Dissecting cell adhesion architecture using advanced imaging techniques

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Key words: adhesion, migration, microscopy, dynamics, cytoskeleton, photobleaching, super-resolution imaging, fluorescence

Abbreviations: AFM, atomic force microscopy; AJ, adherens junctions; ECM, extracellular matrix; EM, electron microscopy; ET, electron tomography; FA, focal adhesions; FB, fibrillar adhesions; FC, focal complexes; FLAP, fluorescence localization after photobleaching; FP, fluorescent protein; FRAP, fluorescence recovery after photobleaching; FRET, forster resonance energy transfer; GFP, green fluorescent protein; IRM, interference reflection microscopy; IR-SPR, infrared-surface plasmon resonance; MMP, matrix metalloprotease; mTFP1, monomeric teal fluorescent protein 1; PALM, photoactivated localization microscopy; ROCK, rho-kinase; SNOM, near-field scanning optical microscopy; SIM, structured illumination microscopy; SF, stress fiber; STED, stimulated emission depletion; STORM, stochastic optical reconstruction microscopy; TIRF, total internal reflection fluorescence; TJ, tight junctions

Cell adhesion to extracellular matrix proteins or to other cells is essential for the control of embryonic development, tissue integrity, immune function and wound healing. Adhesions are tightly spatially regulated structures containing over one hundred different proteins that coordinate both dynamics and signaling events at these sites. Extensive biochemical and morphological analysis of adhesion types over the past three decades has greatly improved understanding of individual protein contributions to adhesion signaling and, in some cases, dynamics. However, it is becoming increasingly clear that these diverse macromolecular complexes contain a variety of protein sub-networks, as well as distinct sub-domains that likely play important roles in regulating adhesion behavior. Until recently, resolving these structures, which are often less than a micron in size, was hampered by the limitations of conventional light microscopy. However, recent advances in optical techniques and imaging methods have revealed exciting insight into the intricate control of adhesion structure and assembly. Here we provide an overview of the recent data arising from such studies of cell:matrix and cell:cell contact and an overview of the imaging strategies that have been applied to study the intricacies and hierarchy of proteins within adhesions.

Introduction

Cell adhesion to other cells and/or to the extracellular matrix (ECM) is a fundamental requirement for normal embryonic development, adult homeostasis and immune function (reviewed in refs. 1 and 2). The cellular structures that mediate interactions with the ECM can take a number of different forms depending upon both the cell type and the tissue environment (see Fig. 1 for overview of adhesion types). The protein composition, localization and proteolytic capabilities of these adhesion complexes all contribute to the classification and function of the structure. Cell:cell adhesion classically plays a role in the stability and integrity of both epithelial and endothelial cell layers. While the structure and components of cell:cell adhesive contacts play different roles to cell:ECM adhesions, both share a large number of common signaling mediators that are responsible for regulating formation, maintenance and dynamics.

Cell adhesion is required for normal development in many different tissues, in the context of formation of specific tissue compartments, maintenance of barrier function and cell migration. In many cases, these adhesive structures are not static but rather they undergo dynamic changes in composition and structure to enable the cells to respond to changing extracellular cues. The regulation of such dynamic changes is under tight spatial and temporal control by numerous signaling proteins that can dictate the type, location and duration of adhesive contact. Recent progress in microscopy techniques has enabled closer observation and dissection of these fundamental events.

Early studies performed on rigid 2D substrates demonstrated different types of ECM adhesive structures exist in single cells at any one time.3 Three classical structures initially described in these studies were Focal Complexes (FC), Focal Adhesions (FA) and Fibrillar Adhesions (FB), each having their own specific characteristics.4–6 FCs are small, transient structures, typically located behind the leading edge of a spreading or migrating cell and are thought to sample the local ECM before disassembling (within minutes) or maturing to form FAs. FAs contain a number of key proteins that are required for stability of the adhesion and transmission of traction forces from the ECM and vice-versa and as such these adhesions have longer lifetimes in the order of tens of minutes. FBs are long, stable structures that run parallel to bundles of fibronectin in vivo and in vitro, and are highly enriched in tensin and α5β1 integrin.7

