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Force Spectroscopy and Beyond: Innovations and Opportunities

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ABSTRACT Life operates at the intersection of chemistry and mechanics. Over the years, we have made remarkable progress in understanding life from a biochemical perspective and the mechanics of life at the single-molecule scale. Yet the full integration of physical and mechanical models into mainstream biology has been impeded by technical and conceptual barriers, including limitations in our ability to 1) easily measure and apply mechanical forces to biological systems, 2) scale these measurements from single-molecule characterization to more complex biomolecular systems, and 3) model and interpret biophysical data in a coherent way across length scales that span single molecules to cells to multicellular organisms. In this manuscript, through a look at historical and recent developments in force spectroscopy techniques and a discussion of a few exemplary open problems in cellular biomechanics, we aim to identify research opportunities that will help us reach our goal of a more complete and integrated understanding of the role of force and mechanics in biological systems.

Our understanding of the natural world was largely limited before the quantitative treatment of forces and mechanics was introduced by Newton and his contemporaries (1). These developments not only established a scientific revolution but also facilitated our ability to engineer the modern world. Yet in many areas of biomedical research, the critical role of force in mediating and guiding molecular interactions is still not fully appreciated.

The development of powerful techniques for applying and measuring forces at the single-molecule level has helped to better establish the role of mechanics in the fields of molecular and cellular biology (2,3). Commonly used single-molecule force spectroscopy (SMFS) methods include tools such as optical tweezers (OTs, Fig. 1 *A*), atomic force microscopy (AFM, Fig. 1 *B*), magnetic tweezers (Fig. 1 *C*), the biomembrane force probe, and more recently centrifuge force microscopy (Fig. 1 *D*) and acoustic force spectroscopy, with descriptions and applications of some of these approaches detailed in recent reviews (4,5).

The application of these tools has led to many new insights into force-mediated biochemistry, from revealing the molecular mechanisms of molecular motors to illuminating the functioning of adhesion molecules on cell surface (3,6). Yet in many ways these tools have still not become fully integrated into mainstream biology—rather, single-molecule force measurements are often still treated as a technique for specialists. Correspondingly, the breadth of biological systems and the range of researchers using these methods has been somewhat limited. Why is this the case, given the key roles that force has already been shown to play? One factor may be the still-significant technical challenges required to perform these measurements, typically requiring labs with significant biophysical expertise and expensive equipment. Another factor may be the difficulty of studying forces not just for single molecules in isolation but in systems of higher complexity such as cells, tissues, and multicellular organisms. In this perspective, we reflect on previous accomplishments that can serve as exemplars for future work and present some opportunities to move the field forward, both in areas of scientific inquiry and opportunities for technological development.

Subcellular

Through astute in vitro measurements, SMFS approaches have played an important role in our modern understanding of cell biology. For instance, much of the early work elucidating the biophysics of cellular molecular motors such as kinesin, myosin, and dynein and deciphering the biophysics

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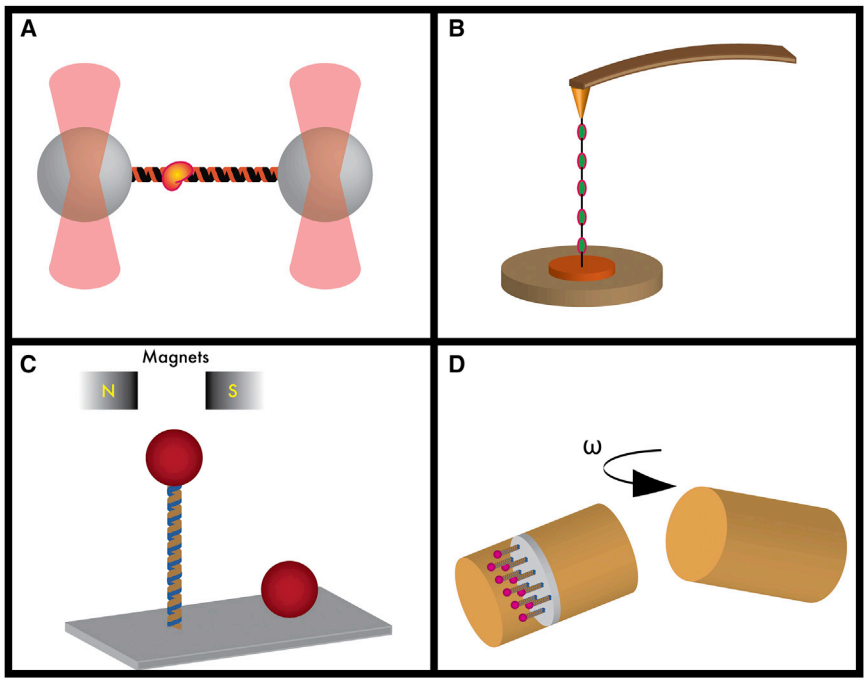

