Histol Histopathol (2014) 29: 000-000

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Probing for protein-protein interactions during cell migration: limitations and challenges

Jeremy Soon Kiat Chan[1]*, Zi Qiang Teo[1]*, Ming Keat Sng[1]* and Nguan Soon Tan[1,2]

[1]School of Biological Sciences, Nanyang Technological University, Singapore and

[2]Institute of Molecular and Cell Biology, Proteos, Singapore

*Authors contributed equally

Summary. Cellular migration is a fundamental biological process occurring as early as embryogenesis to the pathological state of cancer metastasis. Nearly all cellular migrations involve an extracellular signal that is transduced internally by members of a signalling cascade. These signal transduction events are driven by protein-protein interactions (PPIs) that coordinate intracellular activities to enable a cell to migrate. Understanding these PPIs will provide valuable insight into how cellular migration can be modulated perhaps towards a therapeutic end. Histologically, not many techniques are available to demonstrate PPIs. Contrasting agents only demonstrate the presence of a particular protein, and perhaps its co-localisation with another protein. Yet, co-localisation need not necessarily indicate physical interaction between the two proteins. In this review, we highlight four commonly used methods that continue to expand our understanding of PPIs underlying cell migration: co-immunoprecipitation, bimolecular fluorescence complementation, proximity ligation assay and surface plasmon resonance. The methods discussed herein allow for the study of PPIs in a wide variety of biological samples, including cell lysates, live cells, fixed cells and tissues, enabling the quantification of endogenous PPIs and exploration of the nature of PPIs. We also include a rudimentary framework for researchers to decide which experimental method best suits their research goals.

Offprint requests to: Nguan Soon Tan, School of Biological Sciences, Nanyang Technological University, 60, Nanyang Drive, Singapore 637551. e-mail: nstan@ntu.edu.sg or nstan@imcb.a-star.edu.sg

Key words: Co-immunoprecipitation, B:FC, SPR, PLA

The importance of protein-protein interactions (PPIs) and cellular migration

The central dogma dictates that the genome is transcribed into mRNA and subsequently translated into protein (Crick, 1970). James Watson himself, in a keynote address at the Miami Nature Biotechnology Winter Symposium in 2003, referred to DNA as "the script" and proteins as "the actors" in the complex play of life. Although reverse transcription, epigenetic regulation, prions, catalytic and non-coding RNA highlight novel conduits by which genetic information flows, the central dogma still remains one of the most enduring tenets of molecular biology (Mattick, 2003; Shapiro, 2009). Intrinsically, proteins are the crucial workhorses that facilitate the manifestation of a phenotypic end point. The presence, absence, mutation or modification of a particular protein will define the line between normal and pathophysiology. For example, in sickle-cell disease, a single amino acid substitution (glutamic acid to valine) at position 6 in the beta-subunit of the haemoglobin protein promotes haemoglobin aggregation under hypoxic conditions, which leads to the characteristic sickle-shaped erythrocytes and anaemia (Kassim and DeBaun, 2013). However, not every event in biology can be easily attributed to a single protein. A large majority of proteins do not function in isolation but rather require interaction with other proteins (homomeric or heteromeric) to exert an effect on cellular behaviour (Gandhi et al., 2006). The study of PPIs thus becomes

paramount to understanding the molecular underpinnings of health and disease. However, the detection and interpretation of these PPIs is challenging. The cell-type specific, time- and context-dependent expression of proteins and their binding partners, as well as their sub-cellular localisation, post-translational modifications and conformational changes before and after binding are all critical parameters that act in a combinatorial manner to allow for subtle fine-tuning of biological responses at all levels of physiology (Chautard et al., 2009; Zinzalla and Thurston, 2009).

The complexity of studying PPIs is further compounded by the individual nature of each PPI that occurs. PPIs are graded between the extremes of stable and transient interactions (Phizicky and Fields, 1995). Stable PPIs are those found among proteins belonging to a functional complex (e.g., haemoglobin, nucleoporins, integrin-ECM) and that form by default, i.e., the ideal complementary contacts among binding partners are intrinsically coded for by amino acid sequences, and thus, interactions occur with negligible extrinsic influences such as post-translational modifications. Correspondingly, stable PPIs are often retained over a longer time-course and are not easily disrupted. Such PPIs are less challenging to detect using conventional molecular techniques.

In contrast, transient PPIs typically require specific stimuli to encourage exposure of ideal complementary contacts before binding can occur. As implied, transient PPIs are not durable, because successful formation of the PPI results in a conformational change or interactions with other factors that undermine the precondition for binding (e.g. G-protein activation and deactivation, kinase phosphorylation and phosphatase dephosphorylation). Thus, transient PPIs play an important role as molecular switches that enable stringent and precise control of intracellular signal transduction. The temporary nature of such PPIs can pose tremendous difficulties in their detection. Nonetheless, a transient PPI can be detected with less difficulty in a particular cellular context if it occurs more frequently. Most biological events require the coordination of stable and transient PPIs for correct execution. Cellular migration, the focal biological event of our review, is no exception to this rule.

Nearly all cellular migrations involve an extracellular signal that is transduced internally by transient PPIs among members of a signalling cascade. These upstream initiators and effectors of migration are extremely diverse and remain the subjects of intense study and discovery. In contrast, downstream responses such as relinquishment of stable cell-ECM contacts and replacement by less stable (more transitory) cell-ECM contacts at the leading and withdrawing edges of the migrating cell appear more generic and are better understood (Palamidessi et al., 2013; Wehrle-Haller, 2012). Repeated iterations of these changes in PPIs eventually dictate when and where a cell should migrate to. During prenatal development, definition of the germ layers of an early embryo already requires protein-directed cellular migration (Enders et al., 1978; Lawson and Pedersen, 1987; Poelmann, 1981). Rolling adhesion, subsequent extravasation and chemotactic movement of circulating immune cells to the site of infection within tissue also depends on appropriate PPIs between cells and the extracellular matrix (ECM) (O'Neill et al., 2000; Pribila and Shimizu, 2003; Yong and Khwaja, 1990). Wound repair and angiogenesis, which are key processes in the maintenance of organ integrity, can only occur correctly when keratinocytes and endothelial cells receive the appropriate signals for migration (Kim et al., 1994; Decline and Rousselle, 2001; Master et al., 2001). Such migration events during normal physiology also have parallels in pathology, and several diseases arise from or are exacerbated by deregulation of normal cell migration. Cancer, one of the most widely-diagnosed diseases of the 21st century that accounted for up to 13% of worldwide deaths in 2008 according to the World Health Organisation (WHO), sustains its nefarious growth by establishing its own network of blood vessels through de novo angiogenesis (Bergers and Benjamin, 2003; Chung et al., 2010). The most aggressive of cancers culminate their malignant progression in invasion and metastasis, which, is in essence a migratory incident (Hood and Cheresh, 2002; Roussos et al., 2011; Sethi and Kang, 2011).

It becomes apparent that PPIs and their associated signalling networks largely control migration in varying contexts. Precisely probing for the PPIs that occur during cellular migration can thus reveal pertinent therapeutic interventions to promote or prevent migration. Although many techniques have been developed to test for PPIs, herein we feature the most commonly and easily used experimental methods, old and new, that have contributed to and are currently expanding our understanding of these interactions in cell migration. We broadly categorise these methods into probe-based and direct-labelling techniques and evaluate each technique's advantages, limitations and future potential in elucidating PPIs that are pertinent to cell migration.