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Submitted: 04/04/11; Accepted: 06/13/11
DOI: 10.4161/cam.5.4.16915
Hemidesmosomes represent a further class of cell:matrix adhesion structure and are found in specialized epithelial cells such as keratinocytes. These are small, integrin-based adhesions forming river-like plaques that maintain structural links between the keratin intermediate filament and the underlying basement membrane zone. Typically, these structures comprise of plectin proteins that associate directly with the intermediate filaments and form a bridge between β4 integrin and the cytoskeleton to permit firm anchorage to the underlying ECM. Two other classes of cell:ECM adhesion structures, podosomes and invadopodia differ further in their ability to act as local ECM degradation sites by recruiting matrix metalloproteinases (MMPs). Podosomes typically appear in cells of monocytic origin, such as macrophages or osteoclasts, whereas invadopodia appear in malignant cells.

In addition to forming adhesions with the surrounding ECM, many cell types also form specialized adhesions with neighboring cells. Cell-cell adhesion is key to the formation of intact epithelial and endothelial cell layers in vivo, and conveys critical mechanical stability and polarity for assembly of cells within complex 3-dimensional tissue architecture. Epithelial sheets contain an apical membrane that faces the lumen or is the free surface of the epithelium, and a basolateral surface that interacts with the neighboring cells and the basement membrane. This asymmetric organization is referred to as apical-basal polarity and is a characteristic trait of all epithelial cells. Cell-cell adhesions are mediated by different types of junctional complexes, including tight junctions (TJ), adherens junctions (AJ), gap junctions and desmosomes. These junctions comprise transmembrane proteins with extracellular domains that mediate interactions between neighboring cells and intracellular surfaces that facilitate interaction with signaling molecules and cytoskeletal proteins. In polarized epithelial cells, the junctional complexes are asymmetrically localized. For example, TJ are located at the apical-basal border and act to separate the apical and basolateral membrane domains, hold adjacent cells together and create an impermeant fluid barrier between cells. AJ are located basal to the TJ and are considered as primary determinants of cell-cell adhesion. The mechanisms by which cells localize proteins to develop cell-cell junctions to create the intracellular asymmetry are currently poorly understood. Most of our understanding of these molecular mechanisms stems from genetic studies in model organisms and biochemical studies in cultured cells. However, the application of new imaging approaches to study these events in vivo is beginning to provide novel insight into regulation of cell adhesion within complex tissues and whole organisms.

An important aspect of both cell-matrix and cell-cell adhesions is that they modulate and are regulated by cell contractility. Cellular contractile forces are generated through myosin generated tension in the actin cytoskeleton and are essential for tissue morphogenesis, homeostasis and cell migration. Actin-myosin contractility modulates cell migration through focal adhesion assembly and stress fiber formation. Focal adhesions contain proteins such as vinculin and talin, which have mechanosensing capabilities and transmit force from the extracellular matrix to the cytoskeleton. Adherens junctions are also particularly sensitive to contractile forces. Actomyosin activity is required for maintenance of cell-cell contacts and e-cadherin, a component of adherens junctions, has been demonstrated to act as a mechanosensor at cell-cell contacts.

Another common feature of cell-matrix and cell-cell adhesions is that they both signal intracellularly. Focal adhesions are known to recruit many proteins though integrin engagement to create intracellular signaling platforms associated with clustered integrin cytoplasmic domains. These platforms contain adapter proteins, kinases phosphatases and other receptors. These signaling platforms are also connected to the cytoskeleton and signaling through these platforms can result in rapid changes to the cytoskeleton thereby affecting cell-shape and cell movement. Cadherin containing adhesion complexes also recruit and act through signaling molecules such as PI-3-kinase, Src family kinases, Rac and ROCK. Cadherins also recruit β-catenin to junctional complexes, release of β-catenin allows it to translocate to the nucleus and activate transcription (reviewed in ref. 20).