FIGURE 1 Schematics for SMFS techniques. (*A*) OTs use focused laser beams to trap dielectric particles, such as microbeads. Mechanochemical information is teased out by applying tensile force to molecular constructs tethered to these particles. (*B*) Atomic force microscopes use flexible cantilevers to apply direct mechanical forces to molecules of interest, whereas (*C*) magnetic tweezers employ magnetic forces. (*D*) The centrifuge force microscope rapidly rotates to apply centrifugal forces to a sample, which typically consists of hundreds to thousands of tethered beads being pulled and observed in parallel.

of genomic underpinnings such as the mechanism of action of RNA polymerase in DNA transcription and nucleic acid unwinding by helicases, mechanistic insights into the molecular machinery participating in the replication forks, and the role of histones in DNA packaging was carried out using OTs (4,7). Similarly, AFM—often referred to as molecular force probe when used to interrogate single molecules using force and a member of a broader set of scanning probe microscopy approaches—has been used to measure the strength of covalent bonds and catch bonds, to address problems in protein folding and nucleic acid folding, to image chemical groups within the three-dimensional (3D) structure of a protein complex, to identify transiently populated intermediates on the protein folding energy landscapes, and to study cases in paleoenzymology (4,6,7). With improved imaging speeds, it is now possible to measure protein-unfolding rates using AFM that occur on timescales that can be sampled by molecular dynamics simulations (8). Developments in force-probe instruments have been complemented well by improving surface and attachment chemistries—a critical component of any SMFS assay. Minimizing nonspecific interactions between single molecules under study and surfaces, maximizing the number of specific interactions, and maximizing the number of bona fide single tethers in an assay remain as challenges. Passivation approaches have ranged from the simple—use of physisorbed bovine serum albumin as a blocking agent—to the more elaborate, such as the use of dichlorodimethylsilane (9). Moreover, specific linkages can be made distinguishable by embedding precalibrated, well-characterized single-molecule behaviors as markers. With such a confirmation signature, one can identify single molecules with high fidelity (10–13). There also is a perpetual need for improving anchors. To this end, development of covalent tethering approaches has expanded the types of interactions that can be interrogated using SMFS (14,15). Efforts have also been initiated in increasing the number of interactions that can be interrogated simultaneously. Some successful examples include multiplexing OT measurements, developments of parallel magnetic tweezers, and flow-based approaches (5,16–18). Addressing the need for ever-greater amounts of multiplexing capabilities, in 2010, Halvorsen and Wong developed a centrifuge force microscope designed for high-throughput SMFS (19–22). More recently, acoustic force has also been exploited to achieve parallelization (23). With rapid improvements in multiplexing, we might be better placed to answer some fundamental questions in biomolecular mechanics. What is the role of static and dynamic heterogeneity in biomechanical interactions, e.g., how many subpopulations with differing mechanical properties exist for a given interaction? Under what conditions is the use of the ergodic hypothesis—biomolecular fluctuations averaged over time approximate fluctuations averaged over large numbers of molecules—to explain biomechanical properties justified? Energy-landscape profiles have largely been abstractions based on stable states. Could exploration of a greater diversity of metastable states and rare trajectories, afforded by higher multiplexing, result in more detailed shapes of energy landscapes and a better understanding of the kinetics of molecular transitions? Improvements in throughput, spatial and temporal resolutions, and the ranges of loading rates explored, as well as integration with other measurement modalities, could provide the technical

refinements that enable experiments necessary to address these questions.

