Advances in imaging cell–matrix adhesions

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Summary

Adhesion is fundamental to the survival and function of many different cell types, and regulates basic events such as mitosis, cell survival and migration, in both embryonic and adult organisms. Cell–matrix adhesion also regulates the dynamic interplay between cells and surrounding tissues during processes such as immune cell recruitment, wound healing and cancer cell metastasis. The study of cell adhesion has gained momentum in recent years, in large part because of the emergence of imaging techniques that have facilitated detailed analysis of the molecular composition and dynamics of the structures involved. In this Commentary, we discuss the recent application of different imaging techniques to study cell–matrix adhesions, emphasising common strategies used for the analysis of adhesion dynamics both in cells in culture and in whole organisms.

Key words: Cell adhesion, Fluorescence, Focal adhesion, Live imaging, Microscopy, Migration

Introduction

Several different types of adhesions are formed by cells following engagement with the extracellular matrix (ECM). These structures are classified on the basis of various factors, such as protein composition, lifespan and proteolytic properties. Examples of cell–matrix adhesions range from small, short-lived focal contacts to punctate, finger-like projections known as invadopodia (for more details, see Box 1 and Fig. 1). The localisation and timing of the formation of adhesive structures is under the control of numerous different proteins, and adhesion formation is thought to be triggered by activation of the integrin family of ECM receptors. The engagement of integrins with the ECM results in the hierarchical assembly of intracellular signalling platforms in the regions of clustered integrin cytoplasmic domains; these signalling platforms contain adaptor proteins, kinases, phosphatases and other receptors. Integrin activation can result in rapid changes to the indirectly associated actin cytoskeleton, which provides mechanical support for adhesion maturation (for more details, see Box 2). Similarly, the disassembly of adhesions is also tightly spatially and temporally regulated, and is required for efficient cell movement.

Much of what is currently known about localisation and turnover of cell–matrix adhesions has been determined by visualising protein dynamics using a range of microscopy techniques. However, most studies to date have been performed with cells plated on two-dimensional (2D) surfaces. As such, many questions remain unanswered concerning the nature and dynamics of cell–matrix adhesions in complex three-dimensional (3D) matrices. Indeed, the relevance of the different adhesion classifications defined in cells on 2D matrices to those found in cells in 3D environments remains unclear. In this Commentary, we outline several techniques that are commonly used to study aspects of adhesion behaviour in living cells (see Table 1 for a summary), and review specific findings from recent studies that have applied these techniques.

Imaging cell–matrix adhesion dynamics in vitro

Wide-field microscopy

The most widely used method to image fluorescent protein dynamics is wide-field microscopy (also known as epifluorescent microscopy). This technique can be performed with a basic fluorescence microscope that is fitted with a charged-coupled device (CCD) camera and the appropriate excitation and emission filter cubes to distinguish the relevant wavelengths, as dictated by the emission spectra of the fluorescent probes being used. These systems generally allow the user to view cells with both fluorescent and phase-contrast or differential interference contrast (DIC) microscopy methods. This allows the visualisation of the entire cell to determine the localisation of the fluorescent probe with respect to specific structures of interest. Images can be acquired over short or long time periods, and the length of time depends on a number of factors. The more sensitive the camera, the lower the illumination level required to visualise the tagged protein, resulting in a lower risk of cell toxicity and thus enabling a longer viewing time. Additionally, in the case of transfected cells, the expression levels of the protein will also determine the exposure times required to visualise the protein of interest. Acquiring such time-lapse movies allows the user to follow a protein or adhesion marker of interest over time. Using appropriate post-acquisition analysis software, these movies can then be used to calculate, for example, adhesion numbers, rates of adhesion assembly or disassembly, and intensity profile changes in subcellular localisation over time (see Fig. 2 for an example of dynamic changes in adhesions over time). A recent example of this technique is described in a study that used GFP–cortactin as a marker of invadopodia formation and turnover in MTLn3 cells. Data from the obtained movies revealed that the cells that had been treated with small interfering RNA (siRNA) to knockdown focal adhesion kinase (FAK) had greater numbers of invadopodia, and that these adhesions showed higher rates of assembly and disassembly compared with control cells (Chan et al., 2009). A different study used a similar approach to dissect the function of cortactin phosphorylation and its subsequent regulation of cofilin and Arp2/3 to control invadopodia maturation (Oser et al., 2009). By imaging changes in the size and dynamics of invadopodia over time, the authors demonstrated that dephosphorylation of cortactin led to loss of the actin-severing function of cofilin and subsequent maturation and stabilisation of these adhesive structures.

