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Frontiers of microscopy-based research into cell–matrix adhesions

Ohad Medalia[1] and Benjamin Geiger[2]

Focal adhesions (FAs) are highly dynamic multi-protein complexes, through which cells interact with the extracellular matrix (ECM) via integrin receptors. These large assemblies, which typically measure several micrometers in diameter, mediate interactions of cells with external surfaces, and are linked at their cytoplasmic faces with F-actin bundles. Over the last four decades, the molecular diversity of these adhesions and their roles in cell migration and matrix sensing have been extensively studied. Microscopy-based research is considered critical for characterizing and understanding the nature of these assemblies. Here, we review the contributions of, advanced microscopy to the characterization of the functional architecture of integrin-mediated, cell–matrix adhesions.

Addresses
[1] Department of Life Sciences and the National Institute for Biotechnology in the Negev, Ben Gurion University of the Negev, Beer-Sheva 84120, Israel
[2] Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot 76100, Israel

Corresponding authors: Medalia, Ohad (omedalia@bgu.ac.il) and Geiger, Benjamin (benny.geiger@weizmann.ac.il)

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''Knowledge of structure is critical to an understanding of function'' (Dorothy Hodgkin)

This famous quote from Dorothy Hodgkin seems relevant not only to structural studies of macromolecules, but to a more general understanding of the mechanisms underlying the functioning of a wide range of biological systems, such as subcellular organelles, whole cells, tissues, and organisms. Her words are particularly relevant to systems such as the cell adhesion machinery, whose structure, in fact, constitutes its primary function.

Such 'structure-based', mechanistic research is founded on two major factors: firstly, the existence of a well-defined biological question, and secondly, the availability of essential 'enabling technologies'. The fundamental biological question to be discussed in this article concerns the mechanisms underlying cell adhesion, and the primary enabling technologies are light and electron microscopy.

Cell adhesion to the ECM, or to neighboring cells, plays a key role in the assembly of individual cells into coherent, functional tissue. At the same time, these adhesive interactions are critical to the generation of adhesion-mediated signals that regulate multiple cellular processes, including cell division, migration, differentiation and death. These two processes, the scaffolding and signaling, are closely interrelated. As discussed below, different types of cell adhesions contain prominent signaling molecules (e.g. focal adhesion kinases in integrin-mediated focal adhesions, or β-catenin in cadherin-mediated adherens junctions) that can locally affect the stability of the particular adhesion, as well as globally regulate the cell's overall behavior and fate. Given that the adhesion receptors are not themselves signaling enzymes, it appears likely that the local assembly of macromolecular complexes, associated with specific adhesion sites and regulating the precise positioning of the constituent scaffolding and signaling components, is critical to the activation of both scaffolding and signaling activities. Elucidation of the biological processes mediated by cell adhesion requires detailed structural characterization at the highest possible — ultimately, molecular — resolution. The dependence of adhesion research on advanced microscopy is, thus, self-evident.

To illustrate the contribution of advanced microscopy techniques to cell adhesion research, we will present an overview of the major milestones in this field during the past 40 or so years, and highlight the technological developments which enabled them, focusing on different 'eras' in cell adhesion research.

