Imaging methods in mechanosensing: a historical perspective and visions for the future

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ABSTRACT **Over the past three decades, as mechanobiology has become a distinct area of study, researchers have developed novel imaging tools to discover the pathways of biomechanical signaling. Early work with substrate engineering and particle tracking demonstrated the importance of cell–extracellular matrix interactions on the cell cycle as well as the mechanical flux of the intracellular environment. Most recently, tension sensor approaches allowed directly measuring tension in cell–cell and cell–substrate interactions. We retrospectively analyze how these various optical techniques progressed the field and suggest our vision forward for a unified theory of cell mechanics, mapping cellular mechanosensing, and novel biomedical applications for mechanobiology.**

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INTRODUCTION

The cell as a mechanical object

Over the past 30 years, an explosion of scientific ingenuity has resulted in a renaissance for the field of mechanobiology. The earliest predictions about the mechanical nature of the cell can be attributed to Sir D'Arcy Thompson at the turn of the 20th century (Thompson, 1992), although views of cells as mechanical entities were not fully appreciated until the 1990s (Sims *et al.*, 1992; Ingber, 1998). In early studies of tissue engineering where cells were observed within engineered microenvironments, the recognition of cells as sensitive and responsive to their mechanical environment emerged. The study of mechanobiology has progressively entered mainstream biological study and coupled the fields of biology, physics, and engineering (Katta *et al.*, 2015).

One particularly poignant historical example of the shift in the paradigm from the view of cells as bags of biochemical reactions to cells as mechanical structures was the tensegrity model introduced in the early 1990s (Sims *et al.*, 1992). The tensegrity model proposed a pseudoequilibrium state in which the actin-matrix is constantly under tension, microtubules are constitutively under compression, and the intermediate filaments are the linkers between the two allowing for the mediation of these opposing forces (Ingber, 1998). Work from the Ingber group developed this hypothesis, demonstrating a direct connection between stimulation of integrins and cellular reorganization in the direction of the pulling force (Maniotis *et al.*, 1997). Subsequently, studies on cell–matrix adhesion were able to demonstrate definitively that the geometric arrangement of cellular adhesion directly impacts the rates of cell division and proliferation, or cell senescence and apoptosis (Chen *et al.*, 1997). Crucially, fluorescent and transmitted light microscopy were necessary to observe these phenomena and develop the idea of a mechanically integrated cell showing the distributions of each cytoskeletal element and connection to mechanosensitive proteins. New experiments demonstrating a cellular behavior linked to mechanical stimulation (Maniotis *et al.*, 1997), along with downstream effects on cell shape and proliferation (Chen *et al.*, 1997) and the tensegrity model of the cell, were posited to sufficiently explain mechanotransduction. However, it lacked a direct mechanistic explanation of mechanosensing at the molecular level and was flawed in that none of the components of the cytoskeleton were under any type of constitutive force. It became clear the concepts of actin-microtubule tension–compression force balances were unable to explain complex cellular behavior, but it was necessary to understand how phenomena such as cell size, cell shape, and cytoskeletal organization were mechanically integrated to influence cell fate.

Contemporary understanding of mechanically relevant cellular structures has improved dramatically since the original tensegrity model, expanding to encompass linkages among actin, microtubules, and a large number of intermediate filaments as well as

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Abbreviations used: CadTS, Cadherin-TS; ECM, extracellular matrix; FRET, Förster Resonance Energy Transfer; MSD, mean square of displacement; TFM, Traction force microscopy.

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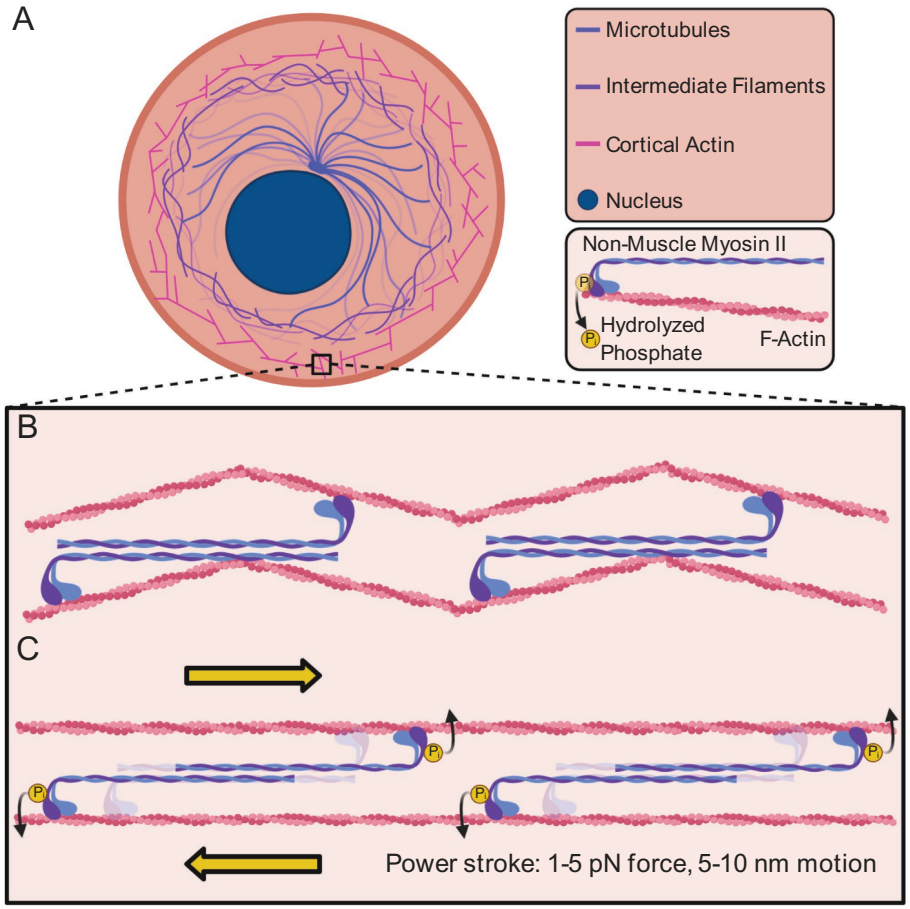