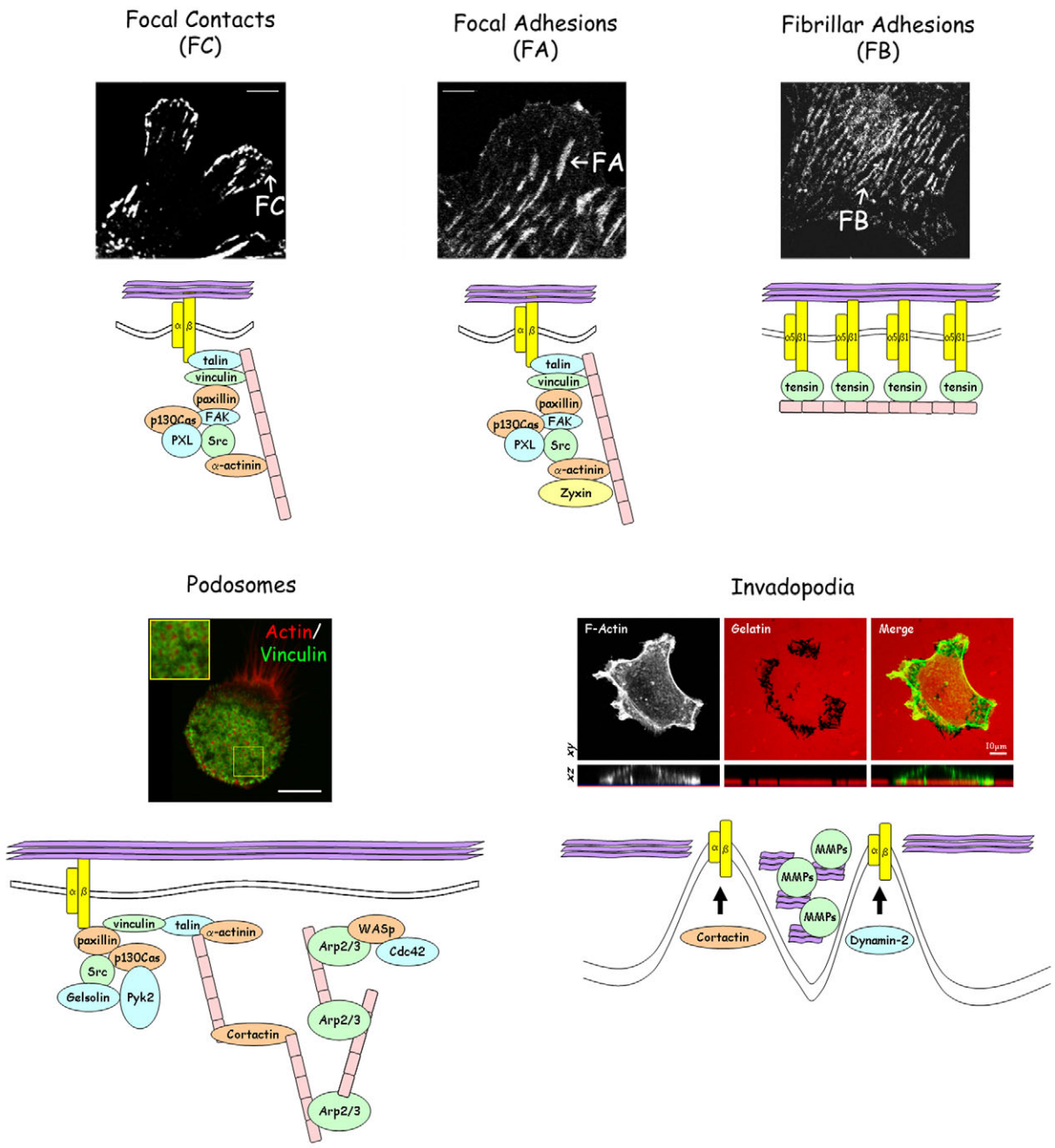

Fig. 1. Examples of adhesion structures and their composition. Confocal images and representative component cartoons of different adhesions are shown. Top images are fibroblasts stained for β3 integrin (focal contact, FC), β1 integrin (focal adhesion, FA) or tensin (fibrillar adhesion, FB). Scale bars: 5 μm. Bottom panels are (left) THP-1 cells stained with actin and vinculin to define podosomes, and (right) A375M cells on TRITC-gelatin stained with phalloidin to show invadopodia. The representative cartoons depict the ECM in purple, integrin (α and β subunits) in yellow and F-actin in pink. MMPs, matrix metalloproteinases. See text for more details.

Spinning-disc confocal microscopy

Spinning-disc confocal microscopy can also be used to image adhesion assembly and disassembly, and is a more sensitive approach with a higher signal-to-noise ratio compared with wide-field microscopy. Samples are illuminated by a standard white light (e.g. mercury–xenon mixture) or, more commonly, by a laser excitation source passing through a radial array of pinholes. This allows optical sections of a sample to be imaged at high speeds, from tens of frames per second to over a hundred frames per second, depending upon the specification of the microscope. Laser light sources can also be used; the addition of an acousto-optical tunable filter (AOTF) allows switching of excitation wavelengths in the order of microseconds. This modification also allows fast imaging of multiple fluorophores within a sample. One drawback of this technique is that the optical section depth is fixed, thus reducing flexibility when imaging samples of different thicknesses. Rates of assembly and disassembly of adhesions can be calculated from resulting movies through

Table 1. Overview of imaging strategies used to visualise adhesions in living cells

Microscope or analysis technique	Principle	Advantages and disadvantages	Recent example of use
Wide-field (epifluorescence)	Sample illuminated by light passing through filter sets; excitations picked up by CCD camera	Widely used method; sensitive CCD camera will limit exposure time required and prevent phototoxicity	(Chan et al., 2009)
Spinning-disc confocal	Light passes through a radial array of pinholes; can image thin optical sections at high speed	Increased sensitivity compared with wide-field; addition of laser light sources and AOTF can increase imaging speeds of multiple wavelengths	(Franco et al., 2004)
Point-scanning confocal	Samples illuminated by lasers of specific wavelengths	Improved *z*-resolution over other methods; new scan heads capable of increasing speed of imaging; laser excitation can result in photobleaching	(Joshi et al., 2008)
Total internal reflection fluorescence (TIRF)	Light passing from solid to liquid phase produces evanescent wave	Images up to depth of 100 nm, making it suitable for imaging adhesions; also enables enhanced signal-to-noise ratio	(Marshall et al., 2009)
Fluorescence recovery after photobleaching (FRAP)	Specific ROI is bleached and recovery of fluorescence intensity in bleached region is measured over time	Speed of recovery gives clues to mode of movement of protein	(Himmel et al., 2009)
Fluorescence loss in photobleaching (FLIP)	Specific region of cell is consistently bleached and not allowed to recover	Not widely used for adhesion study; however, can help define origins of adhesion proteins within a cell	(Hamadi et al., 2008)
Fluorescence speckle microscopy (FSM)	Aggregates of weakly expressed proteins appear as speckles; detected on sensitive CCD camera	Able to monitor movement or turnover of proteins within a structure	(Hu et al., 2007)
Fluorescence correlation spectroscopy (FCS)	Small region of cell is excited many times	Allows user to measure concentration of tagged proteins at forming adhesions	(Digman et al., 2008)
Fluorescence resonance energy transfer (FRET)	Non-radiative resonance transfer takes place when two proteins come into close proximity to each other (<~9 nm)	Different strategies used to measure FRET; relatively easy to perform; analysis of data time-consuming	(Papusheva et al., 2009)