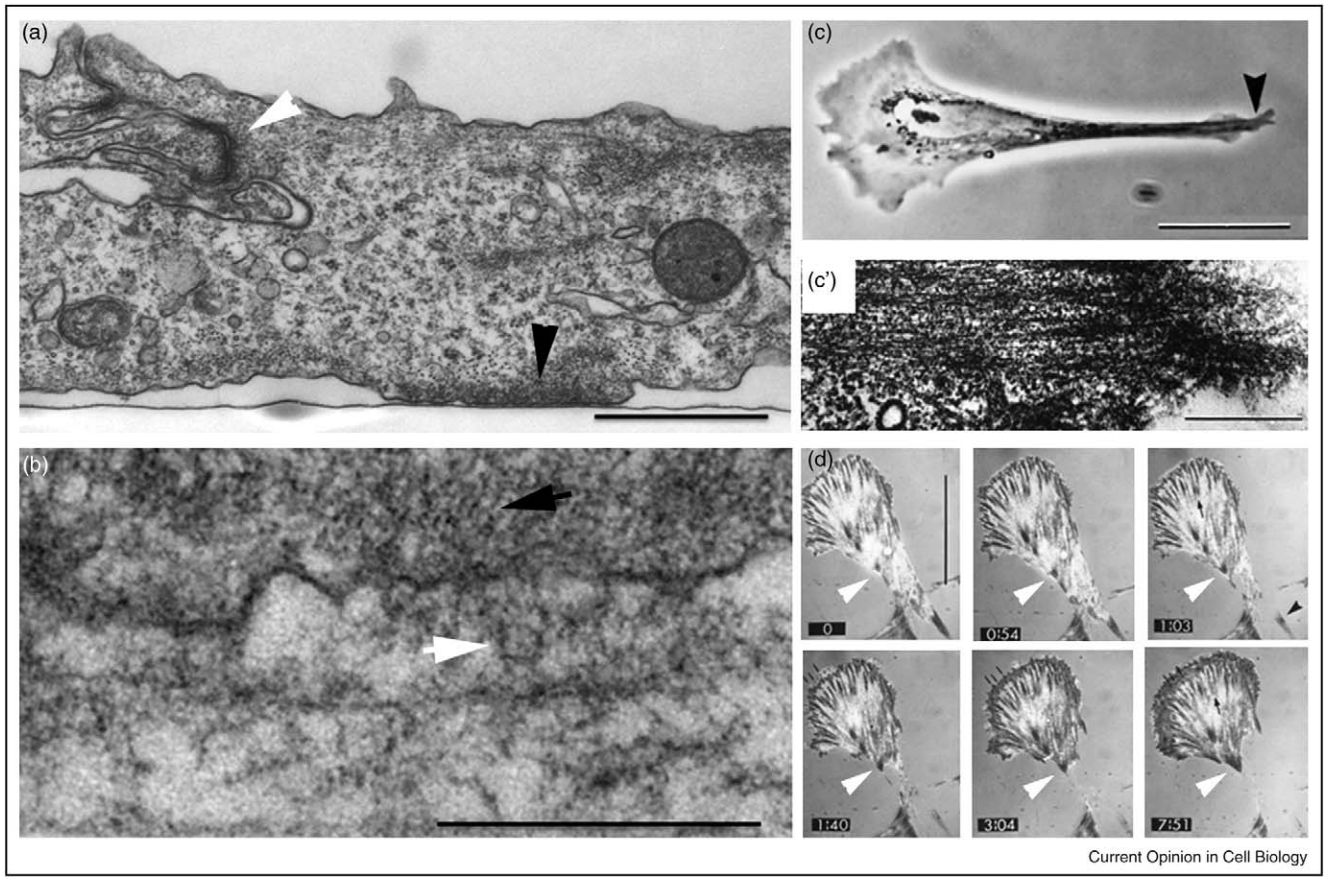
The morphology era

In the early 70s, Michael Abercrombie and coworkers published a seminal series of articles in which they presented a cyclic, three-stage model of cell migration, consisting of protrusion of the leading edge, formation of anterior matrix adhesions, and retraction of the cell, resulting in detachment of the trailing edge [1–4]. These studies were highly insightful, and despite scant knowledge of the underlying molecular mechanisms, and the nature of the associated cytoskeletal networks, highlighted the physical properties of these adhesions, and their functional significance. These early observations were, however, mostly morphologically based, and conducted with the state-of-the-art tools of the time,

including standard transmission electron microscopy (TEM), phase contrast light microscopy, and interference reflection microscopy (IRM). The typical results obtained using these approaches are outlined in Figure 1.

TEM images of chemically fixed, cultured cells revealed discrete areas [known as focal adhesions (FA) or focal contacts], typically elongated and several micrometers in length, in which the plasma membrane runs parallel to the overlying matrix, with a gap of 10–15 nm between the plasma membrane and the ECM (Figure 1a). Multiple cytoskeletal filaments (mostly microfilaments), closely associated with the FA cytoplasmic surfaces, were noted, though their anchorage in the FA membrane was poorly resolved. Tangential sections through FAs enabled a clearer view of the relationships between the cytoplasmic cytoskeleton and the external matrix (Figure 1b), showing that the two fibrillar systems are closely co-aligned, and suggesting a mechanical linkage between them [5]. Sections cut nearly parallel to the substrate along the trailing edge of migrating cells (e.g. the phase contrast image in Figure 1c, and the correlated TEM image in Figure 1c') demonstrate the convergence of cytoskeletal fibers toward an electron-dense area, most likely corresponding to a