A common feature of all adhesions is the large number of proteins that must be coordinated in order to mediate downstream signaling and cell behavior. Dissecting out these complex and promiscuous interaction networks within these structures represents a major challenge to cell biologists. Here we provide an overview of some of the microscopy techniques (summarized in Table 1) that have been recently employed in the field to tackle these fundamental questions and how these methods can be used to better understand the detailed structures and dynamics of adhesions.

**The Molecular Architecture of Adhesions**

Much is now known about the components of adhesion complexes, but the details of how these components are assembled into macromolecular structures remains unclear. Recent studies have suggested that adhesions contain distinct micro- or sub-domains within them, which may coordinate the functions of specific protein complexes. Based on the classical resolution limitation of light microscopy as defined by Ernst Abbe (see below), proteins can be co-localized to adhesions but distinct sub-domains on the nanometer scale are impossible to define. Recently, a number of new techniques have been described which enabled these adhesion sub-domains to be better resolved. These have been collectively termed ‘super-resolution’ techniques as they break or bypass the optical limitations of standard microscopy methods.

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**Figure 1 (See opposite page).** The architecture of adhesions. (A) A representative cartoon of adhesion types and their location within an epithelial cell monolayer. These adhesions are also found in other cell types as discussed in the main text. Note that podosomes are not depicted on this diagram since they are not found in epithelial cells. (B) Representative cartoons showing the components and currently known architecture of the adhesion types discussed in this review. Adhesion components shown here have been demonstrated using various dynamic and super-resolution microscopy techniques.
Figure 1. For figure legend, see page 352.
Light microscopy is restricted by its optical resolution, which is limited to approximately half the wavelength of the light used (Abbe’s defraction limit). This limits observations to objects that are 200–350 nm apart. Since most biological events occur via macromolecular complexes that range in size from tens to a few hundred nanometers, these events are outside the range of conventional light microscopy. Historically, the only way to achieve resolution in the tens of nanometers range has been electron microscopy (EM). EM is limited in the same way as light microscopy; however, the much shorter wavelength of electrons allows sub-nanometer resolution to be achieved and can thereby be used to identify structures within micron-sized focal adhesions. One approach is to use Cryo-ET, which employs tomography to obtain a 3D reconstruction of a sample from tilted 2D images at cryogenic temperature. Cryo-ET can be used to obtain information about complex biological structures at subnanometer resolution. In one such study, Patla et al. used cryo-ET to visualize the architecture of the actin-cell membrane interface in 3D. This revealed the existence of doughnut-shaped particles (around 20–30 nm in diameter), which are localized below the actin network at the cell membrane interface. These particles are not uniformly distributed but grouped into ‘islands,’ link actin filaments to the membrane and are responsive to changes in contractility. This study shows in detail the existence of focal adhesion sub-structures with potentially important roles in mechanotransduction and demonstrates the value of the information it is possible to obtain using EM. However, EM has a number of limitations as it is expensive, time-consuming, can induce artifacts and the sample preparation process currently precludes its use for the study of live cells. Furthermore, the use of multiple labels is technically demanding. Nonetheless the high resolution that is possible to obtain using EM makes it a very valuable tool in the study of adhesions in fixed specimens.

Traditional light microscopy cannot compete with EM for resolution. However, a new generation of super-resolution light microscopy techniques has been developed that aims to provide resolution down to tens of nanometers while preserving the architecture of the cell and potentially allowing high resolution imaging in live cells. These super-resolution microscopy techniques employ a variety of approaches to spatially restrict either the excitation or emission of a fluorophore in order to improve resolution. Near-field scanning optical microscopy (SNOM) is a near-field super-resolution technique that utilizes an evanescent wave and a very small physical aperture. This aperture limits the evanescent wave (and therefore excitation of fluorophores) laterally as well as axially. This bypasses Abbe’s defraction limit in all three dimensions to bring the resolution down to around 20 nm in x, y and z. In addition to this SNOM has the added benefit of providing topographical information of the sample. The limitation of this technique is that only surface structures can be visualized, but a recent study used SNOM to study E-cadherin localization to filopodia during adherens junction formation demonstrating the potential power of this method in the study of adhesions proteins. The drawback of SNOM and other near-field techniques do not suffer from this limitation and several wide-field super-resolution techniques have been developed recently and used to study both cell-matrix and cell-cell adhesions. Techniques