measuring the incorporation or loss of fluorescent signal of the protein being studied. Increase of signal will be the result of adhesion assembly and growth, whereas adhesion disassembly will result in loss of fluorescent signal. Plotting signal intensity values over time on semi-logarithmic graphs will provide a profile of intensity ratios over time (Franco et al., 2004). Alternatively, data points can be plotted and exponential curve fitting analysis applied to permit a similar type of analysis. These ratios (I_n) are calculated using the formula $I_n(I/I_0)$ for assembly and $I_n(I_0/I)$ for disassembly (where I_0 is the initial fluorescence intensity value and I is the intensity value for the relevant time point). Rates can then be calculated from the gradient of the line of best fit, assuming intensity levels are linear (i.e. very low or no photobleaching) as a function of time. Alternatively, the same data sets can be used to calculate other parameters for the dynamic behaviour of focal adhesions, such as size, location, and number or total percentage undergoing turnover.

There are many examples in the field that have employed these types of analysis techniques, including an early study by Franco et al., who used it to show the effect of calpain proteolysis on talin within focal adhesions (Franco et al., 2004). Knockdown of calpain 2 using siRNA in fibroblasts reduced adhesion disassembly rates from 0.09 min^{-1} in control cells to 0.04 min^{-1}, thus suggesting that calpain proteolysis of talin promotes adhesion disassembly in migratory cells. A more recent study used a similar approach to subsequently show that calpain-induced cleavage of talin leads to Cdk5- and Smurf1-mediated ubiquitylation of the talin head. This then results in degradation of the talin head and, ultimately, in enhanced focal adhesion stability (Huang et al., 2009). The analysis of focal adhesion dynamics using wide-field or spinning-disc

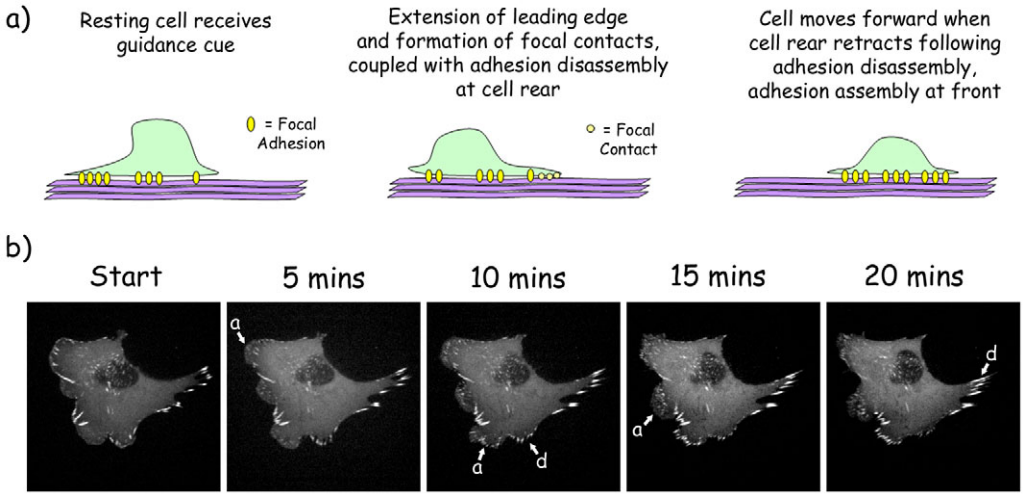

Fig. 2. Adhesion dynamics. (**A**) Representative cartoons and (**B**) still images taken from a time-lapse movie of a fibroblast expressing GFP–vinculin. In B, arrows 'a' and 'd' denote assembling and disassembling adhesions, respectively.

imaging approaches is relatively fast and might therefore represent suitable methods for small-scale screening of siRNA libraries to identify new regulators of adhesion turnover. Proof of principle for this was provided in a recent study, in which hits identified from an siRNA screen for regulators of adhesion size and morphology in fixed cells were verified using live imaging of the common focal adhesion protein paxillin in HeLa cells (Winograd-Katz et al., 2009).

Point-scanning confocal microscopy

In cases in which increased spatial resolution is required, point-scanning confocal microscopy provides a good alternative method (Joshi et al., 2008). Samples are excited through lasers of appropriate wavelength passing through a pinhole, with emitted light being received through a further pinhole. The light arrives at the detector from a narrow focal plane, which means that both noise from out-of-focus light and the *z*-resolution are considerably improved over methods such as wide-field microscopy. Point-scanning confocal systems have additional benefits of variable pinhole and optical zoom, thus providing more flexibility than spinning-disc methods. Speed of imaging depends on the system being used, with most standard systems having the ability to image up to 4 frames/second at a resolution of 512×512 pixels. Acquisition speeds can be increased further in standard confocal systems by using line scanning to acquire a row of pixels along a single axis of the specimen. However, this approach is limited by both the scan area and the excitation pixel dwell time, and can therefore result in low-sensitivity detection of fluorescence. The recent introduction of resonant scan heads has increased the rate of image acquisition again, up to 30 frames/second, thus allowing highly dynamic processes (such as actin assembly or retrograde flow) to be imaged and even movies to be recorded in multiple focal planes. A further benefit of confocal microscopy is the ability to image optical sections within relatively thick specimens (depending on the working distance of the objective) and the use of these images to generate 3D reconstructions, which can provide additional insights into the relative positioning of proteins. The speed of acquisition in the *z*-plane can also be greatly improved using resonant scanning confocal microscopy in conjunction with a fast accurate motorised z-drive (e.g. a Piezo z-drive). The drawback of using confocal microscopy for live imaging, particularly over multiple time points and/or with optical slices at high speeds, is increased photobleaching due to intense laser excitation. Therefore, a balance has to be reached between laser power and the expression levels of the protein(s) being imaged in order for confocal microscopy to be used for longer-term live imaging.