Figure 1



Focal adhesion views of cultured fibroblasts, based on Epon-based transmission electron microscopy. Electron microscopy **(a, b, c, c′)** and interference reflection (IM) microscopy **(d)**. (a) Chicken lens cultured cells, growing on a flat surface, were processed for TEM and cut perpendicular to the plane of the substrate, revealing multiple cytoskeletal filaments accumulating at the adhesion site. The apparent gap between the ventral cell membrane and the underlying ECM (visualized as a dark serum line) is on the order of 10–15 nm. In (a), a cell–cell adherens junction (white arrowhead) and a focal adhesion (black arrowhead) are shown. Bar = 1 μm. (b) A focal adhesion formed by a pig aortic endothelial cell, cut tangentially to the plane of the substrate, showing the approach of microfilaments to the focal adhesion membrane, and the presence of ECM fibers on the other side of the membrane, which appear to be nearly co-aligned with the cytoskeletal fibers. F-actin and ECM are indicated by black and white arrows, respectively. Bar = 1 μm. (c) A migrating fibroblast, shown using phase-contract optics. Direction of migration: from right to left. A well-developed lamellipodium and lamellae (on the left) and a trailing tail, marked with an arrowhead, may be seen. Bar = 30 μm. The indicated area is shown in (c′), using a grazing thin Epon section through a cell. Notice the accumulation of microfilaments converging into a dense area, most likely corresponding to a FA (from the PhD thesis of Wen-Tien Chen). Bar = 0.5 μm. (d) Six frames from an interference reflection microscopy (IRM) movie are shown: FAs appear dark (white arrowhead), and the less tight close contacts, gray. Note the formation of small adhesions under the advancing lamellipodium (toward the upper left corner of the images), the rupture of the trailing edge between 0:54 and 1:03 (minutes:seconds), and the dramatic growth of a new trailing FA, within minutes after the rupture. Bar = 50 μm (from the PhD thesis of Wen-Tien Chen). Notice the lack of detail in all TEM images of specific structures bridging between the actin microfilaments and the membrane.

trailing focal adhesion. In addition to the microfilaments associated with this dense area, microtubules were also frequently seen.

An additional microscopy-based technology used in those early years for studying FA function and dynamics was IRM, which enables the monitoring, in live cells, of the proximity of the ventral membrane to the substrate, and the dynamic reorganization of FAs. These findings are illustrated in the frames from an IRM time-lapse movie (from the PhD thesis of Wen-Tien Chen, and [6] showing the prominence of small adhesions now commonly referred to as focal complexes), the dynamic changes in the position and size of FAs, the presence of gray regions, corresponding to less tight close contacts, the tendency of trailing FAs to rupture as the cell moves forward, and the subsequent growth of a new trailing FA, induced within minutes of the tail retraction.

Taken together, these early studies provided essential insights into the focal nature of matrix adhesions, and the critical role of these adhesions in regulating cell migration. Standard electron microscopy (EM) of that time suggested that cytoskeletal filaments interact with these matrix adhesions; the IRM movies revealed the proximity of the membrane to the matrix at these sites, as well as their dynamics. On the other hand, the molecular properties of FAs, and their ultrastructure, remained entirely unexplored.