| Table 1. An overview of the imaging techniques described in this review |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | Near field      | Wide-field      |                |                |                |                |
| **Principle**  | Evanescent wave limited using a glass coverslip | Evanescent wave limited using a small physical aperture | Photoactivating to photoswitching to localize single molecules | Emission depletion beam to shape the PSF | Moire effect with structured illumination |
| **Resolvable volumes** | x, y | 200–300 nm | 50–100 nm | 20–50 nm | 30 nm (20 nm for iPALM) | 60–70 nm | 100–130 nm |
|                | z             | 100 nm         | 10 nm         | 100 nm (20–30 nm for 3D storm) | 140 nm (10–15 nm for iPALM) | 500–700 nm | 250–350 nm |
| **Simultaneous colours possible** | 3 | 2 | 2 | 2 | 3 | 3 |
| **Examples of recent use in adhesion biology** | Dynamics of p130Cas localization to focal adhesions | Localization of E-cadherin to filopodia during epithelial junction formation | Dynamics of paxillin during focal contact assembly/disassembly | Visualization of striated VE-cadherin in endothelial junctions | Visualization of striated vinculin interspersing actin filaments in podosomes |

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such as photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) use photoactivatable or photoswitchable fluorophores respectively to limit the number of fluorophores emitting simultaneously. These techniques typically operate by photoactivating or photoconverting a very small fraction of fluorophores using a brief pulse of UV or violet light to render that fraction fluorescent. The activated molecules are imaged and localized to their coordinates before removal from the population by photobleaching. In doing so PALM/STORM builds up an image by determining the position of a single fluorophore, thereby bypassing the need to resolve two closely spaced fluorophores and therefore the resolution limits described by Abbe’s law. This process is repeated many thousands of times to build up a composite image of all the single molecule coordinates. PALM was recently used in conjunction with interferometry (interferometric or iPALM) to image focal adhesions in 3D and show for the first time that focal adhesions are vertically stratified into layers of proteins with specific functions. Importantly this study highlighted the role of talin in organizing this stratification by straddling different layers. Due to the time-intensive nature of such image acquisitions very few studies have used PALM to study dynamics of adhesion proteins. However, one such study applied PALM to analyze nanoscale dynamics of paxillin during assembly/disassembly of focal contacts. Previous PALM experiments on live cells assembled the fluorophore position data into a single PALM composite image. However, in this study the position data was assembled into several equal time interval subgroups, which then formed individual frames of a time-lapse movie. In this way the authors were able to achieve a frame rate of 25–60 sec, which may be too long to accurately visualize localization of more dynamic nanoscale events.

By contrast to PALM, stimulated emission depletion (STED) is a technique that controls de-excitation of previously excited fluorophores. STED utilizes a doughnut-shaped stimulated emission beam that de-excites fluorophores outside of the central region of the beam. Fluorescence from this central region is detected to produce images which can currently achieve resolution in the range of 30–80 nm. STED has recently enabled closer analysis of VE-cadherin in the junctions of endothelial cells and has revealed that these structures are not continuous as previously thought, but rather contain discrete puncta and clusters, data that has a wider impact on understanding how these adhesions are regulated. Using STED for co-localization experiments requires the use of different excitation and depletion beams for each dye. This introduces the possibility of drift between beams, particularly in live cells. One group has recently described a method using two dyes that can be excited and depleted using the same beam but that have separable fluorescence signals due to their different fluorescence lifetimes. This also permits addition of a third dye that is spectrally separated (and therefore requires a second pair of excitation and depletion beams) to image three dyes simultaneously using STED. This development will expand the potential applications of STED in adhesion biology and allow localization of multiple proteins to be defined within these structures. Acquisition times for STED images are much shorter than those for PALM (a few seconds per image). Therefore despite a reduction in lateral resolution STED is currently more useful for live imaging of dynamic processes than PALM.

