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Dissecting the Molecular Basis of the Mechanics of Living Cells

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Abstract Cells establish and modulate their morphology and mechanics through the use of structural networks whose components range in size from a few nanometers to tens of micrometers. Over the past two decades, an exciting suite of sophisticated micro- and nanoscale technologies has emerged that permits investigators to directly probe structural and functional contributions of these components in living cells. Here we review underlying principles and recent applications of four such approaches: atomic force microscopy, subcellular laser ablation, micropatterning, and microfluidics. Together, these new tools are offering valuable insight into the molecular basis of cell structure and mechanics and revealing the remarkably broad influence of the mechanical microenvironment on many aspects of cell biology.

Keywords Cellular · Molecular · Structure · Mechanics · Atomic force microscopy · Laser · Microfluidics · Micropatterning · Nanotechnology · Cytoskeleton · Adhesion

Introduction

One of the most fundamental dogmas in modern cell biology is that cell behavior is largely dictated by the types and concentrations of soluble cues in the extracellular environment, such as growth factors, cytokines, and chemotactic agents. Over the past decade, however, an exciting new paradigm has emerged which argues that the physical microenvironment of the cell, including the mechanics, geometry, and three-dimensional topography of the extracellular matrix (ECM), can drive the cell's behavior in equally powerful ways. In particular, the mechanical balance between the cell's ability to exert contractile stresses on the ECM and the elastic resistance of the ECM to that deformation (i.e., ECM rigidity) has been demonstrated to regulate a surprisingly broad range of cellular properties including structure, motility, proliferation, and differentiation. For example, the differentiation trajectory of mesenchymal stem cells (MSCs) may be controlled by dictating either the area of ECM upon which cells are allowed to spread [1] or the rigidity of the ECM [2], and in both cases these physical inputs from the ECM are capable of overriding the influence of soluble differentiating agents in the medium. Moreover, cellular responses to these cues may be intensified or blunted by manipulating biochemical pathways that contribute to the cell's ability to stress the ECM; for example, medium-induced osteogenic differentiation of MSCs may be blocked by inhibiting Rho GTPase-dependent cell contractility [1]. While there are many soluble factors that interact with the structural components in mechanotransduction, in this review, we focus on cell mechanics and structures.

Cells sense, process, and respond to mechanical cues using an integrated set of mechanochemical systems that includes cell adhesion receptors (e.g., integrins), focal adhesion plaques, and cytoskeletal networks; the collective mechanics and dynamics of these systems enable cells to define, stabilize, and modulate their shape [3–6]. The exquisite sensitivity of cell behavior to inputs from the mechanical microenvironment suggests that these cues may

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be incorporated into small-scale technologies designed to interface with cells as a means of controlling cell behavior. In order to accomplish this effectively, it is important to develop a quantitative, engineering-level understanding of cellular structure and mechanics. Moreover, if we are to precisely engineer cells to control how they respond to a physical microenvironment presented by an inorganic interface, we must develop an appreciation for how the molecular-scale structural elements in a cell physically collaborate to produce cellular-scale mechanical properties. Forging these connections between length scales in a quantitative fashion that can be leveraged for the design of new technologies represents a fundamental challenge in cellular engineering.

In this review, we discuss four technologies that are revolutionizing the experimental understanding of the molecular basis of cellular structure and mechanics: atomic force microscopy (AFM), subcellular laser ablation (SLA), micropatterning and microfluidics (Fig. 1). Although all four technologies were originally developed for non-biological applications (e.g., semiconductor processing), over the past decade they rapidly have been finding their way into mainstream experimental cell biology. While AFM and micropatterning manipulate or probe properties of entire cells, SLA and microfluidics both provide access to subcellular structures and mechanics. We discuss the operating principles of all four techniques and review key examples of their applications to problems in cellular structure and mechanics.

Atomic Force Microscopy

The assembly of the cytoskeleton and ECM into three-dimensional biopolymeric networks relies on noncovalent interactions between the constituent macromolecules. Once assembled, these networks develop viscoelastic properties which enable them to resist and transmit mechanical forces. Thus, to understand the connection between molecular and cellular length scales in cellular mechanics, methods are needed that are capable of characterizing the mechanical properties of single macromolecules, subcellular macromolecular assemblies, and whole cells. Atomic force microscopy (AFM) has emerged as a powerful tool with which to access all of these length scales. AFM measures interaction forces between a sample surface and a micron-scale probe (the "tip") affixed to a weak spring cantilever, which is typically microfabricated from silicon or silicon nitride. Because contrast in AFM is derived solely from the interaction force between the tip and sample, this method typically requires no fixation or staining—unlike electron microscopy or immunofluorescence—and may be conducted in physiological media. Thus, the method is perfectly suited to capture dynamic processes in living systems.