The molecular era

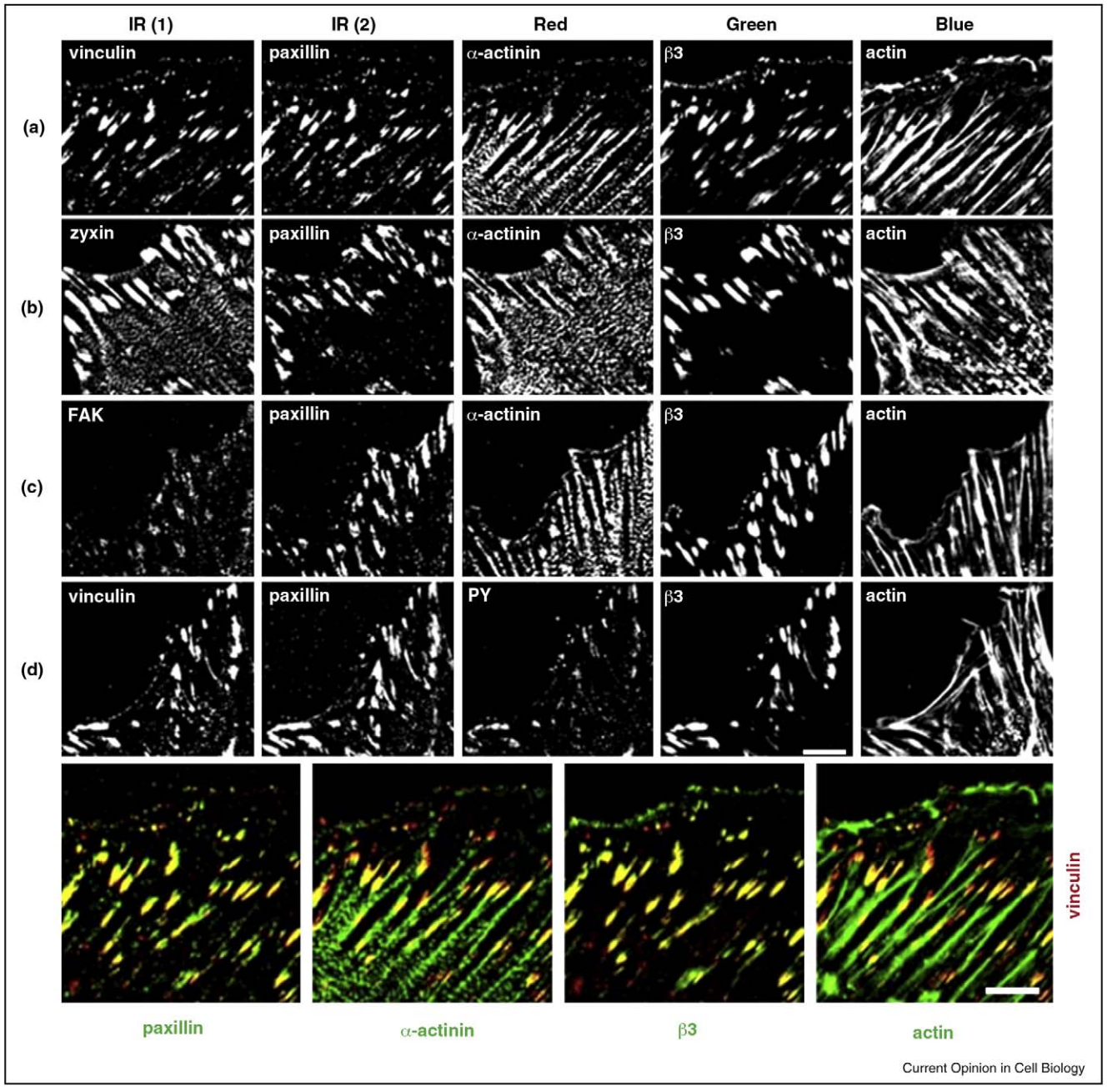
During the mid-to-late 70s, novel immunofluorescence approaches were developed, to elucidate the organization of cytoskeletal molecules such as actin, α-actinin, myosin II, tropomyosin, filamin and intermediate filament components, in non-muscle cells [7–13]. These discoveries provided the first look into the molecular complexity of the cytoskeleton, and the functional diversity of the emerging molecules (e.g. presence of actin crosslinkers as well as motor molecules) suggested that the precise spatial organization of the different components plays an essential role in their function. Early on in this exciting search for novel cytoskeletal proteins, previously unknown components of FAs were discovered and characterized. The first FA component identified using correlated immunofluorescence microscopy and IRM approaches was vinculin [14,15]; other proteins (e.g. talin, paxillin) soon followed [16–18]. Fluorescence microscopy, combined with microinjection of fluorescently tagged proteins, and fluorescence recovery after photobleaching (FRAP), enabled the monitoring of FA formation, reorganization and dynamics, and revealed key features in the complex assembly of matrix adhesions, and their association with the cytoskeleton [15,19]. These include the spatial and temporal relationships between different forms of integrin-based FA-related adhesions, including focal complexes, fibrillar adhesions and podosomes and the differential effect of mechanical perturbation on the distribution of specific FA-associated proteins (e.g. [20]). Furthermore, powerful new approaches for frozen section-based immunoelectron microscopy, by Tokuyasu, Singer and others, enabled the localization of novel components in cells and tissues, with optimized preservation of structure and antigenicity (e.g. [21,22]). This, for example, enabled the differential positioning of specific proteins that apparently co-localized, using immunofluorescence microscopy (e.g. α-actinin and vinculin) at distinct locations, relative to the plasma membrane, suggesting different roles for these proteins in the membrane anchorage of actin filaments.

The accumulation of information about FA composition led to interesting insights into the heterogeneity and corresponding molecular signatures of these cellular structures: namely, the presence of distinct sets of adhesion-associated proteins in different forms of integrin contacts (e.g. FAs, fibrillar adhesions, focal complexes) as well as in different FA structural domains. This is illustrated in Figure 2, where cultured cells, labeled simultaneously for several FA and cytoskeletal molecules (e.g. actin, vinculin, paxillin, α-actinin, integrin), displayed enormous heterogeneity. Using compositional hierarchical clustering approaches [21], this heterogeneity could be resolved into varying compositional signatures, characteristic of specific subdomains within FAs, or of different stages in FA assembly. Specifically, signatures that are enriched with paxillin and contain low levels of actin were located at the edge of focal adhesions. Another signature, containing high levels of zyxin together with paxillin, α-actinin, β3-integrin and actin, was found in focal adhesions, but apparently absent from focal complexes, the latter of which were dominated by another compositional signature with low levels of zyxin. The ultrastructural and functional manifestations of this spatial molecular heterogeneity however, remained unclear.

The molecular complexity era

From the late 70s to date, the molecular characterization of FAs continues to be a challenging endeavor. Attempts to obtain a comprehensive view of the integrin adhesome; namely, the collective of all known components of these structures (see Box 1), point to an extraordinary complexity. From the few tens of molecules assigned to FAs about 10 years ago (e.g. [23,24]), the current number of known adhesome components has reached about 180 ([25] and http://www.adhesome.org), and encompasses both scaffolding molecules, which provide the physical links inter-connecting actin filaments with the integrin receptors (including multiple actin regulators and adaptor proteins), and multiple signaling molecules (e.g. kinases, phosphatases, Rho GTPases, and their regulators), which are capable of modifying post-translationally a wide variety of cellular targets, including many focal adhesion molecules.