Methods for probing PPIs

Co-Immunoprecipitation (Co-IP)

Classically, the study of PPIs has been probe-based and driven by antibody-dependent approaches such as Western blotting, pull-down assays and a combination of these two methods, co-IP. These methods are contingent upon the availability of a specific recognition domain on an antibody that is raised against a particular epitope, which even allows for the specific capture of a protein based on its status (e.g., its activation state or presence of any post-translational modifications). Known as the gold standard in its field of assays, co-IP remains a popular method for the detection of PPIs. The idea of co-IP stems from using a specific antibody to purify and

detect antigens on a small-scale basis. The captured protein is immobilised onto a Protein A or G beaded support via the protein-specific antibody. From this stage, a co-IP procedure would then be able to pull-down any other protein-binding molecules from complex solutions through native interactions (see Figure 1). The power of co-IP comes from the modifiability of the assay to capture transient interactions that would otherwise be missed, or to map specific chains of events during the signalling cascade of interest.

The flexibility of co-IP was demonstrated by Master et al in the characterisation of angiopoietin-1-dependent endothelial cell (EC) migration in angiogenesis (Master et al., 2001). Using antibodies that target phospho-proteins, in this case phospho-Dok-R, the authors demonstrated that the stimulation of EC migration by angiopoietin-1 is dependent on sequential PPIs between Dok-Nck-Pak that are involved in phosphorylation and activation of the indicated proteins. The versatility of co-IP was further extended to demonstrate that phosphorylation on a single tyrosine residue in Dok is necessary to activate this signalling axis to enhance EC

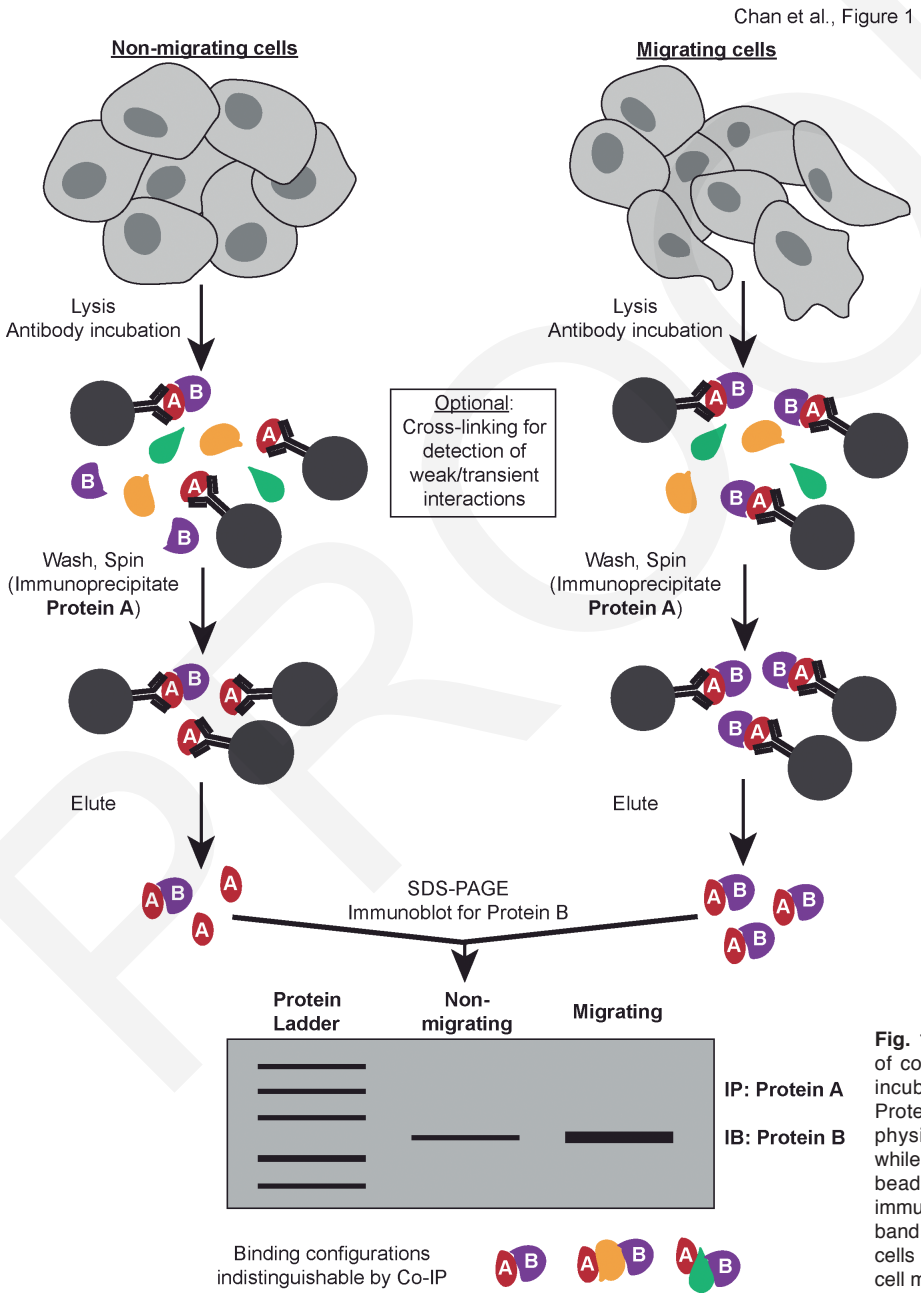

Fig. 1. Schematic depicting the underlying principle of co-immunoprecipitation (Co-IP). Cell lysates are incubated with bead-coupled antibodies specific to Protein A for immunoprecipitation. Protein B which physically interacts with Protein A remains bound while non-interacting proteins are washed off and the beads are pelleted by centrifugation. After elution, immunoblotting for Protein B is performed. A darker band appearing in the immunoblot for the migrating cells indicate that the protein interaction is enriched in cell migration.

migration. By mutating the tyrosine residue in Dok to remove the phosphorylation and activation of Dok, all sequential downstream PPIs were undetectable by co-IP, and the subsequent EC migration was impeded. This result highlights the ability of co-IP as a powerful tool to pinpoint PPIs to the resolution of a single amino acid. Co-IP is thus able to piece together crucial PPIs in a pair-wise manner to account for a migratory event through the use of specific antibodies that detect activated or other post-translationally modified members, within a protein complex. In addition, co-IP can highlight the domain architecture that is necessary for binding, whereas precise mutagenesis enables the detection of key single amino acid residues that anchor an entire protein complex. These versatile properties of a co-IP-based experiment can be particularly useful in peptide drug discovery which is aimed at mimicking native protein interactions that impact cellular migration.