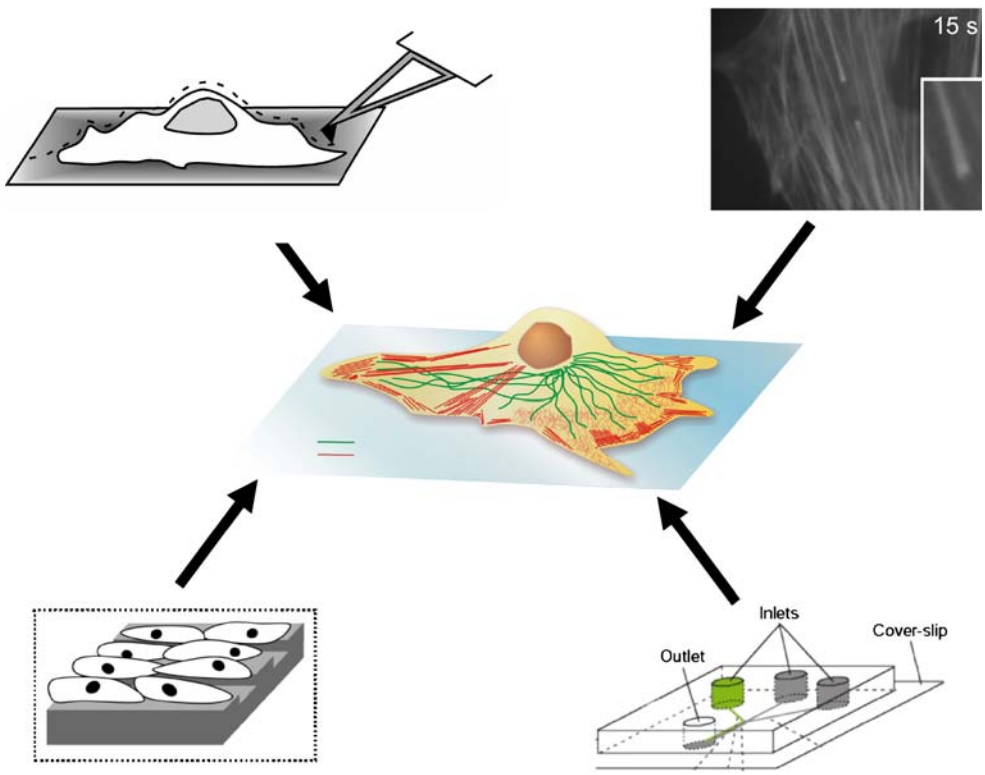

Fig. 1 Approaches to dissecting the molecular basis of the mechanics of living cells. Cellular and molecular mechanics can be studied through a variety of approaches including atomic force microscopy, laser ablation, microfabrication, and microfluidics technology. Image in upper left reproduced from *Traffic* (2001) [7] with permission from Wiley-Blackwell. Image in upper right reproduced from *Biophysical Journal* (2006) [32] with permission from Biophysical Society. Image in lower left reproduced from *Proceedings of the National Academy of Sciences of the United States of America* (2006) [57]. Image in lower right reproduced from *Nature* (2001) [61]

In the classical instrument design, the sample is mounted on a tubular piezoelectric scanner which translates the sample in the horizontal and vertical directions relative to the tip. Interactions between the tip and sample deflect the cantilever, whose position is tracked by a laser beam reflected off of the back of the cantilever onto a quadrant photodiode. Later designs affixed the cantilever to the scanner to free the underside of the sample for inverted optics and enable simultaneous brightfield or fluorescence imaging. Because these tubular scanners are highly susceptible to artifacts introduced by scanner creep, hysteresis and interaxial coupling, more recent designs have utilized piezoelectric stacks that independently control sample motion in all three scan axes. These newer instruments have also incorporated position sensors which enable closed-loop feedback and precise sample localization.

To a first approximation, there are two types of data that may be acquired from the tip-sample interaction with the AFM: images and force measurements. These are shown in Fig. 2 [7]. On the cellular length scale, AFM has been employed to image superficial cytoskeletal structures in living cells that are not easily optically accessible, including cortical actin bundles [8, 9]. In addition to imaging applications, the ability to obtain force measurements in AFM has been used with great success to quantitatively probe the mechanics of biological systems at length scales ranging from single molecules to whole cells. In the area of single molecule mechanics, AFM has been used to measure both the force-dependent unfolding of ECM proteins [10] and cell-ECM adhesion proteins [11] in an effort to understand how these components of the mechanotransduction machinery accomplish mechanochemical conversions. Also, the force-measuring capabilities of AFM have demonstrated great value for quantifying the indentational mechanics of living cells, including cellular elasticities [12], spatial maps of elasticity across the cell surface [13], and transduction of indentational forces into biochemical signals [14].

One of the more innovative recent applications of AFM for the study of cellular mechanics is the measurement of protrusive forces generated by growing actin networks. This problem is of great biological interest because many cells generate lamella and lamellipodia for migration by polymerizing a broad, branched (dendritic) network of F-actin against the plasma membrane at the leading edge of the cell. Parekh et al. [15] recently attacked this problem by nucleating a dendritic actin network from an AFM cantilever and allowing the network to polymerize against a solid support and deflect the cantilever. In order to successfully perform these experiments, the authors needed to confront a key technical challenge: the nanometer-scale cantilever deflections associated with network polymerization are often overwhelmed by thermal drift, which can be

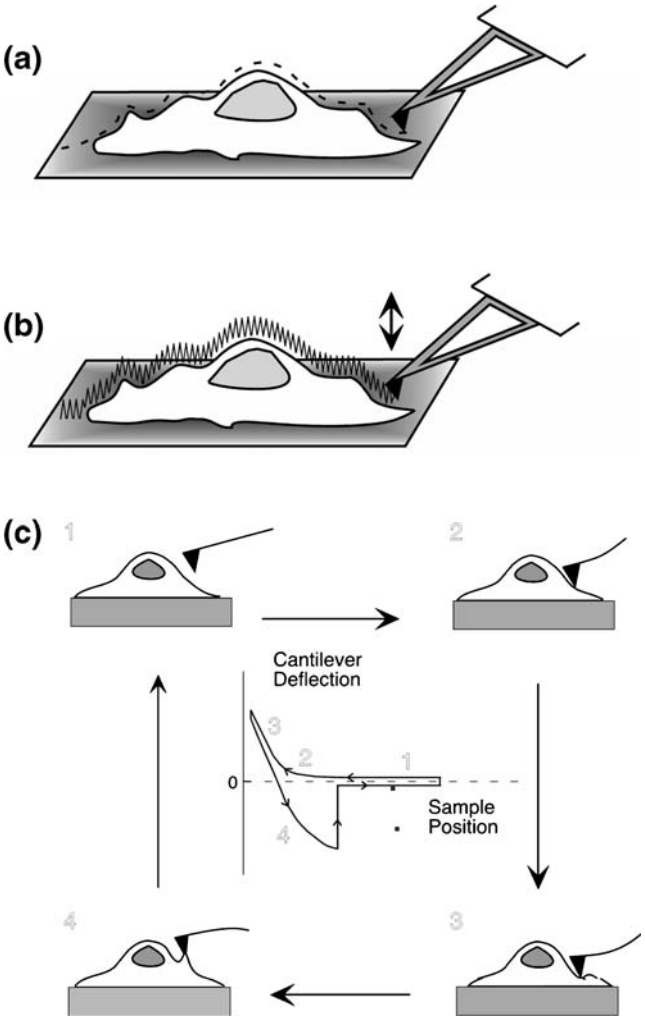

Fig. 2 Atomic Force Microscopy (AFM) imaging and force measurement. **(a)** AFM may be used to obtain images of biological structures, including living cells, by bringing a force probe into contact with the structure and scanning its surface (contact mode). **(b)** To obtain images while minimizing potentially destructive tip-sample contacts, the AFM probe may be oscillated rather than dragged across the surface (tapping mode). **(c)** AFM may also be used to measure tip-sample interaction forces by tracking cantilever deflection as a function of scanner position (force curve). In this schematic illustration of the indentation of a cell, the scanner is retracted, the AFM tip and sample are separated, and the cantilever deflection is zero (*1*). The tip-sample contact leads to cantilever deflection (*2*), and, eventually, cell deformation (*3*). As the scanner reverses direction and pulls away from the cell, tip-cell adhesions produce under-deflection of the cantilever (*4*). Eventually, these adhesions break, once again leading to separation of the tip and sample. Images reproduced with permission from *Traffic* (2001) [7] with permission from Wiley-Blackwell