Figure 2



Molecular complexity of focal adhesions. To illustrate the complex molecular composition of FAs, the same rat embryo fibroblasts **(a–d)**, labeled for a different combination of FA and cytoskeletal components (altogether — 8 different molecules) are shown. Careful comparison of the images, including a computerized clustering analysis, was used to search for molecular signatures (see [20]). 'Actin' indicates F-actin. For simultaneous imaging, cells labeled with five fluorophores, including blue, green, red, and two infrared molecules, were used. 'b3' stands for b3 integrin. To clearly illustrate the spatial relationships between the different molecules, two-color pairs, labeled for vinculin (red) and four other partners (green), are presented at the bottom of the panel. Bar = 10 μm.

One interesting insight, based on *in silico* analysis of the adhesome, involved the presence in this interaction network, of specific motifs that are particularly enriched in this network. Such analysis revealed a few very prominent motifs in the adhesome network, the most conspicuous of which is a scaffolding motif, whereby a signaling molecule and its downstream target, are brought into close proximity by a third molecule [25]. Formation of such motifs during focal adhesion assembly are believed to trigger a wide range of adhesion-mediated signaling events.

Box 1 The integrin adhesome

The collective of molecules, identified over the years as components of integrin-mediated, cell–ECM adhesions, including proteins that are stably associated with FAs, and others that transiently interact with it and affect its function are known as the integrin adhesome [25]. The original adhesome inventory included 156 components, and this number continues to grow. The current version of the adhesome network [23] includes 180 components, including proteins discovered using RNA-interference and yeast two-hybrid screens, as well as localization, microscopy-based studies. The adhesome components are commonly divided into scaffolding molecules including integrins, adaptor proteins, actin binding proteins, and actin that form the physical structure of the adhesion site, and signaling molecules (protein kinases, phosphatases, GTPases and their regulators). Both types of molecules can form an overwhelming number of direct interactions, estimated to be well over 700. It is becoming increasingly apparent that, to accommodate the need for both robustness and dynamic plasticity at the adhesion site, most of these interactions need to be switchable, and subject to regulation, most likely by the FA-associated signaling machinery.

Moreover, analysis of the connectivity characteristics of the adhesome network sheds light on the scaffolding and signaling activities mediated by integrin adhesions [25], and on the mechanisms whereby molecular interactions between adhesome components can be switched on and off [23]. For example, there are several tyrosine-specific protein kinases in the adhesome, which appear to phosphorylate multiple FA-associated proteins, enabling them to interact with the Src homology domain 2 (SH2 domain) of other components. These interactions can also be switched off by specific phosphotyrosine phosphatases [23]. This view of the adhesome network places great functional importance on the characterization of multiprotein complexes that can be detected by combined light microscopy and high-resolution microscopy, enabling the revisiting of adhesion sites, identified by the fluorescence of specific reporter proteins, for a detailed structural analysis. This approach may be complemented by the application of advanced proteomic methods that address the specific interactions between individual, adhesion-related molecules and enable characterization of the mode of interactions between FA components. Yet, such a comprehensive approach for the molecular mapping of FAs strictly depends on techniques that preserve the three-dimensional ultrastructure of the adhesion sites.

Studying the adhesion machinery by cryo-electron microscopy

As indicated above, visualization of the FA substructure requires not only a high-resolution microscopy approach, but also a high degree of structural preservation. Although important information was provided by studies in which specimens for EM examination were prepared by traditional means, the dehydration stage may have resulted in the loss of delicate structures associated with the adhesion site. To demonstrate these effects, we present examples of dense plaques in chicken smooth muscle (the tissue equivalent of FAs) following processing for EM analysis, using: firstly, Epon-embedding plastic sections (Figure 3a); secondly, frozen cryo-sections, after which sections were mounted in methyl cellulose (the Tokuyasu method) (Figure 3b); as well as thirdly, frozen cryo-sections collected on grids, re-vitrified, and examined in the frozen-hydrated state (Figure 3c) [26]. A comparison of the resulting images demonstrates the unique advantages of working in a hydrated environment (Figure 3c): fine structural details within the high-density regions associated with the submembrane plaque of the adhesion sites could only be visualized in the hydrated cryo-sample, suggesting that the dense adhesion plaque is composed of discrete molecular assemblies.