Cellular

Adhesion biology was an early beneficiary of progress in SMFS. Cells can form adhesions with neighboring cells as well as with extracellular matrix (ECM). SMFS approaches have played a vital role in elucidating mechanistic details of mechanotransducers that mediate such adhesions: cadherins, selectins, and integrins (24). The broader role of force in studies of various adult cell types has been reviewed previously (25). Here, we briefly focus on its role in stem cell biology. The role of matrix elasticity in directing differentiation of mesenchymal stem cells was elucidated in a profound study (Fig. 2) by Engler et al. (26). The authors demonstrated that culturing on stiff surfaces induced osteogenic differentiation in stem cells, whereas culturing on surfaces with intermediate stiffness resulted in myogenic differentiation, and culturing on surfaces with low stiffness triggered neurogenic differentiation. Through a battery of force assays, Wen et al. decoupled the influence of matrix porosity and protein tethering from that of matrix stiffness in guiding differentiation (27). Dalby et al. showed that surfaces with a disordered "holey" pattern were sufficient to induce osteogenic differentiation in human mesenchymal stem cells (28). Conversely, McMurray et al. showed that surfaces with "holes" of comparable physical dimensions but presented in a more ordered pattern were sufficient to encourage long-term maintenance of mesenchymal stem cells phenotype (29). Guo et al. suggest changes in cell stiffness to be responsible in determining stem cell fate (30). In a recent review by Smith et al., the role of mechanical cues in guiding stem cell differentiation has been critically appraised (31), albeit the molecular basis of such guidance has eluded complete explication. Continuous physical links from the extracellular matrix to the inside of the nucleus may play a role. For instance, it is known that the nucleoskeleton is connected to the cytoskeleton through the linker of nucleoskeleton and cytoskeleton (LINC) complex, and the cytoskeleton in turn is connected to the ECM through focal adhesions (32). Yet a consensus on the identities of the force sensors and downstream signaling factors that trigger epigenetic changes leading to differentiation remains elusive. New biomechanical tools for studying molecular forces within living cells are needed; the challenges and rewards of in vivo single-molecule studies have been described previously (33). A combination of mechanical and chemical approaches would offer a wider set of engineering controls over purely chemical interventions to guide differentiation of stem cells on specific pathways. Therefore, novel biomechanical approaches that contribute toward answering some of the open questions listed above may also precipitate a new wave of developments in the broader field of cell therapy.

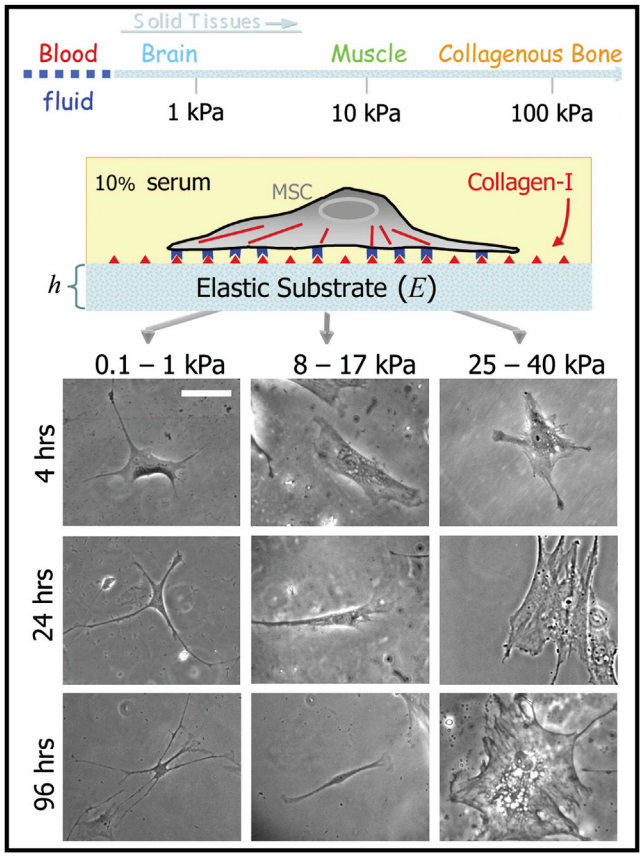

FIGURE 2 Schematic of the assay performed to identify the role of matrix elasticity in governing the differentiation potential of mesenchymal stem cells. A softer matrix, ~0.1–1 kPa, resulted in neurogenic differentiation, whereas a higher stiffness matrix, ~8–17 kPa, resulted in myogenic differentiation. Even higher stiffness, ~25–40 kPa, resulted in osteogenic differentiation. Figure reproduced with permission from Engler et al. (26). To see this figure in color, go online.