Re-epithelialisation and migration of keratinocytes during wound healing is a complex process that requires the coordination of keratinocyte-ECM contacts and re-organisation of the cytoskeleton for directional motility. Sehgal et al used co-IP to highlight that integrin α6β4, once thought to be a minor player in laminin-332 patterning and deposition during keratinocyte migration, in fact plays a central role in enabling directional cell motility (Sehgal et al., 2006). Keratinocytes at the wound edge lay down the laminin-332 tracks on which they travel, and integrins α3β1 and α6β4 on keratinocyte plasma membranes are cognate binding partners of laminin-332 (Nguyen et al., 2000). Integrin α3β1 was first thought to be the major determinant of keratinocyte migration (deHart et al., 2003; Goldfinger et al., 1999). However, in the experimental context of dominant negative Rac-1 and Rac-1 inhibitors, a lack of Rac-1 activity caused circular laminin-332 deposition, which was an impediment to proper keratinocyte migration into the wound. Subsequently, a co-IP experiment clarified that integrin α6β4 is responsible for recruiting and activating Rac-1. In contrast, integrin α3β1 failed to co-precipitate with Rac-1, and Rac-1 recruitment by integrin α6β4 in turn activates cofilin to induce plectin/actin cytoskeletal rearrangements, which permitted integrin α6β4 to pattern laminin-332 deposition in a linear fashion for directional keratinocyte migration. In essence, co-IP has allowed the authors to link a less recognised integrin with Rac-1, and the latter's associated pathways, thus broadening the influence of integrin α6β4in regulating the more subtle points of keratinocyte migration, for instance, the directionality of cellular movement.

Despite the utility and versatility of co-IP in (i) identifying PPIs, (ii) detailing the interfacing residues or unique characteristics of PPIs and (iii) associating PPIs with cellular migration in various contexts, co-IP does have limitations (Phizicky and Fields, 1995). The major limitation of co-IP is the inability to distinguish between direct PPIs or PPIs in a complex. For example, *in vivo* Protein A interacts with Protein B through protein C. However, co-IP results will indicate that protein A interacts directly with B and would yield a false positive result (see Figure 1). Therefore, conclusions of direct PPI that are derived only from co-IP should be verified by another method that overcomes this limitation. Additionally, one crucial parameter is conspicuously missing from the co-IP experiments – the location within a cell where the PPI is occurring. This problem is inevitable because co-IP involves only proteins that are derived from cell lysates, therefore obviating any preservation of cellular structure and thus compartmentalisation of proteins. As such, non-physiological interactions may occur between proteins that are normally located in distinct cellular compartments. It is thus advisable that co-IP be performed in conjunction with immunofluorescence staining of the proteins involved in the PPI being probed to ascertain that the interacting proteins share the same intracellular compartment. In the Dok-R example, immunofluorescent staining followed by confocal imaging was performed to verify that Tek, Dok-R, Nck and Pak indeed co-localised and were found at the cell periphery of migrating ECs.

Given the myriad of proteins that are found in cell lysates, non-specific binding of unwanted proteins to the IP antibody is also possible. To minimize non-specific PPIs, one can enrich proteins in the pseudopodia protruding from the cell body compartment using polycarbonate microporous filters. Pseudopodia and cell bodies are then differentially scraped from the filter surface into lysis buffer for biochemical analysis. Using this method, it is possible to identify novel pseudopodia and cell body proteins, as well as study the spatiotemporal organization of signaling processes that regulate pseudopodium formation and cell polarity (Cho and Klemke, 2002; Tan et al., 2007; Wang and Klemke, 2007). In addition, thorough washing steps are typically involved to disrupt these weak interactions. Researchers can optimise the ionic strength of the buffers that are used in the assay, decrease the amount of target-specific antibody that is used to reduce signal noise, or pre-clear the samples by introducing non-specific antibodies to the cell lysate mixture prior to the actual IP steps.

Another consideration of a co-IP experiment is antibody contamination. The heavy and light chains of the antibody could be co-eluted with the complex of interest and will obscure the results on subsequent reducing SDS-PAGE runs. To circumvent this problem of antibody contamination, researchers may experiment with covalent immobilisation of the antibody to the beaded support. In this setup, beaded supports that are produced commercially can bind the primary amine groups on the antibodies, such that the antibody is permanently bound to the support. Another possibility for crosslinking the antibody to the Protein A/G-attached supports is through the use of crosslinkers, such as bissulfosuccinimidyl suberate (BS3) or its non-water soluble analogue, disuccinimidyl suberate (DSS). In this manner, covalent amide bonds are formed between the

antibodies and the beaded support. These methods aim to reduce the occurrence of antibody contamination during the analysis of results.

Finally, researchers may face the difficulty of ensuring a stable physiological environment for a PPI of interest to occur. This issue is perhaps most apparent when multicolour immunofluorescence staining identifies strict co-localisation of proposed interacting partners that cannot be proven to physically interact via co-IP. The PPI that is formed is likely weak or transient and unable to withstand the mechanical and chemical stresses that are exerted on it during the co-IP assay; this weakness severely limits the sensitivity of co-IP. The incorporation of a fixation or cross-linking step may be necessary prior to cell lysis to stabilise such PPIs. The stringency of wash steps after immunoprecipitation may also need to be reduced. These modifications to a typical co-IP protocol would require optimisation to ensure that false positives do not surface with the reduction of wash stringency, and that epitopes are not masked by fixation or cross-linking.

The continued reliance on these antibody-based methods in many current laboratories is a testament to their reliability, and many of the newer techniques that have been developed to probe PPIs still leverage on antibody specificity. The proximity ligation assay (PLA) is a prime example of one such novel antibody-based technique and will be discussed in greater detail later in this review.

Bimolecular Fluorescence Complementation (BiFC)

BiFC is an *in vivo* direct-labelling technique based on the principle of fluorescence complex formation upon the interaction of two protein molecules (see Figure 2). Two putative interacting partners are individually fused to a fragment of a fluorescent protein. Upon interaction, the fluorescent protein complex reassembles and fluoresces (Kerppola, 2008; Kodama and Hu, 2012). Unlike FRET, BiFC does not involve radiative transfer of energy and therefore the obtained signal is stronger and offers a directly measurable readout. Because PPIs occur within live cells, the compartmentalisation of the interacting protein partners is retained, and the sub-