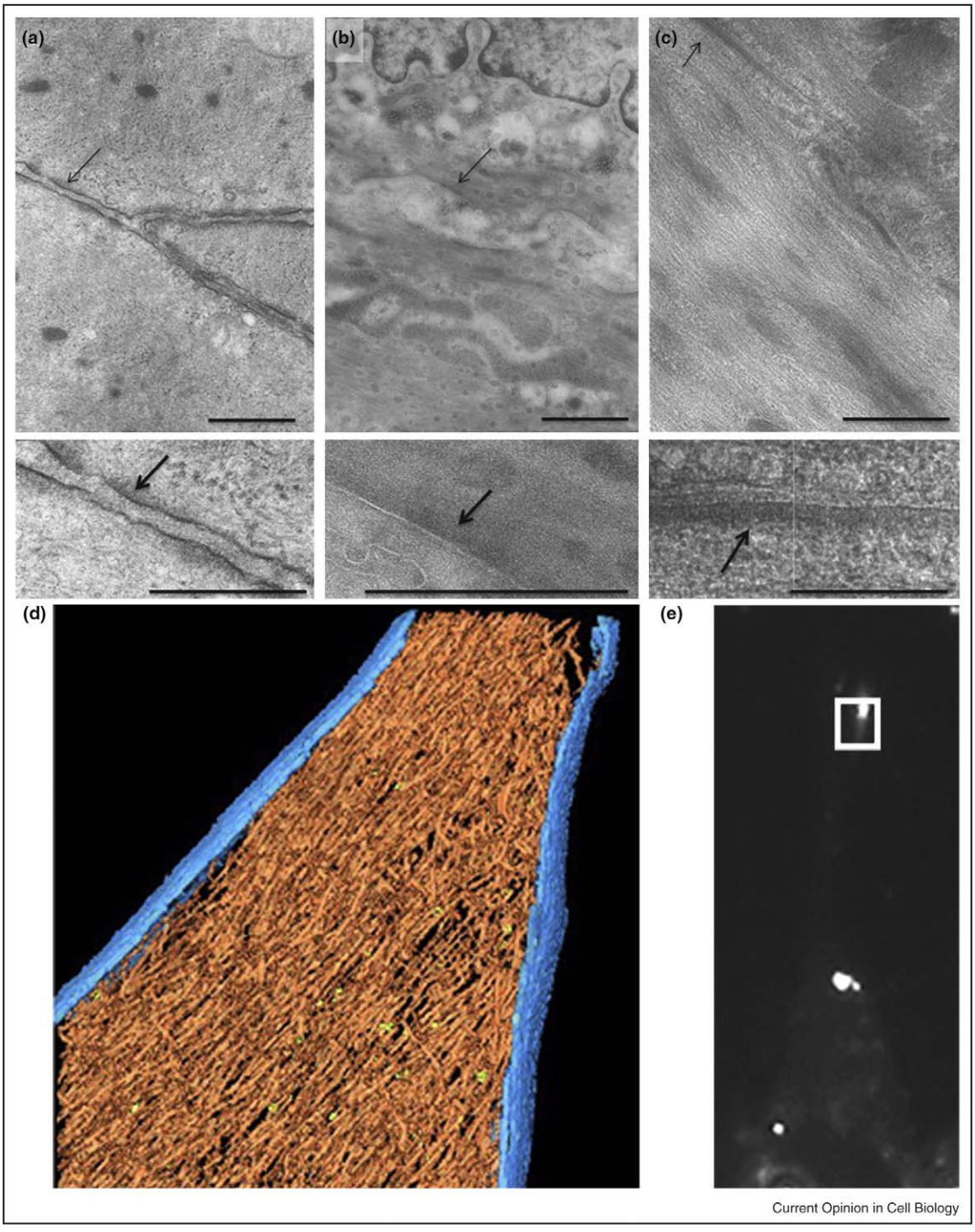
Biological systems in 3D by cryo-electron tomography

Cryo-electron tomography (cryo-ET) is a state-of-the-art technique that offers unique capabilities, enabling the acquisition of 3D information on the macromolecular architecture of cells in an unperturbed state [27,28••,29]. Using this technique, one can visualize cellular states, and reconstruct molecular networks. Given the advantage of vitrification by rapid freezing, biological material can be physically fixed, ensuring close-to-life conditions in samples prepared for cryo-ET [30]. Since neither dehydration nor staining is needed, the delicate cellular landscape is preserved during sample preparation, accurately depicting *in vivo* conditions.

Cryo-ET, like medical computer-based tomography (CT), is capable of retrieving 3D structures of specimens from a series of 2D micrographs. Owing to its large depth of focus, an electron micrograph is essentially a 2D projection of a 3D object, in the direction of the electron beam. As a consequence, features of the sample are superimposed and cannot be separated, as in optical microscopy. Nevertheless, the three-dimensionality of an object can be retrieved by recording a series of projections at varying angles, and computationally synthesizing these projections into a 3D density map; that is a tomogram [31]. In practice, the various projection images are collected by tilting the specimen incrementally around a single axis. This tilt series is then aligned to a common frame of reference, followed by the calculation of the tomogram by projecting the images into a volume, most commonly using a weighted back-projection algorithm [32].