Structured Illumination Microscopy (SIM) is a further technique that enables around a two-fold increase in lateral resolution beyond the defraction limit. SIM requires illumination of the sample with light that is projected through a patterned grating. The coarse interference patterns arising from the emission distribution (known as Moiré fringes) are generated from a range of grid orientations and are then mathematically processed to produce a high-resolution image. The resolution can be further enhanced using 3D-SIM by including additional excitation light modulation to reconstruct the z-axis. One benefit of SIM is that standard fluorophores or dye conjugates can be used for imaging, provided they are stable enough to withstand the repetitive illumination cycles required to gather the data for analysis. A recent study that used SIM to characterize podosome adhesion structures demonstrated that the characteristic vinculin ring is actually a series of linear streaks, which are arrayed so that they intersperse actin filaments radiating out of the dense actin core. These features are not visible using conventional light microscopy, and importantly this data suggests that vinculin may act to directly couple actin to integrin function in these structures. Furthermore, image acquisition rates of around 1 sec have been reported for commercial systems (N-SIM) making this a useful technique for studying protein dynamics in live cells despite the reduction in resolution when compared with STED, STORM and PALM.

Characterizing Adhesion Sub-Domains

The use of super-resolution microscopy has demonstrated that focal adhesions (and probably other adhesion types) are not uniform but instead are stratified and divided into sub-domains that may separate the functions of the adhesion. To elucidate the functions of these strata and sub-domains, the composition of adhesions in response to stimuli must be closely examined in a quantitative manner. Previous studies focusing on the molecular composition of adhesions have primarily used two or three component labeling of fixed cells or FP-tagged proteins in live cells. Various studies have shown heterogeneity in protein composition both between and within adhesion sub-sets, however the ability to investigate the compositions of adhesions at high spatial resolution would allow greater understanding of the roles of specific adhesion sub-complexes. Zamir et al. applied this concept to the study of focal adhesion assembly in fibroblasts and used multi-dimensional cluster analysis of 5-color images to identify typical ‘compositional signatures’ found in the adhesion sites of labeled cells. The authors then produced ‘signature maps’ where each pixel is colored according to its molecular components. This revealed molecular sub-domains in focal adhesions that are selectively modulated by Rho-kinase (ROCK) activity. This study has defined an effective approach for calculating and visualizing variations in composition of sub-cellular structures; however, 5-color imaging is currently limited to studies in fixed cells due to a lack of suitable genetically encodeable tags.
Force Transmission through Adhesions

Mechanical-sensing and mechano-transduction are essential for many cellular processes including tissue homeostasis, wound-healing and morphogenesis. Cells use a variety of strategies to respond to mechanical stimuli and both cell-matrix and cell-cell adhesions have been shown to be involved in mechanosensing and transducing (reviewed in ref. 30). Mechano-sensing requires molecules that can sense changes in extracellular or intracellular tension and transmit these changes to responsive elements within the cell. It has been known for some time that FAs transmit tension from the extracellular matrix to the cytoskeleton and that a number of adhesion proteins act as mechanotransducers including paxillin, talin and vinculin. There is also emerging evidence that formation and stability of cadherin containing cell-cell junctions are force-dependent suggesting common mechanotransduction mechanisms may exist between adhesion types. Myosin II, which controls actin-based cell contractility and rearrangement, is required for focal adhesion and adherens junction formation in response to changes in extracellular mechanical cues. However, the components of the junction complex responsible for this transduction remained poorly defined. The E-cadherin complex acts as one such mechanosensor in epithelial cells and a recent report showed that vinculin is recruited to sites of mechanical tension in epithelial cells where it potentiates E-cadherin-mediated mechanosensing and mediates mechanoregulation of cell-cell adhesion.