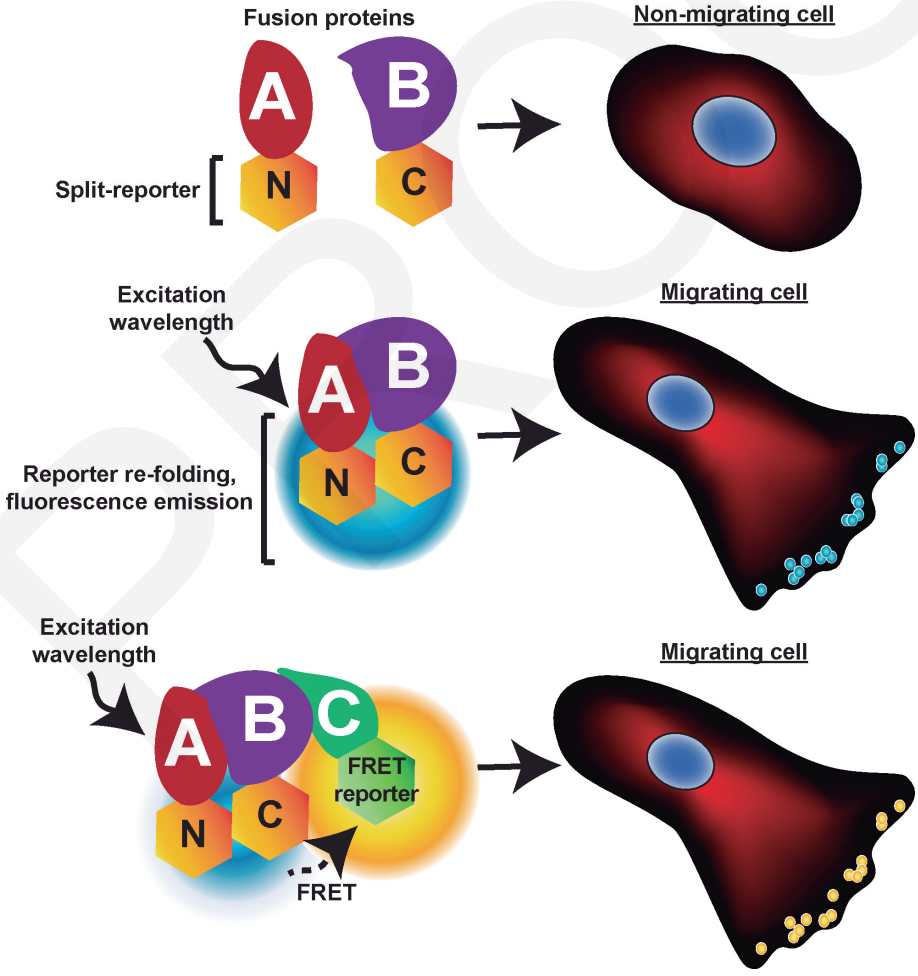

Fig. 2. Schematic depicting the underlying principle of bimolecular fluorescence complementation (BiFC). Cells must first be transfected to exogenously express both proteins of the prospective interaction as fusion proteins. Each protein is fused with either the N- or C-terminus of a split reporter which refolds into a functional fluorophore when the PPI of interest occurs. Appearance of fluorescence signals at the leading edge of a migrating cell confirms the subcellular localisation where PPIs are formed. BiFC can be coupled with FRET to identify higher order interactions involving three or more proteins. Refolding of the fluorophore when two proteins interact results in fluorescence emission that excites a second fluorophore fused to a third interacting partner. The emission of the BiFC fluorophore is quenched while the emission of the FRET fluorophore is enhanced.

cellular localisations of the PPIs can be resolved by confocal microscopy. Thus, if an interaction is detected by co-IP but not observed in a BiFC experiment, it could be an indication that co-IP has picked up a non-native PPI between partners that share distinct sub-cellular compartments in an intact cell. Conversely, the two proteins could interact, but in a different orientation that does not allow the two fragments of the fluorescent protein to assemble into a functional protein.

A wide variety of split-fluorescent reporters are now commercially available, including split-CFP, YFP, GFP and mDsRed, as well as their respective modified variants with superior performance (brighter fluorescence, higher signal-to-noise ratio) (Kodama and Hu, 2012). Using split-fluorescence reporters with non-overlapping emission spectra enables the simultaneous monitoring of multiple complex formations in the same cell. More recently, the repertoire of split-fluorescent reporters has been further expanded with the development of the near-infrared BiFC reporter iSPLIT, which was modelled after the PAS and GAF domains of the bacterial phytochrome RpBphP2 and improved upon by directed evolution (Filonov and Verkhusha, 2013). iSPLIT widens the spectral range and thus the number of split-fluorescence reporters that can be used simultaneously, and it further enables imaging of PPIs in live animals because its excitation and emission spectra lie beyond the wavelengths of the autofluorescent compounds found in animal tissues.

In the context of cell migration, BiFC demonstrated the importance of the integrin-linked kinase (ILK) and p38β interaction in promoting serum-induced bladder cancer cell migration. Yu et al. demonstrated that ILK and p38β elevation in invasive bladder cancer tissues as opposed to that of normal bladder urothelium (Yu et al., 2014). Interestingly, the specific inhibition of ILK activity institutes a selective loss of p38β in bladder cancer cells. ILK phosphorylation of p38β at specific residues was later shown to confer protection against the proteosomal degradation of p38β. Live-cell imaging of bladder cancer cells transfected with p38β and ILK that were fused with the N- and C-termini of Venus, respectively, indicated that ILK specifically interacts with p38β in the cytoplasm. The advantage of using BiFC is twofold. First, an overexpression of the interacting partners tends to accentuate a phenotypic readout, which leads to the ability of BiFC to prove a successful PPI and the subsequent cellular phenotype. Second, in contrast with co-IP, BiFC provides an insight into the sub-cellular localisation of PPIs, and rules out interactions between proteins that do not naturally share an intracellular space or organelle because the assay requires the use of intact, living cells by default. In addition, when a truncated or loss-of-function ILK mutant was fused to the C-terminus of Venus, and subjected to a BiFC assay with p38β, it was definitively shown that the kinase domain is crucial for the interaction with p38β. Similar to co-IP, the option of truncation and mutagenesis of proteins that are involved in a PPI of interest is absolutely viable to identify the protein domains responsible for the PPI. Depletion of either ILK or p38β resulted in a substantial decrease in serum-directed chemotaxis of the bladder cancer cells, thus confirming a causal relationship.

Despite its utility in identifying PPIs in vivo using live cells with sub-cellular localisation resolution, BiFC does have its limitations. The often irreversible reassembly of the fluorescence reporter protein stabilises transient, native PPIs, making it possible to visualise them. However, the drawback to this is that the dynamics of interactions are lost because permanent reassembly of the split-fluorescent probe unnaturally prolongs the very interaction being studied. Furthermore, the absence of a BiFC signal does not necessarily mean that PPIs did not occur. Steric hindrances could lead to a non-optimal orientation of the two fragments of the fluorescent protein to assemble into a functional fluorescent protein, even though the proteins-of-interest have interacted. To work around this, a flexible linker region is sometimes incorporated into the fusion construct, thus allowing sufficient range of molecular motion c to drive refolding of the fluorescent reporter (Kerppola, 2008).

BiFC also assumes that expression of the split-fluorescent reporter that is fused to the putative interacting partners does not interfere with the native structures of the proteins of interest. Alterations in native protein structure can cause residues required for stabilising a PPI to be obscured, which would result in a false negative readout. A fusion protein with abnormal structure may also expose residues to allow for the formation of non-native PPIs that out-compete the native interaction or cause non-native interactions to be interpreted as legitimate. A BiFC experiment should be complemented with immunocytochemistry and co-IP to definitively prove the localisation and existence of a PPI between two proteins of interest.

Another issue to consider in a BiFC experiment is that the extent of expression of the fusion proteins cannot be strictly controlled even when expressed from an inducible promoter. This is a problem that is inherent to any experiment in which overexpression from an exogenous construct is involved and is not unique to the BiFC method. Non-physiological levels of fusion protein expression could strain the cell transcription and translation machinery, which could lead to cell death or the induction of an abnormal cellular state. Alternatively, high non-endogenous expression of fusion proteins may result in fluorescent reporter refolding that is independent of PPI formation, giving a false positive result (Kerppola, 2008). As with any experiment, optimisation of the transfection efficiency or induction of fusion protein expression should be ensured.

The principles behind FRET and BiFC allow both methods to be used concurrently to further increase the number of PPIs that can be observed simultaneously. Most PPI detection methods only detect interactions between two proteins at a time. Yet, some PPIs occur in

ternary complexes and involve three or more proteins that are in physical contact with one another. An example of this situation is the formation of the pSmad2/3-TAP63-p53 complex in cholangiocarcinoma cells that promotes tumour cell migration, in addition to its survival (Lu et al., 2013). However, visualisation techniques for studying such ternary complex formations are lacking.

