dynamics and interplay of a GTPase, kinase and lipid signalling at the leading edge of cells.

Proteases

Proteases play key roles in many cellular processes ranging from apoptosis to cancer metastasis and being able to know when and where proteases are being activated is of great importance to a range of biological and medical fields. A key protease involved in cancer biology is membrane type 1 matrix metalloproteinase (MT1-MMP) that remodels the ECM through proteolysis. A FRET biosensor was developed to monitor activation of MT1-MMP in live cells, which consisted of a protein sequence, which is known to be cleaved by

MT1-MMP sandwiched between enhanced cyan fluorescent protein (ECFP) and YPet fluorophores (Ouyang *et al.*, 2008). When the biosensor is near activated MT1-MMP, the cleavage site would be cut resulting in a loss of FRET in the biosensor. As the catalytic domain of MT1-MMP is expressed on the outside surface of the cell, so too must the biosensor. The authors therefore utilized a vector that targeted the biosensor for the secretory pathway and the C-terminal transmembrane domain of the PDGF receptor to allow it to still be tethered to the plasma membrane. The biosensor was used to show that after EGF stimulation, more active MT1-MMP was at the leading edge.

Another group of proteases, the calpains, are also known to be involved in controlling cell migration through cleavage of a number of substrates at adhesions such as talin and FAK (Franco & Huttenlocher, 2005). Using a similar approach to that of the MT1-MMP probes described earlier, calpain substrate peptides have been used to generate FRET biosensors that monitor the local activation of calpain in intact cells or *in vivo* (Stockholm *et al.*, 2005; Kelly *et al.*, 2009). Although not yet tested in migrating cells, these probes offer the potential to analyse calpain activity within local adhesion environments to understand the potential regulators that control spatial substrate cleavage to promote motility.

Mechanosensors

Recently, FRET has begun to be utilized to monitor mechanical tension within cells using force sensing FRET probes. One such probe was used to study the forces being applied across α-actinin (Meng & Sachs, 2011). α-actinin is an antiparallel homodimer that binds to parallel actin filaments. Venus and Cerulean fluorescent proteins were connected via a spectrin repeat linker that, in its default configuration, results in high FRET. The probe was shown to be force sensing through the *in vitro* addition of single or double-stranded DNA springs that bound to either end of the probe and exerted a force of between 5 and 7 pN, which resulted in a large decrease in FRET as the protein was stretched. This probe named sstFRET was incorporated into the cytoskeletal cross-linking protein α-actinin in between the first and second of its spectrin repeats. Actinin-sstFRET was shown to localize similarly to actinin-GFP in two cell lines despite being a larger protein. Higher FRET was seen at the leading edge of migrating cells and also in actinin when cells underwent osmotic stress perpendicular to the axis of actin filaments, suggesting a supporting role for actinin when the cell undergoes increased stress.

Another example of a mechanosensing FRET probe is one made for the focal adhesion protein vinculin (Grashoff *et al.*, 2010). Vinculin connects active integrins in focal adhesions to the actin cytoskeleton and its recruitment is dependent on sufficient tension being applied at focal adhesions. Traction force microscopy was previously used to estimate tension across focal adhesion components that suggested a range far below previously used methods. A FRET probe was constructed which consisted of an mTFP-Venus (A206K) FRET pair connected via a 40-amino-acid elastic linker and was termed tension sensor module or TSMod. In order to sense tension, this probe took advantage of the sensitivity of FRET efficiency to distance between fluorophores. The module was inserted between the talin binding head domain of vinculin and the actin binding tail domain and was recruited to focal adhesions, which looked similar to a single-fluorophore tagged vinculin. When the TSMod containing vinculin was expressed in cells on fibronectin-coated cover slips it was shown to have lower FRET than a control molecule that contained both fluorophores but only the vinculin head domain. These data were further validated by FLIM data that showed that the lifetime of vinculinTSMod was significantly longer than the head domain-only control. A variant on TSMod was made using Cy3 and Cy5 and this was used to calibrate the sensitivity of the probe using single-molecule fluorescence force spectroscopy that found that the probe was most sensitive between 1 and 6 pN. Using the calibration data and FLIM data it was interpreted that the average force across a stable focal adhesion is $\sim$2.5 pN. The vinculin-TSMod probe was also used to show that small adhesions at the leading edge of migrating cells showed low FRET, indicating high-tension force being applied to vinculin, and in contrast large focal adhesions were shown to have higher FRET indicating lower tension on vinculin within them. This biosensor could be adapted for a wide range of other molecules that are themselves mechanotransducers or force transducers. When taken together this shows that the potential of FRET probes extends even into sensing subcellular forces in real time and with high sensitivity within cells and across proteins.