The resolution of a tomogram is directly dependent upon the angular increment between two adjacent projections, and on the total number of images [33]. Therefore, the aim is to collect as many tilted projections as possible, covering the widest possible angular range. However, due to technical limitations, the tilt series cannot cover the

Figure 3



Electron microscopy-based visualization of the adhesion machinery in smooth muscle tissue and fibroblasts. In chicken gizzard tissue, contractile actin filaments are attached to the plasma membrane at specialized sites, commonly referred to as 'dense plaques', which are closely related to FAs [marked with matching arrows, in the lower magnification image (top), and the higher magnification insert (bottom)]. The same tissue is shown following three distinct processing procedures: **(a)** Epon section after tissue fixation (glutaraldehyde/OsO_4), dehydration and embedding, followed by thin sectioning and heavy metal staining, preserving membranes and some filaments, but showing no substructure in the dense plaque. **(b)** A glutaraldehyde fixed tissue, frozen-sectioned, embedded in methyl cellulose and heavy metal-stained. Overall tissue preservation, including the nucleus and membranes, is good, but filaments are poorly visible, and the dense plaque is largely diffuse. **(c)** A specimen processed by all-cryo-approach [26], including fixation and sectioning as in (b), but the sections, collected on an EM grid are not allowed to dry, but are washed and re-vitrified on the grid. The images (without heavy metal staining) display the various cellular filaments, as

Box 2 Structural analysis of major proteins found in focal adhesions

Several components of the adhesion machinery have already been structurally characterized by X-ray crystallography. With growing numbers of proteins whose structure was solved, and with the improvement of the resolution attainable by cryo-ET, it appears likely that in the near future, structural molecular information could be fitted into cryo-ET images, enabling multi-scale imaging ranging all the way from tissues and cells, to the component molecules. However, high-resolution structural analysis of many components of the adhesion machinery is challenging, due to their high degree of flexibility, and the sheer size of the proteins involved. Below we list a few of the adhesion-related proteins whose structure was solved: details can be found at in the protein data bank (PDB).

The protein	Method	PDB code
Integrin heterodimer	X-ray diffraction,	3F7P, 1QCY, 1L5G, 3K6S, 3K72, 3FCS
Vinculin	X-ray diffraction	1TR2
α-Parvin	X-ray diffraction, solution NMR	2K2R, 2VZI, 2VZC, 2VZD, 3KMU
Talin (fragments)	X-ray diffraction, solution NMR	2X0C, 2KVP, 2KMA, 2KC1, 2KC2, 3G9W, 2KGX, 2DYJ, 2JSW
ILK	X-ray diffraction, solution NMR	3KMU, 3KMW, 3IXE, 2KBX, 3F6Q
FAK	X-ray diffraction	2J0J, 1MP8, 2AL6, 1K04
α-Actinin	X-ray diffraction, electron crystallography	1TJT, 1WKU, 1SJJ

entire spectrum of views, and is limited to ±70°. Consequently, elongation of features along the beam axis is evident, due to a missing wedge in the 3D Fourier space [34]. Moreover, the cumulative electron dose over the entire tilt series must be kept within tolerable limits, typically not exceeding ~6000 e^-/nm^2, to prevent radiation damage to the biological specimen. Hence, to minimize the exposure time and to increase the accuracy of the process, data acquisition must be fully automated, relying on computer control [35••,36].

A further limitation on the use of cryo-ET in eukaryotic cells is its restriction to relatively thin cellular regions. When the object being visualized is thicker than the mean free path of an electron, multiple scattering events substantially degrade image quality, despite the use of a medium-to-high acceleration voltage (300 keV), and an energy filter aimed at minimizing this effect [37•]. Consequently, samples thicker than 1 μm can barely be studied *in toto*, requiring cryo-sectioning before tomographic analysis. Substantial efforts have been made to improve such procedures. While the feasibility of this approach was shown using cryo-sectioned rat liver cells, mouse epidermis, human epidermis, and cardiomyocytes [38•,39–43], it remains technically demanding, and often yields sectioning artifacts [44].

Cryo-ET can be applied to vitrified chicken gizzard tissues that were sectioned as in Figure 3c. This technique enabled us to study the tissue in its hydrated state; therefore, all components of the adhesion machinery can be revealed at a resolution of a few nanometers. Such a methodology will eventually bridge the resolution gap between conventional imaging of tissue sections (see above) via higher resolution structural analysis of cells, *ex vivo*, all the way to molecular resolution, enabling with structural information, obtained by X-ray crystallography (see Box 2).