on the order of microns on the time scale of the experiment. To overcome this, they introduced a second "reference" cantilever with its own laser beam, position detector, and feedback control; they were then able to compensate for drift by using force-feedback to compel the reference cantilever to maintain contact with the surface. With this system, they both passively measured network protrusive forces and imposed defined loads on growing networks to

measure the effect on network growth velocity, much as one might perform a load-velocity measurement on a macroscopic motor. These studies revealed that network polymerization arrests at a threshold load (the so-called "stall force"), consistent with previous optical tweezer measurements with single actin filaments; surprisingly, however, the growth velocity depended on the loading history of the network and not simply the instantaneous load, suggesting that these networks remodel to adapt to applied loads (e.g., by recruiting additional actin filaments) and that these remodeling events are cumulatively "stored" in the structure of the network. In subsequent studies, these investigators modified their approach to measure the dynamic (oscillatory) viscoelastic properties of these growing networks, which enabled them to observe reversible stress-softening phenomena [16].

These results are particularly exciting in light of the parallel and independent efforts of Prass et al. [17] to measure forces associated with cell migration in living cells. Here, the usual tip-sample geometry was altered such that the AFM cantilever was oriented perpendicularly to the sample substrate, in this case a glass coverslip containing a culture of migrating keratocytes. The cantilever was brought in close proximity to the surface so that a cell encountering it in the course of migration would attempt to push the cantilever by extending a process against it, resulting in the cantilever's deflection. Using this approach, the authors measured a whole-cell stall force of approximately 40 nN; this is consistent with measurements that had been obtained with calibrated glass microneedles in previous studies [18].

Subcellular Laser Ablation

Because AFM can only directly interrogate the exterior surface of a living cell, it can only provide limited insight into the mechanical properties of structures deep within the cellular interior. Moreover, properties measured by AFM represent the collective contributions of many different numbers and types of cytoskeletal filaments, and dissecting the contributions of individual filament systems is problematic even with the use of highly specific cytoskeletal inhibitors. Thus, additional technologies are needed for the selective measurement of single cytoskeletal structures in both deep and superficial portions of the cell; subcellular laser ablation (SLA) has emerged as such a method. First introduced for cell biological applications in the late 1970s by the laboratory of Michael Berns [19–23], SLA employs a tightly focused laser beam to irradiate and vaporize nano- to microscale structures in living cells. Specifically, ultrashort laser pulses (e.g., pico- to femtosecond) are focused through a high-numerical aperture objective lens onto an intracellular target that may be visualized by brightfield contrast mechanisms or fluorescence. Upon irradiation, material at the laser focus undergoes nonlinear multiphoton absorption, leading to optical breakdown and material destruction. The extremely high rate of energy delivery and absorption, made possible by the use of ultrashort laser pulses, outpaces the rate at which heat can be passively dissipated to the surrounding material, thereby limiting thermal damage to surrounding structures. Thus, if the pulse energy, pulse width, and repetition rate are chosen correctly, structures in living cells may be selectively incised with sub-micrometer precision without compromising the plasma membrane or killing the cell. Indeed, it was recently demonstrated that delivery of femtosecond laser pulses at kilohertz repetition rates and at pulse energies ranging from 1.4 nJ to 2.3 nJ can produce zones of photodamage as small as ~150 nm [24].

In the context of cell mechanics, SLA has been employed extensively to probe the micromechanical properties of actomyosin stress fiber bundles (stress fibers), the contractile structures that anchor cells to the ECM via focal adhesions and enable them to exert tractions on the extracellular matrix. These tractional forces are critical to cell shape, polarity, and motility, and there is an emerging appreciation that the mechanical balance between tensile forces generated by the cytoskeleton and the compressive resistance of the ECM (i.e., its rigidity) can profoundly influence a surprisingly wide range of cell behaviors including migration speed, proliferation, and differentiation [2, 25–27]. While stress fibers contain actin and a diverse variety of actin-binding proteins, perhaps the most important contributor to stress fiber contractility is myosin II, whose ATP-dependent motor activity permits stress fibers to constrict the ECM, analogous to the relationship between myosin-mediated microfilament sliding and muscle contraction [28]. Evidence for this contractile activity comes primarily from observations of fluorescently-labeled stress fibers in whole cells, which have revealed that a variety of actin-binding proteins including α-actinin and myosin [29, 30] are periodically distributed within the stress fiber, much like the sarcomeres of skeletal muscle. Moreover, treatment of isolated stress fibers muscle contractile agonists reduces the size of the periods, reflecting contraction of the fiber [31].

In an early use of SLA to dissect intracellular structures, Strahs and Berns [21] irradiated stress fibers visible through phase contrast microscopy and followed their retraction and subsequent repair over several hours. By repeating these experiments while pharmacologically manipulating other cytoskeletal systems, they later showed that stress fiber recovery depended on the integrity of the microtubule cytoskeleton, implying functional coupling between microtubules and stress fibers [19]. More recently, SLA has been used to obtain quantitative measurements of tensile loads borne by stress fibers [32] (Fig. 3). Here, the actin cytoskeletons of living endothelial cells were visualized

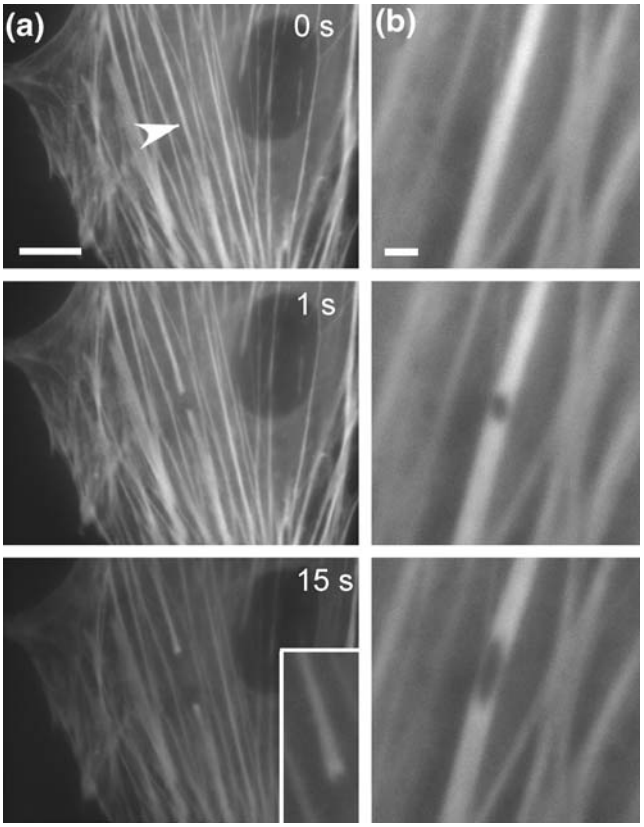

Fig. 3 Subcellular laser ablation (SLA) of stress fibers. **(a)** Incision and retraction of single stress-fibers in living cells. When a stress fiber in this endothelial cell, which has been transfected with yellow-fluorescence protein-tagged actin, is irradiated with a pulsed femto-second laser, the stress fiber severs and undergoes viscoelastic retraction. **(b)** Puncturing of a single stress fiber. Here, the central portion of a large stress fiber is irradiated, leading to a puncture wound that progressively distends as the stress fiber retracts in response to the injury. Bar=2 μm. Images reproduced from *Biophysical Journal* (2006) [32] with permission from Biophysical Society