FRET/FLIM analysis of protein–protein interactions

Given the caveats and limitations of biosensors, measuring interactions between two different proteins in intact cells is best performed using FRET/FLIM. FLIM has been used to study the interaction between β1 integrins and talin as a read-out following receptor activation at adhesions upon treatment of cells with various ECM ligands or small molecule compounds targeting specific integrins (Parsons *et al.*, 2008). Data revealed that activation of integrins with ECM proteins or ligand-mimetic compounds both resulted in localized induction of association between integrin and direct binding partners such as talin. A similar strategy was also used to define and validate a novel inhibitor of β1 integrin activation, sharpin (Rantala *et al.*, 2011). FLIM has also been used to define a molecular pathway leading to transdominant inhibition of β1 integrins by αvβ3 integrin (Worth *et al.*, 2010). In addition to receptor:signalling protein interactions, FLIM has also recently been shown to be a powerful tool for analysing interactions with the actin cytoskeleton. Two

recent publications have employed FLIM to analyse actin-fascin binding in intact cells and *in vivo* (Jayo *et al.*, 2012; Zanet *et al.*, 2012). Fascin has long been known as an efficient F-actin bundling molecule, but using FLIM to analyse this interaction *in situ* revealed a novel nonactin binding role for this molecule in filopodia formation (Zanet *et al.*, 2012). Other examples of the use of FRET/FLIM to analyse spatial assembly of protein complexes include CD44-podoplanin and CD44-ezrin at the leading edge during migration (Legg *et al.*, 2002; Martin-Villar *et al.*, 2010) and LIMK-PAK4/LIMK-ROCK binding in invasive human carcinoma cells (Ahmed *et al.*, 2008; Shea *et al.*, 2008). These studies highlight the power of using FLIM to detect molecular responses or protein–protein interactions at adhesions with precise spatial and temporal resolution that is impossible to achieve using biochemical or immunofluorescence approaches.

Summary and future directions

Considerable progress has been made in recent years in the development of FRET-based biosensors to study different proteins in live cells and this continues apace. Although these probes and analysis methods provide detailed insight into single proteins, or pairs of interacting proteins, one of the next goals is to extend this to analysing multiple protein complex assembly in living migrating cells using FRET. Such analysis is likely to require the use of lifetime analysis of FRET (FLIM) as intensity-based approaches will present considerable complications in terms of data analysis (see previous sections). Moreover, as well as increasing the number of proteins being analysed within a complex, there is also the need to improve upon the spatial resolution of analysis. Coupling FRET-based approaches to study direct protein interactions with superresolution techniques such as structured illumination or stochastic optical reconstruction microscopy methods would present a potentially powerful platform to begin to dissect the precise sites of activation/interaction within discrete subdomains (such as an adhesion site). Such analysis would shed considerable light on the potentially distinct functions for the vast array of adhesion components identified to date and may help define specificity of interactions and changes in partner binding during the cell migration cycle. Finally, the analysis of such activation and interaction events in cells *in vivo* represents a major future goal for a number of researchers both in the analysis of developmental signalling events, but also in animal models of diseases. Such analysis is already being performed in model systems that are optically easier to manipulate, such as drosophila and zebrafish embryos (Kamiyama & Chiba, 2009; Yoo *et al.*, 2010) and more recently in mouse models of cancer metastasis (McGhee *et al.*, 2011; Timpson *et al.*, 2011). Ongoing developments in microscope detector sensitivity and image capture will likely play a role in allowing more detailed analysis of these probes in cells in an *in vivo* setting.

References

Ahmed, T., Shea, K., Masters, J. R., Jones, G. E. & Wells, C.M. (2008) A PAK4-LIMK1 pathway drives prostate cancer cell migration downstream of HGF. *Cell. Signal.* **20**, 1320–1328.

Allen, M.D. & Zhang, J. (2006) Subcellular dynamics of protein kinase A activity visualized by FRET-based reporters. *Biochem. Biophys. Res. Commun.* **348**, 716–721.

Cai, X., Lietha, D., Ceccarelli, D. F., *et al.* (2008) Spatial and temporal regulation of focal adhesion kinase activity in living cells. *Mol. Cell. Biol.* **28**, 201–214.

Day, R.N. & Davidson, M.W. (2012) Fluorescent proteins for FRET microscopy: monitoring protein interactions in living cells. *Bioessays* **34**, 341–350.

Falasca, M. & Maffucci, T. (2012) Regulation and cellular functions of class II phosphoinositide 3-kinases. *Biochem. J.* **443**, 587–601.

Franco, S.J. & Huttenlocher, A. (2005) Regulating cell migration: calpains make the cut. *J. Cell Sci.* **118**, 3829–3838.

Grashoff, C., Hoffman, B.D., Brenner, M.D., *et al.* (2010) Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. *Nature* **466**, 263–266.