Total internal reflection fluorescence microscopy

Total internal reflection fluorescence (TIRF) microscopy is an ideal method to image cell–matrix interactions. It is dependent on the production of an evanescent wave that results from light passing from a high to low refractive index (such as from a glass cover slip to aqueous media). As the standing wave can only penetrate a very short distance, only a limited region can be imaged, to a depth of approximately 100 nm. This leads to high signal-to-noise ratios due to loss of background signals, as well as high-sensitivity detection at a single focal plane, thus making TIRF an ideal technique to study adhesion structures and their protein recruitment within cells on 2D surfaces. However, TIRF is not useful for imaging adhesion structures in cells within complex 3D matrices. Moreover, by virtue of the low total volume of the cell (and therefore fluorophore) that is illuminated in TIRF microscopy, the relative intensity levels that are achieved can be considerably lower compared with those seen using wide-field or confocal *z*-scan approaches. Recent examples of the use of TIRF include a study by Marshall et al., which demonstrated that a cofilin–TagRFP (red fluorescent protein; see Box 3 for further discussion of fluorescent proteins) construct localised specifically to the proximal end of focal adhesions (Marshall et al., 2009). Other studies have employed TIRF to detect specific subcellular localisation of events during cell spreading and migration that lead to adhesion formation or membrane targeting (Choi et al., 2008; Manneville, 2006; Partridge and Marcantonio, 2006).

Photoactivation and photobleaching

A number of different methods are used to determine the kinetics of protein movement within cells, as discussed below.

Photoactivation

The use of photoactivatable (PA) GFP fluorescent tags is proving to be a very useful tool in the study of adhesion biology. These tags are transfected and expressed in the same way as their normal GFP counterparts. However, PA-tag fluorescence is only visible following a pre-'activation' step. For example, activation of PA-GFP requires a burst of 405 nm light before being imaged using a 488 nm laser. Therefore, these tags allow the user to select for a certain population of tagged protein in the cell at a particular point and to follow the fate of this protein after the activation step. As the PA-GFP-tagged protein appears 'dark' before commencing an experiment, it can help to coexpress a second fluorophore-tagged molecule of a different wavelength (e.g. with an mCherry tag) to identify structures or organelles of interest. This provides not only a guide to pinpoint the subcellular site that is to be photoactivated, but also a reference against which the intensity of the PA-GFP protein can be compared to over the time course of the experiment. Similarly, photoconvertible fluorescent protein tags such as Kaede and Dendra might also prove to be useful tools for identifying and tracking specific populations of adhesion proteins. This class of fluorescent tag is visible as a GFP signal, but can be irreversibly converted to an RFP signal following a burst of 405 nm light (Miyawaki et al., 2005). Although potentially powerful in their applications, practically speaking, PA fluorophores can be technically difficult to manipulate as low levels of sample illumination (e.g. from a mercury arc lamp) before commencing the experiment can result in high levels of unwanted background fluorescence (due to photoactivation), making it difficult to subsequently analyse PA-fluorophore-tagged protein redistribution. PA fluorescent proteins have been used to investigate the kinetics of actin dynamics (Osborn et al., 2006) and of adhesion protein behaviour (Betzig et al., 2006). A more recent study used a PA-GFP–α5 integrin construct to investigate the effect of Rab25 binding to α5β1 integrin on tumour cell invasion. Photoactivation of vesicles that were positive for Rab25 led to the appearance of PA-GFP–α5 integrin, which within 60 seconds was followed by a fourfold reduction in fluorescence intensity, with PA-GFP–α5 appearing at the plasma membrane. This suggests that Rab25 is involved in recycling α5 integrin to the leading edge during the invasive process (Caswell et al., 2007). An alternative is the use of newer PA vectors that also contain a normal fluorescent tag, thus allowing the location of a specific pool of the PA-fluorophore-tagged protein of interest to be defined. One example of this is a recent study in which zyxin was tagged with PA-GFP at one end and mCherry at the other (Welman et al., 2010). This allowed the authors to follow zyxin by imaging

the mCherry tag before selecting a particular region to be activated, for example, a focal adhesion. Using this approach, they were able to show that zyxin moves laterally from one adhesion to the next.

Fluorescence recovery after photobleaching

Fluorescence recovery after photobleaching (FRAP) is another commonly used technique to quantify protein kinetics in living cells. As with PA-GFP, cells are transfected with a fluorescently tagged protein, imaged live and then subjected to a bleach step, in which a specific point or region of interest (ROI) is exposed to high-intensity burst(s) of laser emission. Cells are then imaged over a period of time and the time taken for recovery of the tagged protein into the bleached ROI is determined. The dynamics of protein movement can be calculated from these recovery rates and from immobile fractions within the bleached ROI, which provide information on both on and off rates of the protein of interest at that particular site. Recovery can be expressed as half-life, which is the time required for the signal intensity to return to half of its full final recovery value. A very fast recovery is characterized by a short half-life (e.g. in the sub-second range), and might hint at a rapid and possibly diffusion-based movement of a protein. Slower recovery with a longer half-life suggests that the protein is stably retained within the adhesion and that a more regulated mode of transport is involved. Additionally, the relative intensity level at the plateau of the recovery intensity curve can be used to determine the mobile and immobile fractions of the fluorescently tagged protein. Although simple in concept, there are a number of considerations that must be taken into account when acquiring and analysing FRAP data [for more detail, see Sprague and McNally (Sprague and McNally, 2005)]. First, the overall reduction in fluorescence intensity due to photobleaching during time-lapse acquisition must be corrected for in the final data set. This can be achieved by plotting a parallel intensity curve for a background ROI (or the entire field of view to avoid bias) and applying this slope to correct the bleach ROI recovery values. Second, the bleach step must be as rapid and efficient as possible to ensure that recovery does not commence before bleaching is complete. When performing FRAP for the first time on a protein of interest (or on a new microscope), a process of trial and error might thus be required, with different laser powers and times, before a satisfactory bleach is achieved. A recent example of using FRAP to study adhesions is the study by Himmel et al., who investigated the kinetics of a talin construct that encompassed its integrin- and actin-binding domains (i.e. IBS-2 and ABS-3, respectively) using a double-scan-headed confocal microscope. This allows simultaneous bleaching and image acquisition, thus preventing the loss of any time that occurs when a single confocal scan head switches from bleach mode to acquisition; this is particularly useful when highly dynamic proteins are imaged. This technology is highly appropriate in FRAP analysis, as synchronised laser scanning, with one laser stimulating or bleaching and the other scanning with high resolution, ensures efficient photobleaching without any delay between bleach and scan modes. In cases in which protein recovery times are rapid, such as within the first few seconds post-bleach, the use of synchronised laser scanning can provide vital information to accurately determine protein half-lives and recovery kinetics. In this particular study, the authors showed that a truncated GFP-tagged talin (a key focal adhesion and integrin-binding protein) has a shorter half-life in C2C12 myoblast cells than full-length talin (i.e. 45.3±2.2 seconds compared with 33.5±3.6 seconds) (Himmel et al., 2009).