using yellow fluorescent protein (YFP)-tagged actin, and selected stress fibers at the cell-ECM interface were irradiated and severed with femtosecond laser pulses. Irradiated stress fibers retracted in parallel with the axis of the fiber, providing *prima facie* evidence that these structures bear tensile loads. Interestingly, the severed stress fibers obeyed viscoelastic retraction dynamics, with a rapid, elastic recoil over the first 3 to 5 sec, followed by a slow, viscous relaxation over the next 5 to10 sec, analogous to a creep response. When larger stress fibers were irradiated, it was sometimes possible to create a puncture wound rather than an incision, yielding holes with diameters as small as 300 nm that distended into elliptical shapes as the weakened stress fiber relaxed in response to the injury. These puncture-wound experiments also served to directly confirm that SLA can ablate structures with submicron precision in living cells without functionally damaging structures outside of the zone of irradiation. Perhaps the most surprising result to emerge from this study was that the degree to which incision of one stress fiber influenced the cytoskeletal architecture and shape of the rest of the cell depended strongly on the compliance of the ECM on which cells were cultured. For cells cultured on rigid substrates with elasticities on the order of 1 MPa - 1 GPa (e.g., glass), severing a single stress fiber, or even multiple fibers in parallel, did not lead to appreciable cell shape changes. In other words, there was little apparent mechanical coupling between any given stress fiber and other fibers in the cell. Conversely, severing a stress fiber in cells cultured on relatively compliant (~4 kPa) polyacrylamide-based substrates produced a 4–5% elongation of the cell along the axis of the stress fiber, as well as thinning and extension of cytoskeletal structures tens of microns from the site of incision. Parallel studies with traction force microscopy, which enables measurement of cell-ECM tractional stresses and strains by following displacements of fiduciary markers embedded within the ECM substrate, revealed that a single stress fiber contributes to ECM strain across nearly the entire cell-ECM interface and strains the ECM most strongly near the points at which it inserts into focal adhesions. Thus, SLA illustrated direct connections between individual micron-scale cellular contractile structures and the tractional forces distributed over hundreds of square microns.

In addition to stress fibers, SLA has also permitted glimpses into the micromechanical and transport properties of microtubules. In particular, SLA has been employed to target components of the mitotic spindle in dividing cells to investigate how tensile and compressive loads are borne by astral and kinetochore microtubules [33, 34]. This method has also been applied to cortical (cytoplasmic) microtubules; for example, Botvinick et al. [35] photodisrupted cytoplasmic microtubules tagged with a series of GFP variants, including YFP and cyan fluorescent protein (CFP). Following microtubule incision, one of the severed ends depolymerized much more rapidly than the other, as would be expected for newly bared plus and minus ends. Surprisingly, electron microscopy of the ablated microtubules demonstrated that the extent of damage was largely dependent on the choice of fluorescent tag, which the authors attributed to amino acid sequence-dependent differences in fluorophore photochemistry. Microtubules have been postulated to contribute to cell shape and mechanics by supporting compressive loads [36]. To directly visualize relaxation of these compressive forces, a femtosecond laser was used to sever a curved GFP-tagged microtubule in living cells. Following irradiation, the curved microtubule rapidly straightened within 1 to 2 sec, and then depolymerized as would be expected for a compressively-loaded filament [24].

Finally, SLA has been used to investigate the role of local tensile forces on the turnover of focal adhesion

proteins in an effort to identify molecules whose biophysical properties are sensitive to the mechanical microenvironment [37]. In these studies, fluorescence recovery after photobleaching (FRAP) was used to measure the unbinding kinetics of GFP-tagged zyxin and vinculin within focal adhesions. When intracellular contractility was globally dissipated by treating cells with an inhibitor of Rho-associated kinase (Y-27632), the off-rate of zyxin more than doubled, whereas the off-rate of vinculin remained constant, suggesting that zyxin is sensitive to cytoskeletal tension in ways that vinculin is not. To directly confirm that the observed acceleration of zyxin kinetics was due to mechanical tension, SLA was used to sever a stress fiber associated with a specific focal adhesion prior to acquiring FRAP data on that adhesion. Indeed, zyxin turnover was found to be significantly faster in focal adhesions mechanically unloaded with SLA than in untreated focal adhesions. Similar results were obtained when intracellular tension was globally relaxed by culturing cells on increasingly compliant ECM substrates.

Micropatterning

Microfabrication is a field that has contributed to a wide variety of scientific areas including optics, chemical synthesis, and cellular and molecular control. In the field of cell mechanics, microfabrication technology has created avenues for studies such as controlling localized ECM-cell interactions that were often unapproachable before. One major advantage of microtechnology is that the size scale of the fabrication processes and the size scale of the system being studied (i.e., the cell) are approximately the same. Microfabrication can generate devices that range in size from the submicrometer scale to those measuring in the tens of micrometers, corresponding well to the size of single cells, which are often tens of micrometers. Not only is the size scale similar, but many of the materials that are used in microfabrication are naturally compatible or can be modified to be compatible with aqueous cell culture conditions. This has enabled an explosion of microfabrication approaches for cells and molecules. While a wide range of applications for fabrication and biology exists, one particular area that has garnered increasing attention is the study of cell mechanics including cell deformation, structural organization, and extracellular manipulation [38–41]. The findings of a selection of these approaches are described in detail below with respect to merging microfabrication and cell mechanics. We will first, though, discuss the process of microfabrication that enables such technologies to be produced.