Other interesting aspects of stem cell biology include dedifferentiation (inducing pluripotency in adult cells (iPSC biology)) and transdifferentiation (reprogramming one adult cell type to another). In 2006, the field of stem cell biology was revolutionized by identification of a cocktail of just four transcription factors (OCT4, Sox2, Klf4, and c-Myc) by Yamanaka and colleagues that would enable dedifferentiation of mature adult cells back to an embryonic-stem-like pluripotent state (34). After this, various chemical approaches including different sets of transcription factors, small molecules, and even recombinant proteins have been identified that could achieve induction of pluripotency (35–37). However, despite best efforts, the efficiency of these induction approaches has remained low (34). Recent reports of deterministic reprogramming have been challenged by contradictory data (38,39). Moreover, separate studies have highlighted the stochastic nature in the early phase of the reprogramming process (40). The question remains: would engineering mechanical cues in synergy with the chemical/genetic induction pathways have a favorable influence on improving the overall

efficiency of the process? If so, one could modulate these signals as a function of time to have an impact on the kinetics of reprogramming as well. Our understanding of induction of pluripotency may remain incomplete without a fuller elucidation of the forces at play. In addition to an improved fundamental understanding of the process, such investigations may also be useful in hastening translational opportunities using iPSCs. Beyond stem cell biology, taking inspiration from the Human Cell Atlas Project and leveraging improvements in spatiotemporal resolution for fluorescence imaging—super-resolution microscopy—and those in multiplexing force-spectroscopy data, it may be an opportune time for the biophysics community to aspire to the following moonshot: to generate force-map representations with biophysically complete information regarding identities, distributions, and mechanical interactions of every protein within a human cell (41). Whereas efforts such as the Human Cell Atlas Project aim to catalog the identities and distributions of proteins within the cellular milieu, information about their interactions based not only on their thermodynamic and kinetic properties (binding) but also on mechanics (including nonequilibrium dissociations) will provide a fuller picture.

Supracellular

Using force to interrogate biological systems beyond the cellular level of organization—the supracellular level—has provided useful insights (42). For instance, using a combination of genetic and mechanical perturbations, Chanet et al. identified that in response to mechanical constraints, the actomyosin meshworks exhibit a force-orienting mechanism, which may in turn govern cell orientation (43). Such fine control over cell orientation may provide a starting point for mechanical control over organismic morphology. Eastwood et al. developed a technology they called feedback-controlled application of mechanical loads combined with in vivo neurophysiology to tease out the role of touch-receptor neurons in *Caenorhabditis elegans* as a band-pass mechanical filter (44). Through clever use of multimodality approaches, including AFM, laser axotomy, and Förster resonance energy transfer, Krieg et al. identified the central role played by spectrin, an actin-membrane crosslinker, in mediating the sense of touch (45). Although these are examples of cells and tissues taking mechanical cues from their environment, studies have also shown force generated by cells being used to reengineer the ECM around it (46). Tissue-level studies are not limited exclusively to healthy tissue. Shi et al. demonstrated the necessity for two mammary acini, the basic anatomical unit of the mammary gland, to be in mechanical contact with each other to trigger rapid onset of disorganization and invasion of neighboring tissue resulting in metastasis (47). Using an increase in birefringence as a proxy for collagen linearization, Acerbi et al. showed that human breast cancer transformation was accompanied by a concomitant increase in thickening of interstitial collagen (48). Stress fields generated by multicellular tissue in a 3D microenvironment have been measured previously by placing cell-sized, fluorescently labeled oil droplets within growing tissue. Measuring the stress field as a function of deformation of the said oil droplet, Campàs et al. estimated the stress generated by mammary epithelial cells in a 3D matrix (49). Despite progress, the supracellular length scale continues to present its own unique set of challenges to being interrogated mechanically. Specifically, the complex interplay between mechanical cues orchestrating cellular responses, and in turn the cellular chemistry dynamically reorganizing the ECM, present a rather adversarial environment for force sensors to be placed in. Label-free interrogations, such as the previously mentioned birefringence-based approaches, can be nontrivial to generalize across tissue types or calibrate for quantitative stress measurements.