Box 1. Adhesion types

Initial studies revealed that adhesion structures of different sizes can exist in a single cell and at any one time (Izzard and Lochner, 1980). Subsequent research has since revealed that these structures can be classified into different groups depending on their protein composition, size, life span and proteolytic properties (Puklin-Faucher and Sheetz, 2009; Zaidel-Bar et al., 2004; Zaidel-Bar et al., 2007). The integrin family of heterodimeric transmembrane receptors is important in the initiation and stability of all types of adhesive structures. Upon integrin binding to the ECM, talin is recruited to the cytoplasmic face of the plasma membrane, where it binds to integrin cytoplasmic tails. This leads to the recruitment of a cascade of proteins, including vinculin, paxillin and FAK, each playing a part in the adhesion signalling cascade and assisting in determining the life span of the structure (see Box 2). **Focal contacts** (FCs) are the shortest-lived adhesion structures; they are small and typically located behind the leading edge of a spreading or migrating cell. FCs assemble and disassemble within the order of seconds or minutes. As such, they are said to 'sample' the local ECM before either disassembling or moving on to form more stable structures, such as **focal adhesions** (FAs). FAs are larger and more stable than FCs, lasting on the order of tens of minutes. They contain multiple proteins to ensure stability, and traction forces are transmitted from the ECM to the cell and vice versa. Larger and more stable still are **fibrillar adhesions** (FBs). Seen as long, stable structures that run in parallel to bundles of fibronectin in vivo, FBs are highly enriched in tensin and $\alpha 5\beta 1$ integrin (Green and Yamada, 2007). FBs are also sites of localised matrix deposition and fibronectin fibrillogenesis. Two other classes of adhesion structures further differ in their ability to act as local ECM degradation sites. **Podosomes** typically appear in cells of monocytic origin, such as macrophages. These structures are composed of cores of F-actin and actin-binding proteins within a ring of integrins. **Invadopodia** are similar to podosomes in terms of composition, but occur only in malignant cells (Linder, 2007), where they appear as more punctuate, finger-like projections into the ECM, possibly conferring differences in mechanical stability on these cells (Albiges-Rizo et al., 2009).

Fluorescence loss in photobleaching

Fluorescence loss in photobleaching (FLIP) is a method that uses a similar premise to FRAP. However, in this technique, a ROI is repeatedly bleached, thereby restricting any recovery that otherwise would have taken place. Over time, regions outside the bleached ROI will lose fluorescent signal due to movement of proteins from within the bleached ROI to different areas of the cell and vice versa. Monitoring the spread of bleached protein can provide insight into protein transport or into the origin of proteins that are recruited to adhesions. A recent study used FLIP to analyse the translocation of the tyrosine kinase Src from focal adhesions to membrane ruffles upon activation of Src. A fluorescently tagged Src reporter was repetitively bleached within focal adhesions and the level of fluorescence intensity in membrane ruffles monitored over time (Hamadi et al., 2009). The obtained data demonstrate that the fluorescence intensity specifically decreases within the ruffles adjacent to adhesion sites by 35% over 20 seconds, whereas non-specific cytosolic intensity decreases by only 15%. This suggests that rapid and dynamic shuttling of Src between adhesions and ruffles occurs upon Src activation.

Fluorescence speckle microscopy

As a result of developments in CCD technology, the kinetics of molecular movement can now be analysed in greater depth using

Box 2. Proteins involved in adhesion assembly and disassembly

A common series of events takes place upon integrin engagement with the ECM at sites of adhesion assembly (see also Fig. 2). Initially, integrins undergo a conformational switch to an active state, with their complete activation upon talin binding to the β chain of the cytoplasmic tail (Banno and Ginsberg, 2008). Once activated, integrins cluster to provide a platform for the recruitment and assembly of other associated adaptor and signalling proteins. Recent studies in live cells have begun to reveal the complex hierarchy of protein recruitment and release within adhesions (Brown et al., 2006; Digman et al., 2009).

The assembly of adhesions is dependent on the conformation, binding motifs and signalling domains contained within each of the recruited proteins. Not all of these proteins are able to bind the integrin directly, thus establishing a hierarchical chain in which proteins such as talin, paxillin and filamin, which directly bind integrins, can act as linkers to recruit other proteins. For example, vinculin can be recruited to adhesions through binding to talin and this association leads to the recruitment of α-actinin to adhesions, which in turn can bind to paxillin. Paxillin is then able to bind FAK, triggering autophosphorylation of the kinase on tyrosine 397, an event essential for FAK kinase activation. This phosphorylation event also provides a docking site for the SH2 domains of Src family members and their binding leads to phosphorylation of other sites present on FAK, ensuring full activation of its kinase activity and downstream signalling (Tomar and Schlaepfer, 2009). The resulting scaffold of proteins enables amplification of local signalling, including subsequent downstream signalling to the Rho family of small GTPases, resulting in dynamic cytoskeletal rearrangements (Huveneers and Danen, 2009). Experimental evidence also suggests that actin stress fibres can play a direct role in regulating activation and assembly of adhesion proteins through the control of mechanical forces, allowing the adhesion to strengthen (Colombelli et al., 2009).