Hu, X.M., Wu, H.W., Zhang, Z.S., Su, C., Zhao, W., Ma, L., Zhou, J.L. & Wu, G.L. (2001) [Analysis of the mitochondria-related protein of Schistosoma japonicum and its antigen epitopes]. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* **19**, 19–21.

Itoh, R.E., Kurokawa, K., Ohba, Y., Yoshizaki, H., Mochizuki, N. & Matsuda, M. (2002) Activation of Rac and Cdc42 video imaged by fluorescent resonance energy transfer-based single-molecule probes in the membrane of living cells. *Mol. Cell. Biol.* **22**, 6582–6591.

Jayo, A., Parsons, M. & Adams, J.C. (2012) A novel Rho-dependent pathway that drives interaction of fascin-1 with LIM kinase1/2 to promote fascin-1/actin binding and filopodia stability. *BMC Biol.* **10**, 72, doi: 10.1186/1741-7007-10-72

Kamiyama, D. & Chiba, A. (2009) Endogenous activation patterns of Cdc42 GTPase within Drosophila embryos. *Science* **324**, 1338–1340.

Kelly, J.C., Cuerrier, D., Graham, L.A., Campbell, R.L. & Davies, P.L. (2009) Profiling of calpain activity with a series of FRET-based substrates. *Biochim. Biophys. Acta* **1794**, 1505–1509.

Klarenbeek, J.B., Goedhart, J., Hink, M.A., Gadella, T.W. & Jalink, K. (2011) A mTurquoise-based cAMP sensor for both FLIM and ratiometric read-out has improved dynamic range. *PloS One* **6**, e19170.

Kritzer, M.D., Li, J., Dodge-Kafka, K. & Kapiloff, M.S. (2012) AKAPs: the architectural underpinnings of local cAMP signaling. *J. Mol. Cell. Cardiol.* **52**, 351–358.

Legg, J.W., Lewis, C.A., Parsons, M., Ng, T. & Isacke, C.M. (2002) A novel PKC-regulated mechanism controls CD44 ezrin association and directional cell motility. *Nat. Cell Biol.* **4**, 399–407.

Lim, C.J., Kain, K.H., Tkachenko, E., *et al.* (2008) Integrin-mediated protein kinase A activation at the leading edge of migrating cells. *Mol. Biol. Cell* **19**, 4930–4941.

Machacek, M., Hodgson, L., Welch, C., *et al.* (2009) Coordination of Rho GTPase activities during cell protrusion. *Nature* **461**, 99–103.

Martin-Villar, E., Fernandez-Munoz, B., Parsons, M., Yurrita, M.M., Megias, D., Perez-Gomez, E., Jones, G.E. & Quintanilla, M. (2010) Podoplanin associates with CD44 to promote directional cell migration. *Mol. Biol. Cell* **21**, 4387—43s99.

McGhee, E.J., Morton, J.P., Von Kriegsheim, A., *et al.* (2011) FLIM-FRET imaging in vivo reveals 3D-environment spatially regulates RhoGTPase activity during cancer cell invasion. *Small GTPases* **2**, 239–244.

McKenzie, A.J., Campbell, S.L. & Howe, A.K. (2011) Protein kinase A activity and anchoring are required for ovarian cancer cell migration and invasion. *PloS One* **6**, e26552.

Meng, F. & Sachs, F. (2011) Visualizing dynamic cytoplasmic forces with a compliance-matched FRET sensor. *J. Cell Sci.* **124**, 261–269.

Miyawaki, A. (2011) Development of probes for cellular functions using fluorescent proteins and fluorescence resonance energy transfer. *Annu. Rev. Biochem.* **80**, 357–373.

Mochizuki, N., Yamashita, S., Kurokawa, K., Ohba, Y., Nagai, T., Miyawaki, A. & Matsuda, M. (2001) Spatio-temporal images of growth-factor-induced activation of Ras and Rap1. *Nature* **411**, 1065–1068.

Morton, P.E. & Parsons, M. (2011a) Dissecting cell adhesion architecture using advanced imaging techniques. *Cell Adh. Migr.* **5**, 351–359.

Morton, P.E. & Parsons, M. (2011b) Measuring FRET using time-resolved FLIM. *Methods Mol. Biol.* **769**, 403–413.

Ouyang, M., Lu, S., Li, X.Y., *et al.* (2008) Visualization of polarized membrane type 1 matrix metalloproteinase activity in live cells by fluorescence resonance energy transfer imaging. *J. Biol. Chem.* **283**, 17740–17748.

Papusheva, E., Mello de Queiroz, F., Dalous, J., Han, Y., Esposito, A., Jares-Erijmanxa, E.A., Jovin, T.M. & Bunt, G. (2009). Dynamic conformational changes in the FERM domain of FAK are involved in focal-adhesion behavior during cell spreading and motility. *J. Cell Sci.* **122**, 656–666.