Historically, for SMFS experiments, physical models have been successful at describing both the material properties of soft mesoscopic materials such as DNA (e.g., using equilibrium models such as the worm-like chain or freely jointed chain) and the kinetics of inter- and intramolecular transitions (e.g., using Kramers'-style models such as Bell-Evans, Dudko-Hummer-Szabo, and Friddle-De Yoreo) (50–54). As we progress toward studying complex heterogeneous systems such as cell-substrate interactions at the supracellular level, our need to develop higher-level phenomenological models to explain the data also becomes urgent. Because cellular mechanics involve many different length scales, which in turn offer different boundary-condition constraints on models, multiscale dynamic modeling could be one appropriate approach. Additionally, multiplexing SMFS measurements results in large data sets, necessitating the need for better computational approaches to handle this data. Although efforts are in their infancy, groups have already started exploring the possibility of utilizing machine learning—computational pattern recognition without explicit programming—to model force spectroscopy data (55,56). Similarly, for cellular measurements, computer-vision tools have been exploited to generate 3D digital atlases, e.g., for *C. elegans* (57). An early example of how such tools would be useful in systems analysis was the use of Cellprofiler in conjunction with other packages to study the process of induction of pluripotency (58). Through retrospective analysis, Smith et al. identified an increase in proliferation rates for mouse embryonic fibroblasts in conjunction with a decrease in their cellular area as a proxy for their reprogramming potential (59). The authors propose a combination of an "elite" deterministic model and a stochastic model to explain their data. Though alternative models have been proposed and a consensus eludes the scientific community, the study shows mechanistic insights that could be teased out through the use of sophisticated and automated image analysis (60). In closing, supracellular length scale offers perhaps the most fertile ground for

progress in the broader field of biomechanics. From developmental to cancer biology, there are a plethora of problems at this length scale that would benefit from biomechanical interrogations. New and emerging tools, both hardware and software, are required to address this need.

On the horizon

In this section, we briefly discuss recent innovations in the field and work just on the horizon. For instance, using DNA as a molecular spring to interrogate proteins has been proposed previously (61). Scaffolded DNA origami in particular could provide a powerful toolbox to accelerate development in the field. Studies have used DNA as a construction material to generate nearly arbitrary shapes, both in two- and three-dimensional geometries, with high fidelity (62,63). Pfitzner et al. showed that using DNA origami instead of the more traditional double-stranded DNA tethers in single-molecule force measurements, i.e., stiffer tethering, helps reduce thermal noise (64). More recently, efforts have focused on utilizing origami-based structures as force spectrometers (65,66). For instance, Funke et al. developed a hinged DNA-origami structure, shown in Fig. 3, to study internucleosome pair potentials (65). For this assay, the vertex angle between the two leaves of the hinged structure was controlled by the interaction force between nucleosome pairs and was measured by cryo-electron microscopy and Förster resonance energy transfer. Nickels et al. reported using DNA-origami structures as anchor points to position single-stranded DNA handles that can host guest molecules under study (67). Exploiting the single-stranded DNA handles as entropic springs, the authors reported applying pN-level forces on DNA Holliday junctions. The utility of the approach was highlighted by the measurement of transcription factor TATA-binding-protein-induced distortion and its impact on DNA tension. With the possibility of performing measurements in solution, future developments in DNA-origami-mediated force spectroscopy may open the door to interrogations of an Avogadro's number of interacting molecules. Clever reporter design could lead to ultra-high-throughput measurements in search of rarely occupied states in an energy landscape or rare trajectories. Such high levels of multiplexing have been inconceivable until recently.