Recently, Shyu and colleagues established a BiFC-based FRET (BiFC-FRET) visualisation technique to study the formation of ternary complexes in living cells (Shyu et al., 2008). The BiFC-FRET technique encompasses the use of two mutant CFP and YFP proteins, known as Cerulean and Venus, respectively. In general, two proteins are conjugated to the two non-fluorescent fragments, as in the original BiFC technique. When an interaction successfully occurs, the two non-fluorescent fragments fuse to create the Venus fluorescent protein. In the presence of a third protein, which is fused to the Cerulean fluorescent protein, an interaction with the initially formed heterodimeric protein would bring Cerulean (FRET donor) into close proximity to Venus (FRET acceptor) and allow a successful FRET to occur. The visualisation of FRET in this case will be evident only upon the formation of a ternary complex. In their report, the team identified the formation of ternary complexes such as Jun-Fos-NFAT1 and Jun-Fos-p65 (Shyu et al., 2008).

An alternative method to detect and study ternary complex formation is known as the FRET-Fluorescence lifetime imaging microscopy (FLIM) assay (Kinoshita et al., 2007). This assay also relies on a FRET-based theory: two proteins that are part of a ternary complex are each conjugated to a chromophore. Changes in FRET signals in the presence of a third protein are then measured using the FLIM method. FLIM is designed to measure the fluorescence lifetimes of fluorescent proteins. When FLIM is applied as a downstream assay to FRET, protein-protein interactions and conformational changes of proteins in living cells can be spatially and temporally measured with high resolution. Whereas FRET occurrence is tracked by the decreasing lifetime of the donor chromophore in the presence of an acceptor chromophore, FRET-FLIM provides a certain advantage over traditional intensity-based FRET assays and has previously been discussed by Wallrabe and Periasamy (Wallrabe and Periasamy, 2005).

Because such techniques as BiFC-FRET and FRET-FLIM are still relatively novel, studies on ternary complex formations are still in their infancy stages. Future publications using such techniques will elucidate their role in cell migration.

Proximity Ligation Assay (PLA)

The need for a sensitive and specific assay to detect PPIs led to the development of the *in situ* PLA. PLA combines the robust and versatile properties of antibodies with a split-reporter assay to generate a selective and specific method to detect PPIs. This method is similar to immunostaining (immunohisto- and immunofluorescence staining) in principle and allows for the detection of endogenous proteins down to single-cell resolution. This ability allows researchers to study PPIs in their most natural environment, within a cell, which is paramount in the context of cell migration.

In immunostaining, primary antibodies are used to target the proteins of interest, followed by the application of fluorophore-conjugated secondary antibodies to visualise the proteins of interest. This process provides information on the expression and co-localisation of the suspected interacting protein pairs. However, the limitation of microscopy resolution is a barrier to concluding whether the partners are indeed interacting or merely in close proximity. *In situ* PLA overcomes this limitation by replacing the secondary antibodies with a pair of proximity probes (antibodies conjugated to oligonucleotides) (see Figure 3). This pair of oligonucleotides acts as a template for the hybridisation and ligation of subsequently added connector oligonucleotides to form a circular DNA molecule. The hybridisation and ligation of the connector oligonucleotides is only possible if the proximal probes are close to each other (40 nm if employing secondary antibodies or 30 nm if primary antibodies are directly used), which underscores the most crucial innovation of the assay. The circular DNA acts as a template for infinite rolling circle amplification (RCA), a process performed by a DNA polymerase. This generates an extended single-stranded DNA molecule containing concatemeric copies of the original circular DNA sequence that remains attached to the proximal probes (Soderberg et al., 2006). These amplification products (AP) are then visualised using fluorescently labelled oligonucleotides that hybridise to a unique sequence on the AP. Because the AP contains numerous repeats of this sequence, the binding of the detection oligonucleotides generates a clear, localised fluorescence signal that is easily distinguishable from background readings. This feature greatly enhances the resolution of the assay. Because each PPI corresponds to one AP signal, the quantification of the number of interactions and the precise location of each interaction pair becomes possible, which provides further insights into cellular biology (Soderberg et al., 2006).

As mentioned in the BiFC section of this review, BiFC was used by Yu and colleagues to demonstrate the interaction between ILK and p38β *in vivo* to substantiate its relevance to bladder cancer migration potential (Yu et al., 2014). Nonetheless, to validate their findings and ensure that endogenous ILK and p38β indeed interacted in a serum-responsive manner, in situ PLA was their method of choice. In agreement with their BiFC observations, a sharp increase in the number of cytoplasmic PLA signals (each representative of an ILK-p38β interaction) was observed when TSU-Pr1 cells were stimulated with 10% FBS. Treatment with the ILK inhibitor QLT-0267 prevented complex formation

between ILK and p38β (as evidenced by the marked reduction of cytoplasmic PLA signals) and halved the serum-directed chemotaxis of serum-stimulated TSU-Pr1 cells (Yu et al., 2014).

In a study on wound-healing, PLA performed on primary human keratinocytes and mouse wound tissue sections also revealed the importance of ANGPTL4 in modulating interactions between keratinocytes and their ECM (Goh et al., 2010). ANGPTL4 is a secreted factor that is up-regulated by PPAR (Mandard et al., 2004) and hypoxia (Belanger et al., 2002) and has known implications in lipid and glucose metabolism (Oike et al., 2005) as well as breast cancer metastasis (Minn et al., 2005). It is cleaved post-translationally to give rise to N- and C-terminal fragments (nANGPTL4 and cANGPTL4) that are known to possess distinct functions. Goh and colleagues identified cANGPTL4-vitronectin and cANGPTL4-fibronectin interactions that occur in the wound bed and epithelium via PLA. cANGPTL4 binding to vitronectin (or fibronectin) did not obviate cognate integrin α5β1 binding as visualised by triple PLA, wherein a PLA signal is only detected when all three members of the proposed tripartite complex interact. ANGPTL4 was subsequently found to stabilise vitronectin and fibronectin against matrix metalloprotease degradation, which in turn potentiates the activation of integrin-dependent intracellular signalling pathways that promote keratinocyte migration (Goh et al., 2010).

PLA offers researchers a convenient way to detect PPIs that occur between endogenous proteins of intact cells and tissue sections. This method preserves PPI localisation and ensures that only native interactions are highlighted, which positions PLA as a technique of high specificity. With the incorporation of an innovative amplification reaction that strengthens the signal output, PLA allows for detection sensitivity at the impressive resolution of a single PPI. PLA also allows for the