Parsons, M., Messent, A.J., Humphries, J.D., Deakin, N.O. & Humphries, M.J. (2008) Quantification of integrin receptor agonism by fluorescence lifetime imaging. *J. Cell Sci.* **121**, 265–271.

Pertz, O. (2010) Spatio-temporal Rho GTPase signaling—where are we now? *J. Cell Sci.* **123**, 1841–1850.

Rantala, J.K., Pouwels, J., Pellinen, T., *et al.* (2011) SHARPIN is an endogenous inhibitor of beta1-integrin activation. *Nat. Cell Biol.* **13**, 1315–1324.

Rottner, K. & Stradal, T.E. (2011) Actin dynamics and turnover in cell motility. *Curr. Opin. Cell Biol.* **23**, 569–578.

Sato, M., Ueda, Y., Takagi, T. & Umezawa, Y. (2003) Production of PtdInsP3 at endomembranes is triggered by receptor endocytosis. *Nat. Cell Biol.* **5**, 1016–1022.

Scales, T.M. & Parsons, M. (2011) Spatial and temporal regulation of integrin signalling during cell migration. *Curr. Opin. Cell Biol.* **23**, 562–568.

Seong, J., Lu, S. & Wang, Y. (2011) Live cell imaging of Src/FAK signaling by FRET. *Cell. Mol. Bioeng.* **2**, 138–147.

Shea, K.F., Wells, C.M., Garner, A.P. & Jones, G.E. (2008) ROCK1 and LIMK2 interact in spread but not blebbing cancer cells. *PloS One* **3**, e3398.

Stockholm, D., Bartoli, M., Sillon, G., Bourg, N., Davoust, J. & Richard, I. (2005) Imaging calpain protease activity by multiphoton FRET in living mice. *J. Mol. Biol.* **346**, 215–222.

Timpson, P., McGhee, E.J., Morton, J.P., *et al.* (2011) Spatial regulation of RhoA activity during pancreatic cancer cell invasion driven by mutant p53. *Cancer Res.* **71**, 747–757.

Venkateswarlu, K., Oatey, P.B., Tavare, J.M. & Cullen, P.J. (1998) Insulin-dependent translocation of ARNO to the plasma membrane of adipocytes requires phosphatidylinositol 3-kinase. *Curr. Biol.* **8**, 463–466.

Wang, Y., Botvinick, E.L., Zhao, Y., Berns, M.W., Usami, S., Tsien, R.Y. & Chien, S. (2005) Visualizing the mechanical activation of Src. *Nature* **434**, 1040–1045.

Wehrle-Haller, B. (2012) Assembly and disassembly of cell matrix adhesions. *Curr. Opin. Cell Biol.*, **24**, 569–591

Worth, D.C., Hodivala-Dilke, K., Robinson, S.D., King, S.J., Morton, P.E., Gertler, F.B., Humphries, M.J. & Parsons, M. (2010) Alpha v beta3 integrin spatially regulates VASP and RIAM to control adhesion dynamics and migration. *J. Cell Biol.* **189**, 369–383.

Yoo, S.K., Deng, Q., Cavnar, P. J., Wu, Y.I., Hahn, K.M. & Huttenlocher, A. (2010) Differential regulation of protrusion and polarity by PI3K during neutrophil motility in live zebrafish. *Dev. Cell* **18**, 226–236.

Yoshizaki, H., Aoki, K., Nakamura, T. & Matsuda, M. (2006) Regulation of RalA GTPase by phosphatidylinositol 3-kinase as visualized by FRET probes. *Biochem. Soc. Trans.* **34**, 851–854.

Yoshizaki, H., Mochizuki, N., Gotoh, Y. & Matsuda, M. (2007) Akt-PDK1 complex mediates epidermal growth factor-induced membrane protrusion through Ral activation. *Mol. Biol. Cell* **18**, 119–128.

Yoshizaki, H., Ohba, Y., Kurokawa, K., Itoh, R.E., Nakamura, T., Mochizuki, N., Nagashima, K. & Matsuda, M. (2003) Activity of Rho-family GTPases during cell division as visualized with FRET-based probes. *J. Cell Biol.* **162**, 223–232.

Zanet, J., Jayo, A., Plaza, S., Millard, T., Parsons, M. & Stramer, B. (2012) Fascin promotes filopodia formation independent of its role in actin bundling. *J. Cell Biol.* **197**, 477–486.

Zhang, J., Hupfeld, C.J., Taylor, S.S., Olefsky, J.M. & Tsien, R.Y. (2005) Insulin disrupts beta-adrenergic signalling to protein kinase A in adipocytes. *Nature* **437**, 569–573.