Microfabrication is a technique that is used to produce many products including integrated circuits and biological sensors. One major tenet of this approach is to use planar masks to create detailed structures and then build up larger components by repeating this approach over multiple layers. Hard lithography, which is used to fabricate computer chips, uses this layer-by-layer process to create the final configuration. Soft lithography is a complementary process that is an extension of hard lithography, where the final configuration of the form serves as a mold to make a reverse-form system. Polymers such as polydimethylsiloxane (PDMS) are poured on the mold and solidified to create this reverse configuration. Although there have been many advances in this generalized approach including techniques such as microcontact printing, capillary lithography [42, 43], and nanoimprint lithography [44–46], a significant number of these approaches use the core approach of utilizing masks.

One example of merging microfabrication and biology, which is described below, is in creating a stamp for patterning proteins. While this is just one example, many of the microfabrication processes have similar general approaches. Microfabrication and stamping have been combined in a wide variety of applications from the patterning of cells and proteins to colloidal assembly [47–51]. This process is shown in Fig. 4. In this process, a silicon chip is first coated with

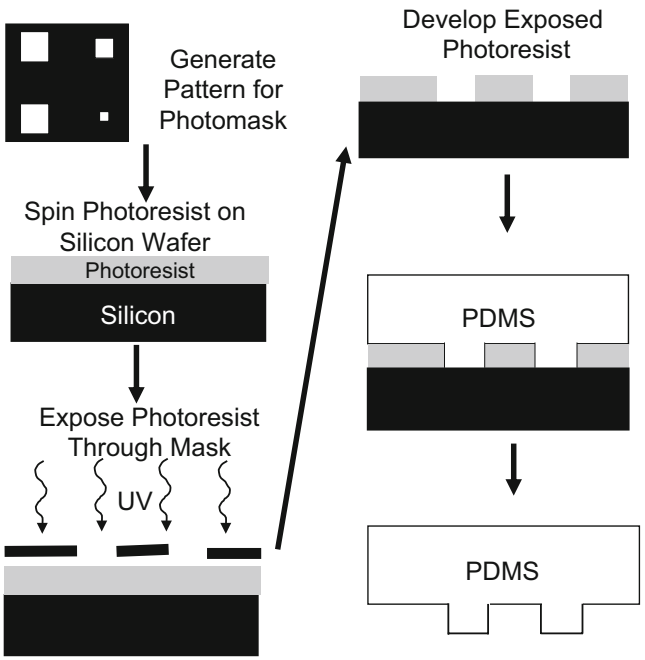

Fig. 4 The microfabrication process for soft lithography. A mask is generated using techniques such as high-resolution printing. Then, photoresist is spun in a thin layer onto a silicon wafer and heated to solidify the resist. The mask is placed on top of the photoresist, and then the system is exposed to ultraviolet light. The mask is then removed and the photoresist is developed, removing the areas which were not shielded from the light. Liquid polydimethylsiloxane is poured onto the fabricated features. The polymer is heated to generate a solid, flexible slab which is then peeled from the mold, creating a reverse-template of the system made entirely of the polymer. Adapted from Singhvi et al. [48]

photoresist using a spin coater. The rotational speed of the chip and the properties of the photoresist define the thickness of the photoresist layer (usually in the micrometer range) and ensure an even distribution of photoresist over the chip surface. The photoresist reacts to illumination sources such as ultraviolet light and will then either cross-link (negative photoresist) or destabilize (positive photoresist). A mask is used to transfer a pattern onto the photoresist through blocking or by allowing the illumination source to react the photoresist on the surface of the wafer. The mask can be designed in many ways, such as with mask writers, for high-resolution features. An inexpensive alternative is to utilize a high-resolution printer, which can create a transparency with printed features (usually black) provided the resolution of the features is above ~20 micrometers. This mask is then placed in close contact with the photoresist on the top of the chip. This system is exposed to UV light which enables the photoresist to react. The areas where the black print is positioned will effectively block light and will not react. The mask will then be removed, and the wafer will be submerged in a photoresist developer and rinsed. In the case of positive photoresist, the resulting features will be the opposite of the exposed areas since the UV exposed areas will now be absent. This part of the process will create the "mold" with features on the silicon wafer. After this point, the mold is used to create multiple copies of the reversed system. One way to approach this is through the use of a polymer such as PDMS. PDMS (Sylgard 184) is a two-part polymer that is mixed at a 10:1 ratio of base to curing agent while in a liquid form. This mixture is then poured onto the top of the mold. The polymer is subsequently heated and will cure into a solid, yet flexible, material. The polymer can then be peeled off of the wafer, resulting in an inverse mold. The specific details of these protocols can be found in multiple publications [52–54]. One significant advantage of this process is that the creation of the new polymer forms can be repeatedly accomplished after the initial mold has been fabricated within a short time frame of less than 2 h for each form. The final result of the process described above is a polymer system with embedded features; these can be useful in a range of applications. A few selected examples are discussed below. Note that this approach can also be used for developing microfluidic devices, which will be discussed in the final section.

Microfabrication techniques have been used to facilitate the examination of questions germane to cellular and molecular mechanics that may not be answerable by other previously established means. These techniques have been used, for example, to fabricate a bed of microneedle-like posts to measure the response of single cells from a mechanical perspective [55]. The microfabricated structures were columns whose deflection could be determined by using beam bending equations and knowing the geometry and compliance of the system. Cells were cultured on the tops of the posts and allowed to spread over them to connect the microfabricated structures to the cell through their focal adhesion complexes. The cells that exerted force on the independently moving posts could be imaged, and their subcellular tractions could be determined. The ability for cells to attach and spread on these microposts was further controlled chemically through serum, lysophosphaticlic acid, and the expression of constitutively active RhoA. One of the significant cellular mechanics findings was that the force increased with the size for adhesions larger than 1 μm^2. These studies demonstrated that coordinated signaling occurred between biochemistry and mechanics in cell adhesion and structural response. Thus, this fabrication approach allowed for the design of scientific studies that could be used to understand cellular mechanics.

Another example that leveraged the unique advantages of microfabrication focused on measuring subcellular displacements through flexible substrates. This was accomplished while controlling the location and geometric spreading of cells [56]. In this study, quantification of cellular traction forces was undertaken through the use of adhesive islands of variously defined size and shape. These islands were located on the surface of a polymer gel that contained embedded fluorescent beads that acted as fiducial markers. Smooth muscles cells were seeded onto geometrically constrained regions (2,500 μm^2 squares and 25 or 50 μm diameter circles), and were subsequently cultured on the gels. This geometry control was accomplished through the use of a thin membrane pierced with microholes corresponding to the geometry of interest that was placed on the top of the flexible gels. Collagen was then used to coat the top of this system so that position of the actual ECM only occurred only in the areas where the microholes were located. After the cells assumed the shape of the patterned islands, the displacements of the microbeads were tracked to create a map of the traction forces exerted by the individual cells. The cells that spread on the square islands exhibited traction forces at their corners in both the absence and presence of the contractile agent histamine. The cells on the round islands did not have tractions directed in any particular direction, although strong tractions were found in the direction of the protrusions. This microfabricated system enabled a real-time approach for examining mechanical forces within living cells in both constrained and unconstrained environments.