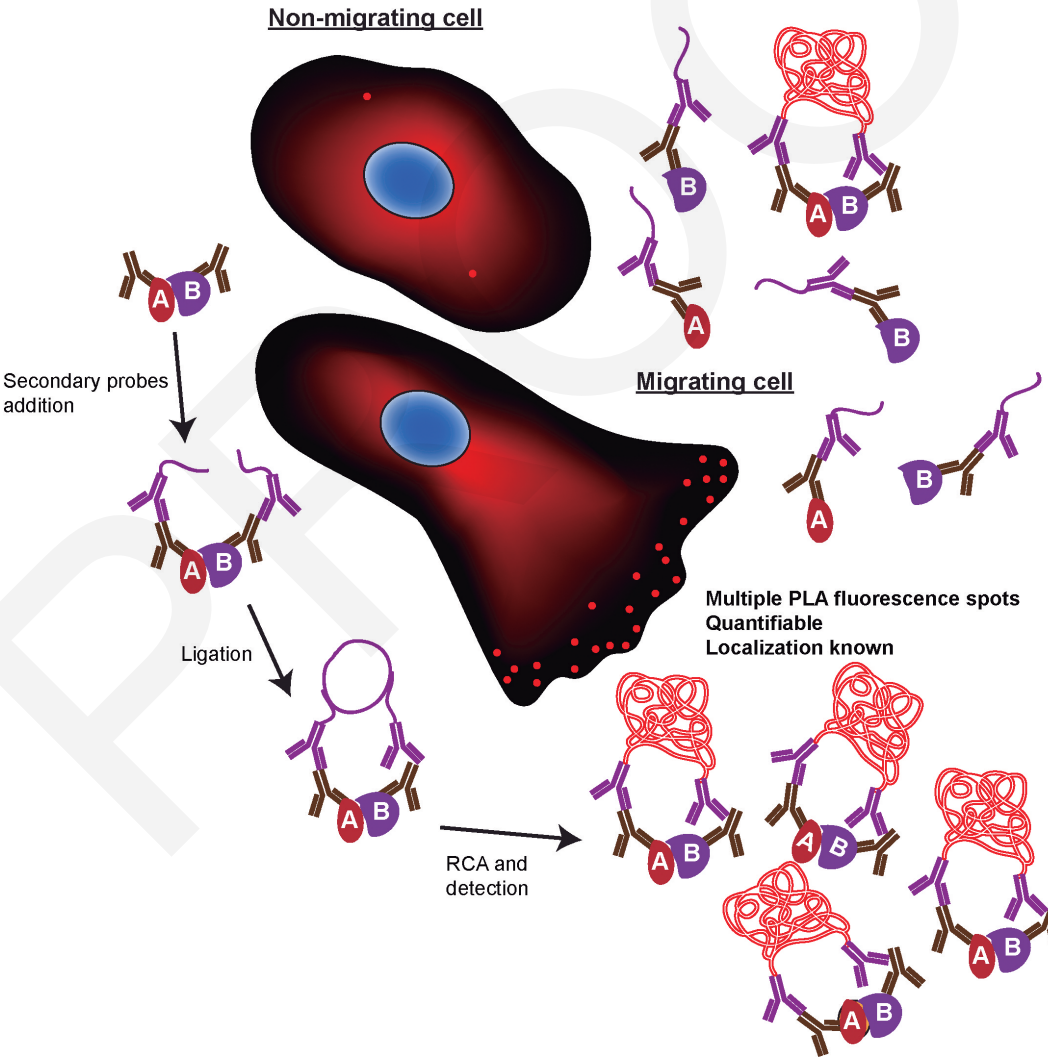

Fig. 3. Schematic depicting the underlying principle of the proximity ligation assay (PLA). Cells are fixed and permeabilised before incubation with antibodies specific to the proteins involved in the prospective PPI of interest. Secondary probes are added, consisting of secondary antibodies conjugated to oligonucleotide sequences. Subsequent ligation and rolling circle amplification (RCA) reactions are only permitted when the oligonucleotide sequences are within 40nm of each other. The amplification products (AP) of RCA contain a unique sequence to which fluorescent detection oligonucleotides bind to, producing a spot signal representative of individual PPIs of interest.

detection of transient PPIs in cells and tissues, up to sub-cellular levels because fixation of the cells is involved. This technique provides a relative but objective quantification of PPIs, which is a characteristic that is not readily achieved when using other techniques. However, PLA also has its own limitations. Being an antibody-based assay means that the success of PLA is still dependent upon ensuring ideal antibody binding conditions and is vulnerable to the stresses of wash steps which may compromise the sensitivity towards transient PPIs. Hence, this requirement emphasises the need for positive and negative controls for each PPI being tested. For example, when probing for an interaction between cANGPTL4 and vitronectin with PLA, Goh et al also probed for vitronectin and integrin β5, which is a well-established cognate interaction (Goh et al., 2010). As a negative control, one can consider probing for proteins that do not share the same cellular compartment under physiological conditions (e.g., integrin β5 and nucleoporin).

Another limitation is that PLA has a low upper limit on the number of interactions that can be simultaneously detected. The triple PLA experiment for the identification of the cANGPTL4-integrin α5β1-vitronectin ternary complex most likely required careful optimisation of antibody binding conditions and of the time that was allowed for amplification and fluorescent-labelled oligonucleotide binding. Increasing the number of PPIs that are being simultaneously probed could be complex and liable to failure. Additionally, permeabilisation of cells to allow for access by the primary antibodies means that PLA is used as an end-point experiment and is not amenable to live-cell imaging.

Surface Plasmon Resonance (SPR)

The concept of SPR has previously been described (Helmerhorst et al., 2012). Briefly, surface plasmon is an electromagnetic wave that propagates along the surface of a thin metal layer. To achieve SPR, a beam of polarised light is shone through a prism at a precise angle where it undergoes total internal reflection at the prism/metal/medium interphase and results in the excitation of the surface plasmon to generate the SPR. The angle of the light is extremely sensitive to changes in the reflective indexes of the medium that is adjacent to the metal layer; therefore, valuable statistics on protein interactions can be obtained by directly measuring the intensity of the reflected light. Experimentally, the protein of interest (ligand) is bound to a sensor chip that normally has a gold polymer interface. The suspected protein (analyte) solution is then allowed to flow over the sensor chip. When binding of the ligand and analyte occurs, the reflective index of the medium changes, and this change is dependent upon the molecular weight and number of proteins that are bound, among others. The characteristics of the individual interactions, whether they are transient or stable, strong or weak, fast or slow directly impact the changes in intensity of the reflected light that is measured and yields real-time and highly sensitive information on protein interaction kinetics and equilibrium constants.

One recent article demonstrated the use of SPR to analyse the binding of plasminogen (PIg) to its postulated receptor, thrombomodulin (TM), in promoting endothelial migration and invasion (Chen et al., 2013). This experiment was performed by measuring the binding kinetics of PIg and TM using a Biacore sensor, and the readout was given in terms of the association and dissociation rate constants. High values for the association and dissociation rate constants indicated that the PIg-TM binding and dissociation events occurred rapidly.

One potential drawback of the SPR technique is the possibility of the protein-of-interest losing its native configuration upon its attachment onto the sensor chip surface, or that its orientation on the chip may confer steric hindrance that could prevent analyte binding. To address this problem, researchers may have to consider alternatives, such as switching the ligand to become the

Table 1. Summary of methods for probing PPIs during cell migration.

		Co-IP	BiFC/FRET/FLIM	PLA	SPR
Experimental requirement	Probe-based	✓		✓	✓
	Direct labelling		✓		
	Intact cell (PPI localisation retained)		✓	✓	
	Cell lysate (PPI localisation lost)	✓			✓
	Live-cell imaging		✓		
Nature of PPIs	Stable	✓	✓	✓	✓
	Transient	✓*	✓	✓*	✓
	Post-translational modifications involved	✓		✓	✓
Readout	Semi-quantitative	✓	✓		
	Quantitative			✓	✓
	Affinity and kinetics				✓

*: denotes modification to protocol may be necessary.

analyte or optimising the attachment so that minimal binding heterogeneity to the sensor surface occurs. The presence of low-affinity or non-specific surface sites can also lead to an increase in non-specific binding effects at the surface of the sensor chip. Similarly, the pH and molarity of the buffers that are used will also influence the PPIs and questions remain as to whether these conditions reflect the endogenous context. Similar to co-IP, no subcellular localisation can be inferred from the interactions.