Adhesion disassembly can occur at different rates depending on its type, composition and where it is located within the cell. FCs both assemble and disassemble quickly to allow continuous protrusion of a leading edge. FAs are more stable structures and can be localised to the leading edge, under the cell body or at the trailing edge, thus disassembling at different rates depending on the local environmental cues. Several factors have been shown to influence FA disassembly. One recent study reported a role for FAK in mediating p190RhoGAP activation in migrating fibroblasts. This activation results in decreased levels of active Rho and in increased Rac activity, thereby decreasing myosin-dependent tension at the leading edge and promoting Rac-dependent adhesion instability (Tomar et al., 2009). Additionally, the calcium-dependent protease calpain is known to cleave talin at adhesion sites, leading to increased rates of adhesion disassembly (Franco et al., 2004). Calpain can also regulate formation of invadopodia in cancer cells by regulating Src signalling (Cortesio et al., 2008). Microtubules have also been proposed to mediate adhesion disassembly through a number of mechanisms. A number of studies have demonstrated that microtubule tips can repeatedly target adhesions, at both the leading edge and the rear of a migrating cell, although the mechanisms governing these targeting events remain unclear (Broussard et al., 2007). This targeting drives adhesion disassembly through a combination of local Arg-mediated inhibition of Rho activity, and FAK- and dynamin-dependent endocytosis (Ezratty et al., 2005; Miller et al., 2004).

fluorescence speckle microscopy (FSM). This technique is used to analyse fluorescently tagged proteins that are expressed at very low levels in live cells. Such fluorescently labelled proteins appear as speckles and can be detected with a combination of a wide-field (or TIRF) microscope and a CCD. Visualisation of these speckles enables the monitoring of the movement, turnover or flow of tagged protein groups within cellular structures. Although it is relatively simple to acquire data with sufficiently sensitive detectors, the caveat of this technique lies in the analysis of the resulting data. This requires the use of complex mathematical modelling methods and FSM is thus currently not as widely used as other methods described here (Danuser and Waterman-Storer, 2006). Initially, this technique was used to study actin dynamics within newly formed lamellipodia in migrating cells (Waterman-Storer et al., 1998), but more recently it has also been used for investigating adhesion proteins (Hu et al., 2007). This later study revealed that integrins move more slowly in adhesions (~0.1 μm/minute) than other focal adhesion proteins, such as FAK (0.15 μm/minute), talin (0.25 μm/minute) and α-actinin (0.27 μm/minute). These data can then be combined with data showing the kinetics of adhesion-associated actin, thus permitting the determination of correlations between protein movement and actin-binding capabilities. This hierarchical control of motion within these groups of proteins relative to actin has shed considerable light on the functional relevance of the numerous actin-binding and associated proteins within focal adhesions (Hu et al., 2007).

Fluorescence correlation spectroscopy

More than 150 different proteins have been shown to be recruited to adhesions (Zaidel-Bar et al., 2007) and there has been increasing interest in investigating and quantifying protein–protein interactions at these sites. One method to study protein–protein interactions is fluorescence correlation spectroscopy (FCS), which allows measurement of the concentration of fluorescently tagged proteins at developing adhesions. FCS requires the continuous laser excitation and detection of a small region of a cell over a large number of time intervals. Post-acquisition software is then used to determine diffusion rates and aggregation and flow velocities based on intensity correlation over time. Two different fluorophores can be used in these cross-correlation analysis experiments, which then makes it possible to calculate the relative rates of protein transport, association or formation of adhesion complexes (Brown et al., 2006). One study used FCS to elegantly define detailed protein velocity maps for actin and the adhesion-related proteins α-actinin, α5-integrin, talin, paxillin, vinculin and FAK (Brown et al., 2006). The data showed differences in the stability of the linkage between integrin and actin among different cell types and on the same cell type grown on different ECM densities. From these data, the authors proposed two potential key points of linkage (or 'clutch' sites): one at the level of the integrin and the other at the level of α-actinin or actin. These data suggest that the strength of association between cell and ECM increases as actin and adhesions become more organized, showing the importance of studying the hierarchical organization of factors that regulate adhesion signalling and dynamics.

Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) is a technique that allows detection of fluorescent molecules with overlapping spectra that are in very close proximity, thus inferring 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 with the shorter wavelength, comes into close proximity with the acceptor protein (typically <9 nm), a non-radiative resonance energy transfer takes place from the donor to the acceptor fluorophore, leading to three measurable outputs. The intensity of acceptor increases, whereas that of the donor decreases and is coupled with a reduction in donor fluorescence lifetime. Any of these outputs can be used to obtain information regarding interactions between proteins of interest.

There are a number of methods to perform and measure FRET. The most commonly used involve the transfection of proteins that are fluorescently tagged with, for example, CFP as the donor and YFP as the acceptor, because these fluorophores can be imaged on a wide-field or confocal microscope (see Box 3 for further discussion of fluorophores). By calculating the ratio of intensity of both fluorophores in each pixel across each image, FRET can be measured, assuming that the levels at which each tagged construct is expressed are the same, thereby resulting in equal intensities. This is commonly known as ratiometric FRET. 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 those induced by interactions with effector binding partners, that lead to induction or loss of FRET between the donor and acceptor fluorescent proteins. This approach was originally used to study the Rho family of small GTPases. It made use of intramolecular FRET biosensors encoding either one or two domains of specific GTPases known to be important for interaction with their target molecules to dissect their localised activation following certain biological stimuli (Pertz and Hahn, 2004). More recently, FRET experiments have been used to investigate the simultaneous activation of multiple GTPases in a cell undergoing membrane protrusion (Machacek et al., 2009).