Although a multitude of papers have been published to describe other technologies in the general area of microfabrication, we present one more example, namely the use of microfabricated channels to examine the response of mesenchymal stem cells to mechanical stimulation as shown in Fig. 5 [57]. The goal of this work was to examine mesenchymal stem cells with respect to the engineering of

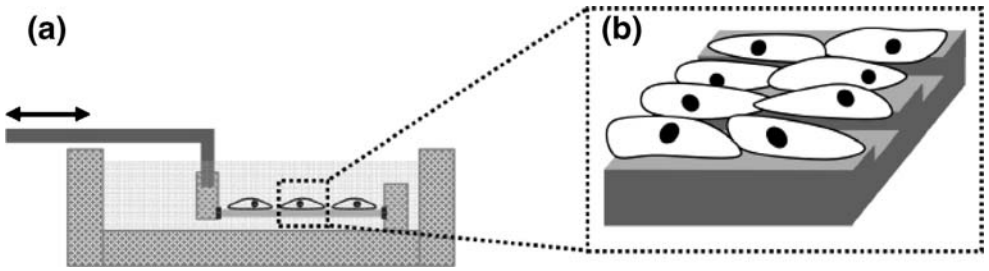

Fig. 5 Using microfabricated polymer structures to investigate stem cell mechanical response. (**a**) Mesenchymal stem cells are cultured on top of long thin grooves that are microfabricated on a polymer slab for applying mechanical strain. (**b**) The physical constraints of these microfabricated grooves induce the cells to align themselves in the direction of the grooves. The cells are then mechanically stimulated by applying a strain on the polymer substrate. The subsequent mechanical response of the cells can be observed and compared to the normal alignment of the attached cells through this approach. Adapted from Kurpinski et al. [57]

new tissues. The manner by which mechanical forces actually cause changes in the genetics of these cells is a subject of intense debate. In order to examine this, the authors microfabricated long, thin grooves for cell culture using soft lithography. The authors found that cells aligned on these grooves, and then independently aligned in the direction of a cyclic, uniaxial, mechanical strain. Furthermore, when the cells were mechanically stimulated with the grooves aligned parallel to the cyclic mechanical strain, the cells revealed an increase in calponin 1, a decrease in cartilage matrix markers, and an increase in proliferation. However, when the cells were cultured on grooves perpendicular to the strain, these biochemical changes were significantly decreased. Thus, using these microfabricated structures helped determine the effects of mechanical strain on stem cell responses with respect to the direction of the strain and resultant cellular orientation.

Microfluidics

Microfabrication technology has enabled many different fields to proceed in novel directions, but one specific technology in this domain can control fluid flow related to cellular and molecular response; this field is microfluidics. Microfluidics can enable researchers in cellular and molecular mechanics to specifically control components related to cell structure and mechanics including the cytoskeleton and extracellular matrix as described below. Microfluidic devices can be fabricated using the same approach as described previously, yet this technology presents researchers with control over an aqueous environment suitable for studying cells and molecules. Microfluidics enables spatiotemporal control over the chemical milieu of living cells by exploiting the unique properties and characteristics of low Reynolds number flow. While the ability to use microfluidics to impose mechanical forces on cells is limited due to the size of the fluidic channels (i.e., the shear force on a single attached cell in a microfluidics channel is often less than 1 dyne/cm^2, and physiological shear is in the range of 15 dynes/cm^2), cell structure, which has been discussed previously and is critical in cellular and molecular mechanics, can be significantly affected though using structural modifying agents. As the cytoskeleton is a filament system that can be affected by biochemical manipulations, a microfluidic device that can control both the time and space location of such stimulation can enable novel approaches to answer challenging scientific questions that were unapproachable before the development of this technology. For example, if local domains of a single cell have their structure modified through chemicals such as cytochalasin-D, which depolymerizes the actin cytoskeleton, the mechanical response of that cell will be significantly altered. This type of local manipulation can be accomplished through the use of microfluidic systems and is described in more detail below. Not only can the internal environment of the cell be altered with microfluidic systems, the extracellular environment can be controlled as well. This can have a significant impact on the response of the cell and its structure, as the cell attaches to substrates and the ECM through the heterocomplex of linked proteins within the focal adhesion complex as discussed previously. While it has been shown that the focal adhesion complex is linked through transmembrane proteins called integrins, there is recent evidence of the involvement of other transmembrane proteins that link the extracellular matrix to the intracellular cytoskeleton such as syndecan-4. Microfluidics is poised to become one uniquely useful approach for examining the structural and chemical features of these linking proteins as they provide mechanical stability to adherent cells.

To build microfluidic devices, an approach similar to the previously described microfabrication process can be used employing silicon wafers, masks, photoresists, and PDMS. However, the final polymer device in this system has a specific configuration that must be formed for the microfluidics device. One possible configuration would be to have intersecting channels molded into the top of a PDMS slab in a Y-shaped form, where two inlet streams converge into one central outlet stream. A similar system with three inlet streams is shown in Fig. 6(a). After the intersection

configuration is fabricated, the PDMS slab must have holes created at the end of each channel with a cylindrical punch for the addition and removal of liquids. The PDMS slab would then be inverted and brought into contact with a clean glass coverslide; the two surfaces naturally adhere together, but also could be induced to do so via plasma oxidation or heating, which would more strongly bind the two surfaces together. Closed channels are now formed with the bottom of the channel being the transparent glass. Fluid is introduced into the two inlets and removed through the outlet by pipetting solutions into the punched holes at the end of the channels. To control flow speed through this fluidic system, gravitational height differences can be utilized as additional fluid is pipetted in, the difference in fluid height between the inlet and outlet will cause an increased flow toward the outlet to equalize the liquid heights. Cells can be cultured in the microfluidic channels using approaches similar to those already developed in biological studies for culturing on glass surfaces such as coating the glass with extracellular matrix (e.g., fibronectin). One significant advantage of the glass bottom is that the processes occurring inside the channels can be visualized at high resolution on a conventional inverted optical microscope. Thus, the common tools for imaging analyses (e.g., green fluorescent protein, cell motility tracking, and quantum dots) can also be implemented with the microfluidics system. Note that the use of a microfluidic device designed to converge multiple streams into one main channel would not cause any mixing at the point of the converging streams other than that resulting from diffusion along the length of the main channel. As the streams are usually continuously flowing, their interfaces are in contact for only a minimal amount of time, and the opportunity for diffusion is limited. This lack of mixing is attributable to the low Reynolds laminar flow, as the dimensions of the channels are small (usually less than 100 micrometers) and the flow rate is slow (usually less than 100 μm/second). Using this microfluidics approach, the actin cytoskeleton has been altered at subcellular domains, which is observed through monitoring the displacements of the nucleus and mitochondria within a single living cell [Fig. 6(b)]. More specific details and protocols for creating microfluidics can be found in other published manuscripts [58, 59]. A few selected and specific examples of applications that are enabled by microfluidic technology are explained in the ensuing paragraphs.