Although it is known that tension is transmitted via adhesions to the cytoskeleton little is known about the exact mechanisms involved. Further study of mechanical control of adhesions and mechanotransduction via adhesions requires the development of specific tools to analyze tension occurring within the junction. One such recent development is the use of a biosensor based on an elastic domain derived from spider silk inserted within the adhesion protein vinculin tagged at either end with mTFP1 and Venus. Measurement of changes in FRET between mTFP and Venus indicate changes in tension across vinculin. This biosensor is calibrated to measure forces across specific proteins in cells with piconewton sensitivity and the authors showed that tension across vinculin correlates with FA assembly and disassembly in a manner that is controlled separately to vinculin recruitment to focal adhesions. A similar FRET biosensor approach has also been recently used to study tension across the actin-binding adhesion protein β-actinin, but this time using a spectrin repeat as the mechanosensor element. The application of internal osmotic pressure within the cell resulted in increased cytoskeletal tension and increased force across β-actinin. These biosensors have potential to shed further light on tension across other specific molecules found at cell-matrix and cell-cell adhesions.

An alternative approach has been reported using a laser to bleach a periodic pattern into actin filaments allowing retraction of SFs to be followed upon changes in tension. Laser nano-surgery and AFM were applied to release or apply force to stress fibers and study the effects on the actin filaments themselves and on focal adhesion proteins. This study revealed recruitment of zyxin to F-actin stress fibers and focal adhesions in response to mechanical tension and corresponding release of zyxin when tension is disrupted. Zyxin is recruited to the interface between retracting SFs and the plasma membrane demonstrating a potential role for this protein in tension induced FA formation. Quantitative analysis of the mechanics of filament retraction is possible using this technique by tracking bleached striations through the filament during force application or release. Tension upon actin filaments is a general feature of mechano-transduction and therefore this technique could be used to study the role of a variety of adhesion proteins in force sensing and transduction.

The Study of Adhesion Dynamics

Adhesions are dynamic structures and the mechanisms regulating their assembly and disassembly remain poorly defined. Super-resolution imaging techniques are becoming very powerful tools for the study of cell adhesions, but in most cases the acquisition speed is a limitation that precludes the study of dynamics of adhesion proteins in live cells. While live super-resolution imaging will likely be in use in the near future, alternative strategies have been employed to permit these dynamic events to be monitored by light microscopy methods.