Imaging cytoskeletal networks by correlated fluorescence microscopy and cryo-electron tomography

For decades, EM of the actin cytoskeleton was performed using a wide variety of techniques, including the use of standard sections of chemically fixed, resin-embedded cells, detergent-extracted cells subjected to negative staining or critical point drying [45–47], or unroofed cells, subjected to examination by scanning electron microscopy [48]. While these methods provided important insights into the architecture of actin networks [46], the spatial resolution of the structures revealed by these methodologies was limited, especially in the third dimension. Embedding cells in polymer before or after sectioning provided seminal information on cell-adhesive structures. However, this technique could not provide the means for visualizing individual filaments, nor structural information on macromolecular complexes *in situ*. The dehydration steps involved in the sample preparation — even when carefully performed — had a major detrimental effect on the microfilament system, and the use of metal coating or negative staining perturbed the 3D meshes of the actin filaments. This sensitivity may be attributed to the delicate nature of microfilaments, and their dynamic properties. The fast exchange of cytoskeletal proteins, as revealed by fluorescence recovery after photobleaching (FRAP) experiments [49,50], or fluorescence correlation microscopy [51,52], indicates that actin and actin-associated proteins may exchange at half-life times on the order of just a few seconds, rendering them highly susceptible to changes induced in the course of standard EM sample preparation. It is noteworthy that, while the typical exchange rate of FA

(Figure 3 Legend Continued) well as the specific substructure of the dense plaque (arrows). Bars = 1 μm. **(d)** Cryo-electron tomography of a vitrified fibroblast. A surface rendering view of the FA-protrusion shown in **(e)**, inset (bordered in white). Actin is depicted in tan, and membranes in blue. (e) Fluorescent microscopy image of a GFP paxillin-expressing cells, grown on the EM grid.

plaque constituents was on the order of seconds, the apparent reorganization of the adhesion site, as a whole, was relatively slow, on the order of minutes.

Electron tomography of vitrified, but otherwise unaltered, cells, offers advantages over the other approaches mentioned above; therefore, it appears to be a key technique for the 3D reconstruction of actin architecture [28••,53•,54]. This approach also led to the realization that the most destructive step in sample preparation is the dehydration of the specimen. By circumventing this problem, cryo-ET can reveal the actin filament network of intact eukaryotic cells and enable visualization of its interaction with the plasma membrane, without the need for chemical fixation or heavy metal decoration.

A major limitation of cryo-ET is the cellular area that can be covered in a single tomogram: $\sim 2\ \mu m^2$, about 1.5% of the peripheral area in a typical eukaryotic cell. Therefore, localizing specific biological structures such as focal adhesions under conditions of low-dose cryo-EM, constitutes a major challenge. Accordingly, cryo-EM was recently combined with fluorescent-light microscopy imaging, to identify specific cellular components and processes in an unambiguous manner [55••,56,57]. Fluorescence microscopy enables the location and mapping of a desired cellular feature that can later be found and studied in 3D, under an electron beam, by means of cryo-ET. Figure 3d shows a rendering of the FA region shown in Figure 3e. In this experiment, cells expressing a GFP derivative of paxillin, one of the hallmark protein components of the integrin adhesome, were grown directly on EM grids. The grid was then vitrified and the cellular position identified by fluorescence microscopy, prior to cryo-ET image acquisition and analysis. In this reconstruction, a large number (>500) of actin filaments, forming a thick (~250 nm) bundle, may be seen approaching the adhesion site. The acquisition of 3D information at a resolution of 5–6 nm enables their investigation on the level of single filaments, as well as visualization of different binding partners and membrane linkers.

Concluding remarks

Looking toward the current and future prospects in adhesion research, the challenges for advanced microscopy will most likely grow considerably, to meet the needs of adhesion researchers. As knowledge of the scaffolding and regulatory components of the adhesion machinery increases, there emerges a need to map the various components of the adhesion sites, at a molecular resolution. This mission could benefit from further development of correlated microscopy — combining super-resolution optical microscopy (e.g. [58]) with cryo-ET. Conducting such experiments with a large variety of fluorescently tagged FA molecules, could shed light on the molecular mechanisms underlying FA scaffolding and signaling activities. Monitoring physiological changes in the molecular organization of FAs (e.g. during cell spreading or migration), or following pharmacological or genetic perturbation of the adhesion machinery will, most likely, require time-resolved correlated microscopy, combined with powerful quantitative imaging tools. Mapping of molecular interactions within the adhesion site could be based on real-time fluorescence resonance energy transfer (FRET) combined with cryo-ET, and the relationships between mechanical stress and FA function could be studied by combining force traction microscopy with advanced cryo-EM. In summary, correlative approaches combining cryo-ET with a variety of optical methods that are capable of quantifying molecular events in live cells, are a central component in their development. Correlated microscopy, combining data from optical and electron microscopes remains a highly demanding technology, but in view of its power and potential applications, one may expect the development of automated tools for sample registration and handling, which will become broadly accessible.

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- •• of outstanding interest

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