Despite these disadvantages, SPR has many advantages as well. In addition to it being a technique that is label-free, it provides rapid results and is highly sensitive; researchers are able to obtain real-time measurements of protein interactions and can determine the concentration of the analyte without the need for standard calibrations by applying calibration free concentration analysis (CFCA). CFCA provides an actual "active" concentration of the analyte that is compared to other techniques, such as ELISA or spectroscopic methods. This feature is critical, particularly in the pharmaceutical industries where the actual "active" concentration of an analyte is crucial to design an optimised production line. SPR is also the only technique that can provide data on the affinity constants (association and dissociation) of PPIs.

Conclusion

Cellular migration is a fundamental and important process that occurs during numerous biological activities, such as embryogenesis, angiogenesis, wound healing, inflammatory response and cancer metastases. Control of cellular migration is dependent upon PPIs that initiate and effect cytoskeletal remodelling alongside changes in cell-ECM contacts to enable a cell to move on its substratum in a directional manner. Given the multitude of proteins that coexist and interact within a cell at any point in time, it is often difficult to identify PPIs that are relevant to cellular migration. A reductionist approach to this problem is to demonstrate that a particular PPI associates strictly with a migratory event and that PPI formation is necessary and sufficient to promote cellular migration. To this end, probe-based and direct-labelling techniques have been developed to provide researchers a visual, semi- if not fully quantifiable readout of PPIs that drive the migratory events in cell biology. Each of these methods has its merits and shortcomings, but many serve complementary functions when applied in combination. Herein we have highlighted four methods, co-IP, BiFC (pure and hybrid versions), PLA and SPR which continue to enable researchers to gain insight into the molecular mechanisms that underlie cell migration. In summary, the decision to use one method over the other can be summarised through three main factors: (1) experimental requirement, (2) the nature of the PPIs being probed and (3) the preferred experimental readout (see Table 1).

For example, if one chooses to employ co-IP for the identification of PPIs involved in migration, an antibody against proteins of interest must first be available, and the experimental setup, by default, forgoes the sub-cellular localisation of the PPI which could be important in some cases. Co-IP is more suitable for the detection of stable interactions; however, it is possible to stabilise transient interactions with the integration of a cross-linking step in its protocol. Although a researcher may choose to employ BiFC from the beginning because BiFC is directly capable of simultaneously capturing transient PPIs and the sub-cellular localisation where the PPI is occurring, the design of reporter-fusion protein constructs and optimisation of their *in vivo* expression may be costly and time-consuming. Hence, co-IP could be an efficient way for researchers to gain a foothold in demonstrating that the PPI indeed exists before committing to elucidating its localisation via BiFC.

Arguably, the best evidence for migration-related PPI formation is the observation of PPIs that form as migration occurs. Such observations can be made using BiFC and live-cell imaging in real time. Nonetheless, the readout of BiFC is only semi-quantitative because fluorescent labels only allow intensity-based inference of the frequency of PPI occurrence, and conclusions made from cells expressing exogenous, split-reporter-fused proteins may be viewed sceptically. These reservations can be overcome with PLA, which allows each PPI to be counted in non-migratory versus migrating cells which yields a precise quantitative result of whether a PPI is upregulated during cell migration. Moreover, probing is performed on endogenous proteins of unmodified cells, which alleviates any concerns over the non-native effects of the exogenous expression of split reporter-fused proteins.

As has been highlighted in this review, a greater depth into PPIs can be gained by truncating or mutating the proteins that are involved in a PPI of interest. Truncations or mutations that abolish PPI formation can reveal essential domains and residues that hold together entire protein complexes. Should further depth into the interaction be desired, SPR can be used to provide insight into the affinities and kinetics of binding.

To conclude, we have endeavoured to consolidate the advantages, limitations and considerations when using each of these methods to probe for PPIs in cell migration. The techniques discussed here are by no means exhaustive, but should suffice as a sound framework for researchers to decide on their preferred approach.

Acknowledgements. This review is supported by the National Medical Research Council, Singapore (NMRC/1280/2010). J.S.K.C and Z.Q.T are research scholars under the Nanyang President Graduate Scholarship. M.K.S is a research scholar under the Nanyang Technological University Research Scholarship.

References

Belanger A.J., Lu H., Date T., Liu L.X., Vincent K.A., Akita G.Y., Cheng S.H., Gregory R.J. and Jiang C. (2002). Hypoxia up-regulates expression of peroxisome proliferator-activated receptor gamma angiopoietin-related gene (PGAR) in cardiomyocytes: role of hypoxia inducible factor 1alpha. J. Mol. Cell. Cardiol. 34, 765-774.

Bergers G. and Benjamin L.E. (2003). Tumorigenesis and the angiogenic switch. Nat. Rev. Cancer 3, 401-10.

Chautard E., Thierry-Mieg N. and Ricard-Blum S. (2009). Interaction networks: from protein functions to drug discovery. A review. Pathol. Biol. (Paris) 57, 324-333.

Chen P.K., Chang B.I., Kuo C.H., Chen P.S., Cho C.F., Chang C.F., Shi G.Y. and Wu H.L. (2013). Thrombomodulin functions as a plasminogen receptor to modulate angiogenesis. FASEB J. 27, 4520-4531.

Cho S.Y. and Klemke R.L. (2002). Purification of pseudopodia from polarized cells reveals redistribution and activation of Rac through assembly of a CAS/Crk scaffold. J. Cell Biol. 156, 725-736.

Chung A.S., Lee J. and Ferrara N. (2010). Targeting the tumour vasculature: insights from physiological angiogenesis. Nat. Rev. Cancer 10, 505-14.

Crick F. (1970). Central dogma of molecular biology. Nature 227, 561-563.

Decline F. and Rousselle P. (2001). Keratinocyte migration requires alpha2beta1 integrin-mediated interaction with the laminin 5 gamma2 chain. J. Cell Sci. 114, 811-823.

deHart G.W., Healy K.E. and Jones J.C. (2003). The role of alpha3beta1 integrin in determining the supramolecular organization of laminin-5 in the extracellular matrix of keratinocytes. Exp. Cell. Res. 283, 67-79.

Enders A.C., Given R.L. and Schlafke S. (1978). Differentiation and migration of endoderm in the rat and mouse at implantation. Anat. Rec. 190, 65-77.

Filonov G.S. and Verkhusha V.V. (2013). A near-infrared BiFC reporter for in vivo imaging of protein-protein interactions. Chem. Biol. 20, 1078-1086.

Gandhi T.K., Zhong J., Mathivanan S., Karthick L., Chandrika K.N., Mohan S.S., Sharma S., Pinkert S., Nagaraju S., Periaswamy B., Mishra G., Nandakumar K., Shen B., Deshpande N., Nayak R., Sarker M., Boeke J.D., Parmigiani G., Schultz J., Bader J.S. and Pandey A. (2006). Analysis of the human protein interactome and comparison with yeast, worm and fly interaction datasets. Nat. Genet. 38, 285-293.

Goh Y.Y., Pal M., Chong H.C., Zhu P., Tan M.J., Punugu L., Tan C.K., Huang R.L., Sze S.K., Tang M.B., Ding J.L., Kersten S. and Tan N. S. (2010). Angiopoietin-like 4 interacts with matrix proteins to modulate wound healing. J. Biol. Chem. 285, 32999-323009.

Goldfinger L.E., Hopkinson S.B., deHart G.W., Collawn S., Couchman J.R. and Jones J.C. (1999). The alpha3 laminin subunit, alpha6beta4 and alpha3beta1 integrin coordinately regulate wound healing in cultured epithelial cells and in the skin. J. Cell Sci. 112, 2615-2629.