Photoactivation and photobleaching techniques have proven to be important tools for dissecting protein kinetics in adhesion biology. Fluorescence recovery or localization after photobleaching (FRAP and FLAP respectively) in which fluorophores can be selectively ‘turned on’ or ‘off’ allows the user to track a subset of the fluorescently tagged population. These are not super-resolution techniques but have been particularly useful in the study of the dynamics of focal adhesions components during assembly and disassembly, and can be combined with other optical techniques to make them more powerful. ‘Classical’ FRAP studies have provided valuable insight in recent years into the dynamic control of various focal adhesion components. Studies have used FRAP to quantify molecular binding kinetics of FA proteins such as vinculin, talin, β3 integrin and α-actinin within focal adhesions and to demonstrate the complexity of their dynamics and interactions. FRAP studies have also been used to look more closely at specific interactions within focal adhesions. One such study used vinculin deletion mutants to show the link between the active conformation and actin-binding capabilities of vinculin and its increased stability at focal adhesions. A further study analyzed talin domains is similar detail and revealed that helix bundles in the rod domain of talin cooperate to regulate targeting of this key integrin regulating molecule to focal adhesions. FRAP has been used extensively in the study of focal adhesion dynamics, but it can also be applied to the study of other cell adhesion types. For example, dynamics of β4 integrin-GFP at seemingly stable hemidesmosome adhesion sites was recently studied by FRAP in keratinocytes. Data revealed two populations of β4 integrins with distinct mobilities, potentially due to association with different classes of proteins within distinct hemidesmosome types. FRAP can also be used effectively to study protein dynamics within cell-cell junctions although the possibility of bleaching areas away from the junction can complicate data interpretation. A new approach combining
two-photon excitation with fast 3D fluorescence microscopy was recently used to measure E-cadherin dynamics in mature epithelial junctions by FRAP. In this study, the diffusion and turnover of E-cadherin-GFP at mature junctions was measured in order to clarify the contribution of membrane diffusion, endocytosis and exocytosis/recycling to E-cadherin dynamics at mature junctions. In contrast to previous reports which showed a large membrane diffusible E-cadherin fraction in immature junctions, this study revealed that diffusion instead accounts for only 10–20% of E-cadherin and mobility is mainly due to endocytosis/exocytosis exchange processes at these sites. A further study also employed FRAP to demonstrate a role for endocytosis, as well as lateral membrane dynamics in regulating E-cadherin stability in A431 tumor cells. In this case, the authors proposed a role for internalization, lateral membrane diffusion and integrin-dependent Src activation in retarding E-cadherin movement at junctions resulting in altered junctional strength. This study highlights the complex relationship between protein and adhesion stability and suggests that these two events are not always directly coupled. Other studies have successfully combining FRAP/FLAP with other techniques. A recent example of this was a study of the dynamics of p130Cas (a key adhesion and mechano-sensitive signaling protein) using a combination of TIRF and FRAP. In this study p130Cas was shown to localize to focal adhesions throughout their lifetime and rapidly exchange with its binding partners in a p130Cas phosphorylation-dependent manner.

A further challenge in the field is the study of adhesions in cells within 3-dimensional (3D) environments. Cell-matrix adhesions have been detected in 3D in fixed cells, however some controversy exists as to whether FA exist in 3D due to a lack of evidence in live cells. A recent report claimed that adhesions were undetectable in cells expressing GFP-vinculin and GFP-zyxin and embedded in 3D matrices. The authors concluded that if adhesions did occur in 3D, they must be smaller than 0.3 μm and last for less than 1 sec and therefore be undetectable using their experimental and acquisition set-up. However, overexpression of FP tagged adhesion proteins is known to produce high levels of cytoplasmic background, which can mask specific localization of these proteins to adhesions. Indeed, Kubow and Horwitz recently showed that reducing background fluorescence using a less efficient promoter enabled visualization of dynamic GFP-paxillin containing adhesions in cells within 3D collagen matrices. This study highlights the importance of careful interpretation of data from microscopy studies using overexpression, as well as the need for more detailed analysis of adhesion types and dynamics in cells in 3D.

Quantifying Adhesion Dynamics without Using Fluorescent Proteins

The use of FP tagged proteins to study adhesion dynamics is now commonplace. However tagging or overexpressing proteins can affect their interactions and dynamics resulting in potential artifacts. Previous attempts to quantify adhesions dynamics have generally involved tracking and/or scoring of individual structures, which is time-consuming and laborious. Recent studies have described methods to obtain information about adhesion dynamics in an automated or semi-automated fashion without the need for fluorescent probes. One such example is an automated analysis method for quantifying adhesion dynamics in live cells using Interference Reflection Microscopy (IRM). IRM is the imaging of interference patterns created by the thin (a fraction of the wavelength of light) spaces between two surfaces, such as the space between a coverslip and a cell membrane. IRM can therefore be used to generate images of sites of close contact between a cell and the substrate with high contrast and definition. Additionally, the relatively short acquisition times (around 1 min or less) allow visualization of dynamic changes in these contact points. IRM was first described nearly 40 years ago but the recent advance using an automated image processing system to generate adhesion maps has allowed batch analysis of discrete changes in assembly, disassembly, size and sliding of adhesions. This method was applied both to the study of focal contacts in fibroblasts and podosomes in dendritic cells but could be applied to any sites of cell-substrate contact.