An alternative to ratiometric FRET measurements is photobleaching of either the donor or acceptor within a specific ROI, followed by measuring the change in intensity of the other fluorophore within the same region. This can be performed on intra- or inter-molecular FRET sensors, with the latter containing proteins or protein domains coupled to fluorophores on separate plasmids. Of the two, acceptor photobleaching is generally preferred as it results in an increase in donor intensity when FRET occurs, which is an easier readout to measure than the loss of acceptor intensity upon donor photobleaching. The nature of the acceptor photobleaching approach means it is best suited to analysing either a single time point 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 technical), calibrations and corrections for bleaching to avoid false positives. Additional problems that might arise include bleed through from one fluorophore to another and a poor signal-to-noise ratio, thus giving skewed results [for more details, see Berney and Danuser (Berney and Danuser, 2003)]. A recent example of the use of both ratiometric and acceptor photobleaching FRET in adhesion biology was the development of a FRET-based sensor to study conformational changes in the FERM domain of FAK. 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 might not permit FRET. 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, that is, between the FERM and kinase domains, and the YFP tag on the N terminus. The sensor was shown to behave as a 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 (Papusheva et al., 2009).

An alternative way to measure FRET is by fluorescence lifetime imaging microscopy (FLIM). FLIM uses specialised detectors to measure the inherent fluorescence lifetime of the donor molecule (see Box 3) and can be performed using either one- or two-photon illumination sources, depending on the type of FLIM. A reduction in the normal fluorescence lifetime of the donor (typically 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 ROI for statistical analysis. FLIM has a number of benefits over intensity-based approaches, as it is independent of fluorophore concentration and highly sensitive; it is thus able to offer significantly better spatial resolution and improved signal-to-noise ratios (Levitt et al., 2009). However, FLIM requires more specialised (often expensive) acquisition equipment and often highly complex data interpretation, which means that this technique is still much less widely used in FRET studies than intensity-based methods. FLIM has been used to study the interaction between β1 integrin and talin following receptor activation at adhesions upon treatment of cells with various ECM ligands or small-molecule compounds targeting specific integrins

Box 3. Fluorescence and fluorescent probes

As discussed in this Commentary, many different techniques can be used to image adhesion structures. Each technique offers its own advantages and provides a different type of data for the structure or protein of interest. Most techniques involve fluorescent tagging of the protein(s). Fluorescence is the absorption of one photon at one energy or wavelength and re-emission of one or more photons at lower energies or longer wavelengths. Several strategies are available for the labelling of proteins for microscopic analysis; a widely used method is the incorporation of genetically encoded fluorescent protein (FP) tags, such as green fluorescent protein (GFP), into an expression vector or transgenic animal. More recently, spectral variants of GFP have been developed, such as mRFP (red), YFP (yellow) and CFP (cyan) (Shaner et al., 2007). Imaging of fluorescently tagged proteins requires excitation at a specific wavelength, which then leads to emission of light at a higher wavelength. For example, GFP is optimally excited at a wavelength of 488 nm and emits at 514 nm. Fluorescence lifetime refers to the average time the molecule remains in the excited state before emitting a photon. For commonly used fluorescent proteins (that emit photons with energies from the UV to near IR), typical excited-state decay times are within the range 0.5–20 ns. DNA constructs encoding fluorescently tagged proteins can be transfected into the cells of interest and proteins then visualised by microscopy, using a technique appropriate for the nature of the experiment (see main text for details). However, care should be taken when interpreting data obtained from cells in which adhesion or cytoskeletal components are overexpressed, as overexpression can markedly alter the balance of molecules in these complex networks and can result in the generation of artefacts.

(Parsons et al., 2008). Data revealed that activation of integrins with ECM proteins or ligand mimetic compounds both resulted in localised induction of association between integrin and direct binding partners such as talin. A more recent study used FLIM to define a molecular pathway leading to transdominant inhibition of β1 integrins by αvβ3 integrin (Worth et al., 2010). Another recent study used the combination of ratiometric and FLIM analysis of FRET to demonstrate that vinculin acts as a mechanosensor at focal adhesions. A novel vinculin tension biosensor was used to show high tension across vinculin in newly forming adhesions at the front of migratory cells (Grashoff et al., 2010). These studies highlighted 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.

Traction force microscopy

There has been considerable recent interest in defining the role of cell traction forces in generating mechanical signals to drive adhesion dynamics. Attachment of cells to ECM proteins or to neighbouring cells leads to the generation of internal forces through cooperative force generation by actin and myosins; this, in turn, can result in cellular-dependent deformation of the surrounding environment (Wang et al., 2009). One method that has been employed to study this phenomenon in live cells is traction force microscopy (TFM). This method makes use of an acrylamide and/or collagen gel containing fluorescent microbeads. Cells are plated on top of these gels, and phase-contrast and fluorescence images are acquired to mark the position of the cells and the beads, respectively. In earlier TFM studies, cells were then trypsinised and removed from the gel to allow an image of the same site to be taken, thus providing a 'no force' map of the surface. The displacement of the beads, as seen in the differences between these images, can then be analysed using a number of complex algorithms depending on the nature of the gel used (Wang and Lin, 2007). More recently, similar approaches have been applied to both traction force and adhesion dynamics in live cells undergoing migration. One such study reported a method to dissect tractional forces exerted in *x*, *y* and *z* planes in live migrating fibroblasts (Maskarinec et al., 2009). Another study combined the use of FSM and TFM to demonstrate that F-actin speed is inversely related to traction stress near the cell edge, where adhesions are formed and F-actin motion is rapid. By contrast, larger adhesions beneath the cell body, where the F-actin speed is low, show a direct correlation between F-actin speed and traction stress (Gardel et al., 2008). Thus, TFM offers a way to analyse both adhesion protein dynamics and local ECM deformation by intact cells.