Microfluidics have been used in a number of applications for controlling cellular and molecular responses. The ability to have spatiotemporal control over the internal and external environments of cells is essential to understand behaviors including mechanical response. Three examples

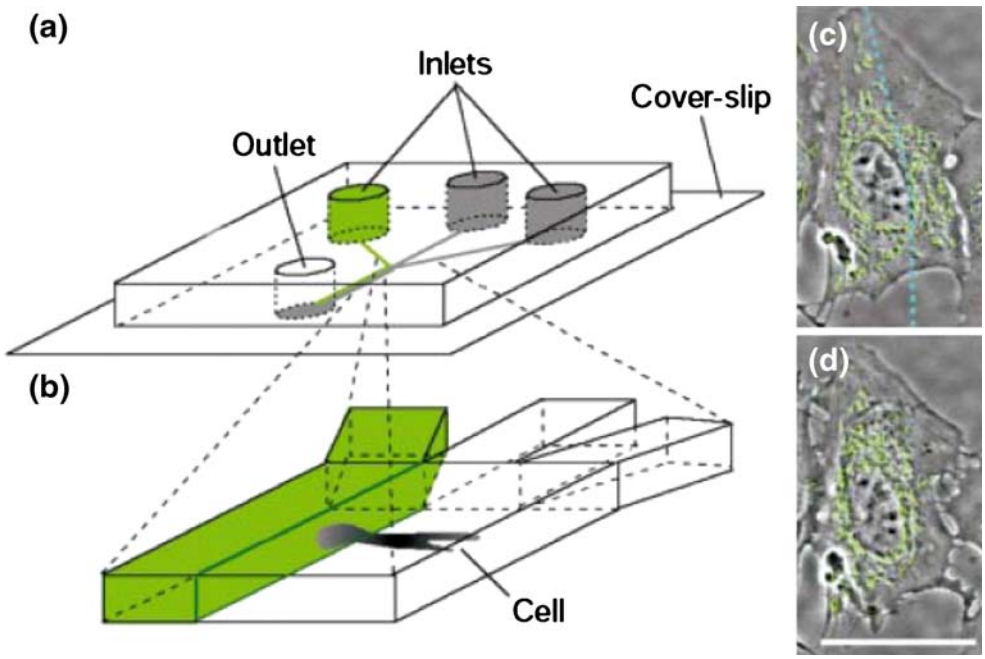

Fig. 6 Using microfluidic devices to control the stimulation of subcellular domains. **(a)** A three-channel inlet microfluidic device is used to stimulate local domains of a single cell. The microfluidic device is made through a conventional microfabrication process. The polydimethylsiloxane slab is placed on top of a no. 1 borosilicate coverslip so that the cells can be viewed with conventional high-resolution optical microscopy. After coating the channels of the device with an extracellular matrix such as fibronectin, the cells are flowed into the channels. **(b)** Streams are then introduced into each of the inlets which do not mix when they intersect due to the low Reynolds number laminar flow. These streams subsequently interact with and stimulate the cells in their local domains. The microfluidic device can be used to disrupt actin filaments within local domains of a single cell using latrunculin A. The mitochondria and the nucleus, which are observed here as fiducial markers within a cell, displace in response to local alteration of the cytoskeleton **(c)** before and **(d)** after treatment with latrunculin A. Bar=25 μm. Adapted from Takayama et al. [61]

are presented here that demonstrate the advantages of microfluidics in spatially and temporally governed studies at the cellular and molecular levels. The first example uses microfluidics to control the internal chemical environment at subcellular domains [60, 61]. This ability is enabled by the microfluidic devices themselves, which limit mixing laminar flow and allow for fluid streams to be separated with control over the spatial position of the streams down to the single micrometer scale. This degree of control is congruent in scale to that of many cells, which can be tens of micrometers in diameter when attached and spread on surfaces such as the glass bottom of a microfluidic device. In this work, a three-inlet stream microfluidic system was used to present membrane-permeable molecules to specific subcellular domains of living cells. This PARTCELL (*Part*ial treatment of *cell*s using laminar flows) microfluidic system was also able to visualize the cells through the thin glass coverslip at the bottom of the channels. One of the scientific avenues pursued was the internal structural alteration of cellular environment. As the internal structure directly affects the mechanical responses of these biological systems, this process allows researchers to ask questions that were previously unapproachable. This approach provided the advantage of tracking the mitochondria while the PARTCELL delivered cytoskeleton depolymerization agents to localized portions of a single cell. The mitochondria, which provided fiducial marker points inside the cell and also are known to be associated with the cytoskeleton of the cell, were tracked as they moved in response to the alterations in the structure of the living cells. The ability to deliver chemicals to cells is allowing studies to be conducted at a subcellular level, a significant advancement from the whole-cell or multi-cell experiments that dominates previous research.

Another example is a microfluidic system that controls the movement of cells by delivering chemicals to induce directed cell motility [62]. Cell motility along surfaces is governed by mechanical and structural components and this device helps to enable examinations of chemotaxis. Neutrophils were induced into a chemotactic response (directed cell motility) using the microfluidic devices, which were able to create complex gradients of interleukin-8. The cells attached and spread on the bottom surface of the glass channel where they were subsequently exposed to gradients of interleukin-8. The array of gradients included an approximate step gradient (a change in concentration, i.e., presence or absence, of interleukin-8) as well as a hill gradient (a gradual slope of decreasing or increasing concentration of interleukin-8). The resulting cell movement was abrogated for the step function when the cells reached the gradient transition area. However, for the hill gradient, the cells continued to move toward the higher concentration, managing to progress beyond the highest concentration in the gradient before reversing direction. The ability to create and maintain these chemical cues for chemotactic studies through microfluidic devices has enabled novel scientific questions to be explored.