Helmerhorst E., Chandler D.J., Nussio M. and Mamotte C.D. (2012). Real-time and Label-free Bio-sensing of Molecular Interactions by Surface Plasmon Resonance: A Laboratory Medicine Perspective. Clin. Biochem. Rev. 33, 161-73.

Hood J.D. and Cheresh D.A. (2002). Role of integrins in cell invasion and migration. Nat. Rev. Cancer 2, 91-100.

Kassim A.A. and DeBaun M.R. (2013). Sickle cell disease, vasculopathy, and therapeutics. Annu. Rev. Med. 64, 451-466.

Kerppola T.K. (2008). Bimolecular fluorescence complementation: visualization of molecular interactions in living cells. Methods Cell Biol. 85, 431-470.

Kim J.P., Chen J.D., Wilke M.S., Schall T.J. and Woodley D.T. (1994). Human keratinocyte migration on type IV collagen. Roles of heparin-binding site and alpha 2 beta 1 integrin. Lab. Invest. 71, 401-408.

Kinoshita K., Goryo K., Takada M., Tomokuni Y., Aso T., Okuda H., Shuin T., Fukumura H. and Sogawa K. (2007). Ternary complex formation of pVHL, elongin B and elongin C visualized in living cells by a fluorescence resonance energy transfer-fluorescence lifetime imaging microscopy technique. FEBS J. 274, 5567-5575.

Kodama Y. and Hu C.D. (2012). Bimolecular fluorescence complementation (BiFC): a 5-year update and future perspectives. Biotechniques 53, 285-298.

Lawson K.A. and Pedersen R.A. (1987). Cell fate, morphogenetic movement and population kinetics of embryonic endoderm at the time of germ layer formation in the mouse. Development 101, 627-652.

Lu D., Han C. and Wu T. (2013). 15-hydroxyprostaglandin dehydrogenase-derived 15-keto-prostaglandin E2 inhibits cholangiocarcinoma cell growth through interaction with peroxisome proliferator-activated receptor-gamma, SMAD2/3, and TAP63 proteins. J. Biol. Chem. 288, 19484-19502.

Mandard S., Zandbergen F., Tan N.S., Escher P., Patsouris D., Koenig W., Kleemann R., Bakker A., Veenman F., Wahli W., Muller M. and Kersten S. (2004). The direct peroxisome proliferator-activated receptor target fasting-induced adipose factor (FIAF/PGAR/ANGPTL4) is present in blood plasma as a truncated protein that is increased by fenofibrate treatment. J. Biol. Chem. 279, 34411-34420.

Master Z., Jones N., Tran J., Jones J., Kerbel R.S. and Dumont D.J. (2001). Dok-R plays a pivotal role in angiopoietin-1-dependent cell migration through recruitment and activation of Pak. EMBO J. 20, 5919-5928.

Mattick J.S. (2003). Challenging the dogma: the hidden layer of non-protein-coding RNAs in complex organisms. Bioessays 25, 930-939.

Minn A.J., Gupta G.P., Siegel P.M., Bos P.D., Shu W., Giri D.D., Viale A., Olshen A.B., Gerald W.L. and Massague J. (2005). Genes that mediate breast cancer metastasis to lung. Nature 436, 518-524.

Nguyen B.P., Gil S.G. and Carter W.G. (2000). Deposition of laminin 5 by keratinocytes regulates integrin adhesion and signaling. J. Biol. Chem. 275, 31896-31907.

O'Neill G.M., Fashena S.J. and Golemis E.A. (2000). Integrin signalling: a new Cas(t) of characters enters the stage. Trends Cell Biol. 10, 111-119.

Oike Y., Akao M., Kubota Y. and Suda T. (2005). Angiopoietin-like proteins: potential new targets for metabolic syndrome therapy. Trends Mol. Med. 11, 473-479.

Palamidessi A., Frittoli E., Ducano N., Offenhauser N., Sigismund S., Kajiho H., Parazzoli D., Oldani A., Gobbi M., Serini G., Di Fiore P.P., Scita G. and Lanzetti L. (2013). The GTPase-Activating Protein RN-tre Controls Focal Adhesion Turnover and Cell Migration. Curr. Biol. 23, 2355-2364.

Phizicky E.M. and Fields S. (1995). Protein-protein interactions: methods for detection and analysis. Microbiol. Rev. 59, 94-123.

Poelmann R.E. (1981). The formation of the embryonic mesoderm in the early post-implantation mouse embryo. Anat. Embryol. (Berl). 162,

29-40.
Pribila J.T. and Shimizu Y. (2003). Signal transduction events regulating integrin function and T cell migration: new functions and complexity. Immunol. Res. 27, 107-128.
Roussos E.T., Condeelis J.S. and Patsialou A. (2011). Chemotaxis in cancer. Nat. Rev. Cancer 11, 573-87.
Sehgal B.U., DeBiase P.J., Matzno S., Chew T.L., Claiborne J.N., Hopkinson S.B., Russell A., Marinkovich M.P. and Jones J.C. (2006). Integrin beta4 regulates migratory behavior of keratinocytes by determining laminin-332 organization. J. Biol. Chem. 281, 35487-98.
Sethi N. and Kang Y. (2011). Unravelling the complexity of metastasis - molecular understanding and targeted therapies. Nat. Rev. Cancer 11, 735-748.
Shapiro J.A. (2009). Revisiting the central dogma in the 21st century. Ann. N. Y. Acad. Sci. 1178, 6-28.
Shyu Y J., Suarez C.D. and Hu C.D. (2008). Visualization of AP-1 NF-kappaB ternary complexes in living cells by using a BiFC-based FRET. Proc. Natl. Acad. Sci. USA 105, 151-156.
Soderberg O., Gullberg M., Jarvius M., Ridderstrale K., Leuchowius K.J., Jarvius J., Wester K., Hydbring P., Bahram F., Larsson L.G. and Landegren U. (2006). Direct observation of individual endogenous protein complexes in situ by proximity ligation. Nat. Methods 3, 995-1000.
Tan N.S., Icre G., Montagner A., Bordier-ten-Heggeler B., Wahli W. and Michalik L. (2007). The nuclear hormone receptor peroxisome proliferator-activated receptor beta/delta potentiates cell chemotactism, polarization, and migration. Mol. Cell. Biol. 27, 7161-75.
Wallrabe H. and Periasamy A. (2005). Imaging protein molecules using FRET and FLIM microscopy. Curr. Opin. Biotechnol. 16, 19-27.
Wang Y. and Klemke R.L. (2007). Biochemical purification of pseudopodia from migratory cells. Methods Mol. Biol. 370, 55-66.
Wehrle-Haller B. (2012). Assembly and disassembly of cell matrix adhesions. Curr. Opin. Cell Biol. 24, 569-5681.
Yong K. and Khwaja A. (1990). Leucocyte cellular adhesion molecules. Blood Rev. 4, 211-25.
Yu L., Yuan X., Wang D., Barakat B., Williams E.D. and Hannigan G.E. (2014). Selective regulation of p38beta protein and signaling by integrin-linked kinase mediates bladder cancer cell migration. Oncogene 33, 690-701.
Zinzalla G. and Thurston D.E. (2009). Targeting protein-protein interactions for therapeutic intervention: a challenge for the future. Future Med. Chem. 1, 65-93.

Accepted February 19, 2014