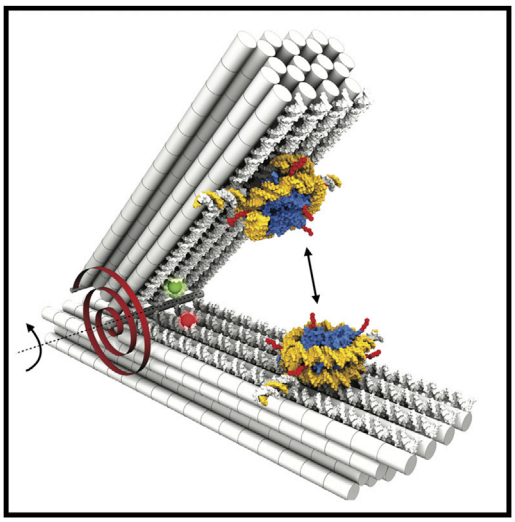

FIGURE 3 Cartoon representation of a self-assembled force spectrometer. Such a system has been used to measure distance-dependent pair potential between a histone pair. Figure reproduced with permission from Funke et al. (65). To see this figure in color, go online.

Within the field of structural biology, an intriguing example of biomechanical perturbation was reported by Hekstra et al. (68). The authors report using high-intensity electric fields to perturb protein crystals and subsequently probe the dynamics of recovery using time-resolved x-ray crystallography. Such a pulse-probe approach, termed electric-field-stimulated protein mechanics, could help uncover the structures of previously unknown force-induced states and folding intermediates. At a longer length scale, could we imagine coupling information gleaned from deep-tissue imaging to mechanical perturbation? In the longer term, photo-triggered nanoactuators could potentially be utilized for therapeutic applications (69). Magnetic fields could also be used—Serwane et al. incorporated biocompatible, magnetically responsive ferrofluid microdroplets as local mechanical actuators within growing zebrafish embryos (70). In the future, a system like this could be generalized to actuate a variety of mechanical responses within biological tissue in situ.

Finally, retracing a similar arc of history as physics, biophysicists are beginning to utilize an improved understanding of biomechanics to engineer the world around us. Korin et al. engineered micron-sized shear-activated nanoparticle aggregates and loaded them with a thrombolytic drug for improved "clot busting" (71). Release of the drug is triggered by the decreased lumen at the site of the clot, making such approaches very precise. The field of sustainable energy sources provides another example. Recognizing the potential of *Bacillus* spores as a transducer for converting chemical potential to useful mechanical work, Chen et al. engineered evaporation-driven engines and generators (72,73). A recent modeling study suggests that within the US alone, the generators engineered using this technology would be able to produce ~325 GW of power, a figure equivalent to almost 70% of the total electrical energy generation rate for the US in 2015 (74).

Richard Feynman said, "What I cannot create, I do not understand." And by that yardstick, our understanding of the role of force in biology, despite impressive progress, is still limited. Could we utilize our knowledge of mechanics between components that we have already studied to design a de novo structure with prescribed mechanical properties? We could aim to decipher the emergent mechanics of complex macromolecular assemblies based on information regarding individual members of such assemblies (75). We are only beginning to scratch the surface in mapping the communication between the mechanical and chemical

circuitry of a cell (24). Will we be able to predict the temporal evolution of a cell's behavior, under chemical or mechanical perturbations, based on such a mapping? To realize such goals, we will have to utilize available force-spectroscopy tools in novel and clever ways, in addition to developing new tools and mathematical frameworks. To promote the full adoption of such tools among the broader community of biologists and expand the field of force spectroscopy, we should also reduce barriers of complexity and cost. "Instrument-free" approaches, such as molecular devices that use gel-based readouts, or inexpensive high-throughput approaches, such as centrifuge-based force spectroscopy, could potentially alleviate at least some of these challenges. Going beyond technologies for scientific discoveries, engineering therapeutic interventions may be within the reach of molecular biophysicists. For instance, approaches such as DNA self-assembly could be harnessed to design ways to orchestrate molecular interactions at cell surfaces. Understanding physiological responses in response to force-mediated intervention would provide a read out for such approaches. We encourage readers to be creative and to think beyond the standard systems that have been studied, the standard tools that have been used, and the standard problems that have been examined. Forces are everywhere, and with the remarkable methods that have been and are being developed, many new opportunities are emerging.

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