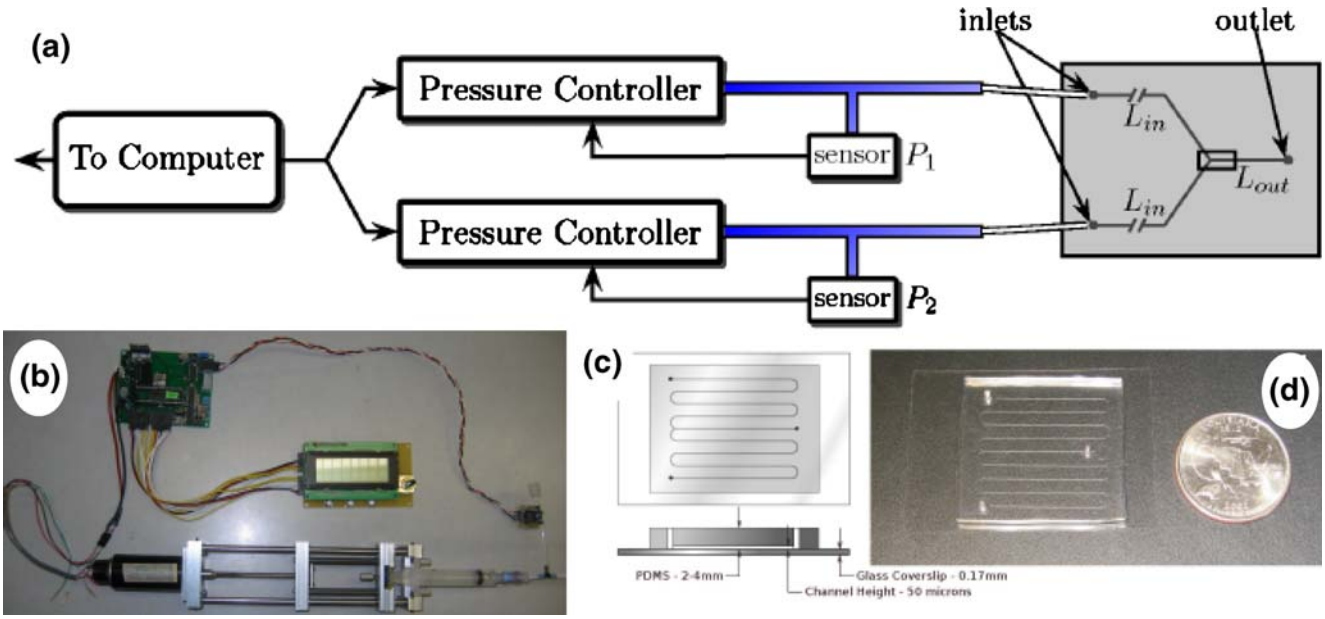

Fig. 7 A pressure-regulated automated-feedback microfluidic system. (**a**) A serpentine microfluidic system is regulated by a custom fabricated closed-loop pressure control system. (**b**) The pressure at each inlet is controlled via a motorized stage with continual feedback monitoring of pressure levels. This provides a very significant advantage over conventional syringe pump systems, which typically have little or no feedback controls. This provides the ability to position the interface through a computer-control system for short time switching (less than 0.1 sec) or long duration experiments without the need for constant monitoring of the system. (**c**) The serpentine channels intersect to create a laminar flow interface and (**d**) are centimeters in length while being micrometers in height and width. Adapted from Kuczenski et al. [63]. Reproduced by permission of *The Royal Society of Chemistry*

Although there are many other examples of microfluidic approaches in terms of cell structure and mechanics, one advance that has recently been made is the ability to provide fast switching and external long term control of the interface position of the chemical streams. This was achieved through the construction of an automated-control pressure-feedback microfluidic system (Fig. 7) that enables both short duration (e.g., short switching of subcellular chemical stimulation) and long duration experiments without any need for continuous monitoring of the microfluidic system. This advance in technology can be directly applied to many existing microfluidic approaches, including the two previous examples. Historically, hydrostatically driven flows controlled by fluid column heights presented challenges for fast and high precision movement of the interface. Furthermore, commercial syringe pumps used in volumetric displacement-driven flows had discontinuities in their position due to the innate characteristics of stepper motors. In our experiments, we designed and employed a serpentine microfluidic channel governed by an automated-pressure control-feedback system [63]. The interface position of the two intersecting laminar streams could be dynamically adjusted with a response time below 0.1-sec results indicative of speed and accuracy of control previously unattainable at the interface position. This has significant implications for experiments in the biological domain on short time scales (e.g., calcium signaling) and long time scales (e.g., differentiation).

Conclusions and Future Work

The field of cellular and molecular mechanics is a rapidly expanding area that is constantly reinventing itself. An understanding of the interrelationships of the cytoskeleton, the focal adhesion complexes, and the extracellular matrix is of critical importance in understanding molecular, cellular, and mechanical response perspectives, and is vital to promoting advances on the fronts of this multifaceted field of study. One of the exciting directions being pursued in this area is the development of novel technologies to pursue questions that are unapproachable using conventional techniques. We have briefly discussed four major areas that can be used in exploring these biological questions, each of which has distinct advantages. The AFM is an excellent tool for imposing specific mechanical force as well as mapping the structural response of single cells and molecules. Laser ablation is an approach that can be used to alter the internal environment of living cells at highly precise small volume areas, which can help parse out the importance of structural elements in cells. Microfabrication techniques enable researchers to probe mechanics questions such as traction forces and the effects of these forces on cell fates such as the proliferation rate. Finally, microfluidics can be used to alter the internal structure and extracellular interactions of cells, which can dictate the array of possible cellular responses in a variety of cell types from endothelial cells to neurons. Future directions in the area of cellular and molecular mechanics will continue to involve new technologies that will interface with biological systems. Our understanding of mechanics fostered by such applications will be furthered by examining these discoveries with respect to new perspectives as well. One might, for example, choose to pursue these studies while evaluating the cells in terms of materials science and polymer physics research. One of these complementary research areas that is currently gaining increasing interest is the concept of examining cellular functions as living smart materials. While materials such as shape memory alloys and polymers adapt to external stimulation in a directed manner, the cell also has the ability to respond to diverse stimulations, including mechanics, through structural and chemical adaptations in directed, yet often in unexpected or unexplored, ways. These adaptations can have many characteristics of robust systems, but are inherent within the system (i.e., the cell) rather than having to be manufactured into the final product (i.e., the material). This opens new possibilities to apply biomimetic approaches at the cellular and molecular scales, and builds upon previously successful larger scale biomimetic system approaches that have been used, for example, with gecko feet. These new approaches and perspectives will continue to expand the field of cellular and molecular mechanics in exciting and novel directions. The key is to use mechanics as one of the major foundations for exploring the living and adaptable characteristics of the cell.

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