A further new method to study cell-cell and cell-substrate adhesion in a high throughput manner has recently been described that is based on infrared surface plasmon resonance (IR-SPR) spectroscopy. SPR utilizes surface electromagnetic waves that propagate in a direction parallel to a metal/vacuum interface. This wave is very sensitive to changes in the boundary between metal and the external medium, for example adherence of cells to a gold film surface. IR-SPR produces an evanescent wave that penetrates a few microns into a cell layer and propagates approximately 70 microns along the cell layer. SPR analysis can be used to follow the formation of initial cell contact with the matrix, cell spreading, formation of inter-cellular contacts and formation of a monolayer. IR-SPR is a label-free method that allows quantification of adhesion dynamics in large numbers of cells (more than 10^5 cells simultaneously) with high temporal resolution (here reported as 25 sec per spectra acquisition) and as such holds great promise as a technique for non-invasively screening for novel regulators of cell adhesion in future.

Recently AFM has also emerged as a useful tool to study the dynamics of adhesion formation in live cells. A recent report used a combination of AFM, correlative fluorescence microscopy and micro-patterned surfaces to directly quantify biophysical processes in podosomes in live cells; particularly dynamic changes in podosome height and stiffness. While this study did not correlate findings to the mechanosensing activity of podosomes, its seems likely that AFM studies in live cells will be a useful tool in the future for studying mechano-sensing and mechanotransduction in podosomes and other adhesions. An alternative approach was taken in another study to combine the use of fluorescence and Atomic Force Microscopy (AFM) to provide topographical analysis of focal adhesions marked with GFP-paxillin. As AFM relies on a scanning probe to physically map out the surface topography, fixed cells were ‘de-roofed’ prior to analysis. The combined AFM and fluorescence images revealed that, as might be predicted, paxillin is predominantly localized at the ventral surface of the adhesion, whereas F-actin is largely at the membrane-distal region.
Conclusions and Future Perspectives

Several novel imaging approaches to studying adhesion behavior and composition have recently emerged. Super-resolution techniques such as STORM, STED, SIM and iPALM allow imaging with resolution down to tens of nanometers. These techniques are immensely powerful and have already yielded significant advances in our understanding of adhesion architecture. Most super-resolution techniques are time-consuming and unsuitable for dynamic events—future improvements to speed of acquisition and analysis would make these techniques more powerful and allow the cell-biologist access to single-molecule analysis of dynamic adhesion events. Further development of systems that incorporate multiple techniques will permit analysis of several parameters in parallel and allow improved correlation between multiple aspects of adhesion. A significant advance in this direction had been the new Bessel beam plane illumination microscope developed by Planchon et al. This microscope uses scanned Bessel beams with a combination of structured illumination and/or 2-photon excitation to create light sheets of less than 0.5 μm. Planchon et al. have demonstrated 3D resolution as low as 0.3 μm with acquisition speeds of 200 image planes per second in live cells. Although this technique does not provide axial resolution in the range of super-resolution techniques such as STORM or STED, the extremely quick acquisition times and low cellular toxicity allow imaging of dynamic cellular events at sub-micron resolution.

Most of the studies using super-resolution microscopy have so far investigated molecular architecture of focal adhesions and other cell-matrix adhesion in 2D systems. These studies have revealed the existence of sub-domains within focal adhesions and hinted at the possibility of these sub-domains having discrete functions. So far cell-cell adhesions have not been shown to contain sub-domains or stratified layers. However since they, like focal adhesions, are involved in signaling and mechanotransduction in addition to their classical role in adhesion it seems likely that they may be organized into signaling, mechanotransduction and adhesion sub-domains. Combining siRNA screens with high-resolution imaging may also provide insight into specific regulators of adhesion sub-domain assembly or maintenance.

Acknowledgments

The authors would like to thank Gareth Jones (King’s College London) for advice and suggestions on the manuscript figures, and The Royal Society and National Institutes of Health Research Comprehensive Biomedical Centre for funding.

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