Visualising adhesions in whole organisms

Imaging adhesion dynamics in an intact organism is extremely challenging. The high resolution required for imaging small structures such as an adhesion is, at present, not achievable for mammals. Indeed, much controversy remains as to whether the adhesion types and classes that have been so carefully defined in isolated cells in vitro bear any resemblance to those found in the same cells within animals. Currently, the majority of in vivo adhesion and migration studies within mammals focus on following single cells or groups of cells that have been fluorescently labelled or genetically manipulated (Sahai, 2007; Sahai et al., 2005; Wolf et al., 2007). One benefit of applying multiphoton microscopy to these studies is the ability to simultaneously and non-invasively image the structure of collagen surrounding the cells by second harmonic generation (SHG) (Campagnola et al., 2001). SHG occurs as a by-product of the imaging laser light passing through the highly polarisable, ordered collagen fibrils and results in emerging light that has a wavelength proportional to the square of the incident light. Thus, SHG images can be collected on a separate detector and overlaid with fluorescent images to visualise interactions between cells and matrix in vivo (Sahai et al., 2005; Wolf et al., 2007). Aside from mammals, other studies of migration and adhesion in vivo have been performed in live *Drosophila*, *Xenopus* embryos and zebrafish, with the last being ideal for fluorescence studies as they are essentially transparent. As these organisms are thinner and smaller than mammals and also relatively easy to manipulate, better resolution can be achieved with spinning-disc or conventional confocal microscopy. Recent studies of border and immune cells in *Drosophila*, and neurons in zebrafish have revealed exciting new information regarding cell migration and adhesion within intact organisms and the signalling mechanisms responsible for driving these events (Bianco et al., 2007; Koster and Fraser, 2001; Prasad and Montell, 2007; Robles and Gomez, 2006; Stramer et al., 2005).

The dynamics of membrane protrusion formation have recently been analysed in detail in live migrating neutrophils of zebrafish embryos. This study made use of fluorescent phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] and phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] lipids and GTPase reporters to demonstrate that PtdIns(3,4,5)P_3 lipid signalling is activated at the leading edge of migrating neutrophils, and that this activation is not coupled to activation of Rac at the leading edge (Yoo et al., 2010). Although this study did not directly measure adhesion dynamics, it will probably pave the way for future applications to study adhesion protein behaviour and protrusion dynamics in vivo. A more direct study of the dynamics of adhesions in vivo came from another recent report that investigated the dynamics of proteins within the more stable adhesive myotendinous junctions of *Drosophila* embryos and larvae (Yuan et al., 2010). Using FRAP, integrins within these structures were shown to be dynamic, despite the stable nature of the adhesion, and regulated through clathrin-dependent endocytosis. This study highlights the importance of protein dynamics within both transient and long-term adhesive structures, as turnover of components is important for the correct function of both adhesion types. In addition to their role in muscle junctions, the dynamic behaviour of cadherins has also been studied in epithelial cells in *Drosophila* embryos. Both FRAP and photoactivation techniques were employed to study the dynamics of both E-cadherin and actin at adherens junctions (Cavey et al., 2008). The authors show that there are two populations of actin at cell junctions that in turn control E-cadherin stability and dimerisation. The homophilic interaction of E-cadherin on adjacent cells was found in stable actin patches regulated by α-catenin and was not required for junction stability. By contrast, highly dynamic contractile actin networks were responsible for tethering cadherin molecules and restricting lateral movement at junctions. The dynamics of E-cadherin complexes have also recently been demonstrated in vivo in mouse models using similar technical approaches. This study showed that E-cadherin underwent rapid recovery at cell junctions within solid tumours at rates that are three times greater than those seen in vitro (Serrels et al., 2009). This dynamic recovery was associated with increased migration rates of cells at the edge of tumours, and could be suppressed in vivo using the Src inhibitor dasatinib (Serrels et al., 2009). These

studies of cell–cell adhesion protein dynamics highlight the potential of similar analysis in investigating cell–matrix adhesions in vivo in the future.

A number of studies have used mouse knockouts or disease models to analyse the importance of adhesion in various pathological settings. For example, a number of studies have used live intravital imaging to follow the fate of tumour cells, and stromal or inflammatory cell populations in mouse xenograft or inflammation models (Deane and Hickey, 2009; Sahai, 2007). Although these studies did not aim to directly analyse adhesion dynamics, the use of genetic knockouts or RNA interference has made it possible to partially dissect the involvement of adhesion proteins in cell growth, adhesion and migration. Ongoing improvements in the resolution of intravital imaging methods might eventually allow the imaging of the dynamics of specific adhesion-associated proteins in single cells.

Conclusions and future perspectives

With a range of techniques currently being developed and used successfully for studying cell–matrix adhesions within an in vitro setting, naturally, the next step in imaging is towards in vivo analysis of adhesion formation. The past decade has seen an explosion of new techniques for the analysis of protein function, dynamics, localisation and interactions at sites of cell adhesion. With both microscopy companies and biologists alike now investing considerable time and effort into developing new imaging systems, the next decade also looks set to bring new and exciting technology to the adhesion and migration field. The expansion of data- and software-sharing sites is also providing invaluable resources for laboratories that are starting out in the field and seeking technical advice. The current challenges largely concern the optimisation of existing techniques to improve both spatial and temporal resolution to allow further delineation of the fast kinetics of adhesion proteins in live cells. Exciting progress is being made in the development of commercially available super-resolution microscopy techniques, such as interferometric photoactivated localization microscopy (iPALM), stimulated emission depletion (STED) microscopy, structured illumination microscopy (SIM) and stochastic optical reconstruction microscopy (STORM). These techniques go beyond the diffraction limit of resolution and, as such, can be used to analyse localisation of proteins within structures with precise detail to the 10 nm scale. Moreover, designing systems to combine multiple techniques will allow the sequential or parallel analysis of a number of parameters, thus making it possible to directly relate the formation of a single adhesion to the kinetics of the local protein population. 3D imaging and in vivo microscopy are also areas of great interest currently, and it is likely that the next major advances in this field will result in high-resolution imaging of adhesions during live cell adhesion in complex 3D matrices and within whole organisms.

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