FIGURE 1: Force generation within all nucleated cells. Although there are many molecular motors within cells, cellular force generation in most cells results from myosin II on actin. (A) Cells contain many cytoskeletal filament networks including microtubules, intermediate filaments, and actin. (B) Actin filaments are polymerized throughout the cell in relaxed state. (C) Myosin II contracts the cytoskeleton, generating force on the actin during activation. ATP provides the energy source for stretching of the actin filament by myosin II. Each Myosin power stroke provides between 1 and 5 pN of force and moves down the actin filament by 5–10 nm (Finer *et al.*, 1994; Takagi *et al.*, 2006). Created with BioRender.com.

mechanical integration of the plasma membrane, cytoskeleton, and nucleus. Rather than an equilibrium system of tension–compression elements perpetually sustaining the structure of the cell, we now know the dynamic and energetically fluctuating nature of the cytoskeleton. Cellular forces are generated by motor proteins and filament polymerization; these forces are spatially distributed and temporally regulated throughout the cell with a complicated set of accessory proteins and feedback networks (Fabry *et al.*, 2001). Cell force generation and translocation is ultimately driven by myosin inside the cell, which individually generates piconewtons of force or nanometers of displacement, as shown in Figure 1, but myosins and actin can work in concert for larger magnitudes of force.

Despite decades of study, no singular model has been sufficient in capturing the diverse biomechanical environment of human tissues. Thus, a number of questions remain: how do molecular structures ultimately function on a whole-cell level, and how are these processes conserved in cell monolayers and organoids? Can we reformulate existing models and methodologies to encompass the complexity seen in diverse cellular systems? Are mechanical models of biological processes a purely academic study or are there meaningful medical applications that can be derived from this approach?

To answer these questions, we aim to briefly examine the past accomplishments that have driven the field of mechanobiology to where it is today in the context of the technologies that made it possible to do so. Additionally, we will briefly address the role of biochemical mechanotransducers that affect downstream processes such as transcription and translation. Finally, we will discuss recent studies that demonstrate the developing role of mechanotransduction for future biomedical applications.

EARLY STUDIES IN MECHANOBIOLOGY

Substrate modifications

Some of the first mechanistic studies to examine cellular mechanobiology included cells grown on reduced stiffness substrates compared to glass or tissue culture plastic. It was shown that substrate stiffness was inextricably linked to cellular locomotion (Pelham and Wang, 1997), cell morphology (Chen *et al.*, 1997; Pelham and Wang, 1997), and downstream cellular signaling (Chen *et al.*, 1997). By comparing cells on polyacrylamide gels of varying stiffness, Pelham and Wang demonstrated that the size of focal adhesions, distribution of the cytoskeleton, and cell shape were significantly altered on softer gels (Pelham and Wang, 1997). In addition to a reduction in cell size on soft cells, localization of the focal adhesion protein vinculin was altered. Inhibition of the actin cytoskeleton on stiff substrates showed irregular focal adhesions similar to those observed on soft substrates (Pelham and Wang, 1997). Furthermore, the inhibition of tyrosine phosphatases allowed cells plated on soft substrates to recover the same cellular geometries, cytoskeletal structures, and vinculin activity that are seen in cells plated on stiff substrates. Thus, the authors discovered evidence of a mechanosensing feedback loop within the cell (Pelham and Wang, 1997). This concept of mechanical feedback was further reinforced when Chen *et al.* demonstrated that forcing cells to adopt a controlled shape by patterning the substrate surface results in stark differences in cell proliferation and survival (Chen *et al.*, 1997). Cellular geometry affects some of the most tightly controlled signaling pathways in the cell, modifying focal adhesions, proliferation, and apoptosis.

Recent studies with modern substrate engineering approaches have begun to elucidate the more nuanced effects of cell–substrate interactions on terminally differentiated cells. Cells exert piecewise contractile forces with a similar mean displacement step size on engineered nanopillars with 1.2 nm for single pillars and 2.5 nm for pillar pairs (Wolfenson *et al.*, 2016), highlighting both the nanometer step size and the focal adhesion complex illustrated in Figure 2B. The cellular response to extracellular stiffness also has implications in gene expression and cellular specialization (Engler *et al.*, 2006). Engler *et al.* modified only the stiffness of the surface on which mesenchymal stem cells were plated and showed that cell morphology and downstream transcriptional markers associated with lineage

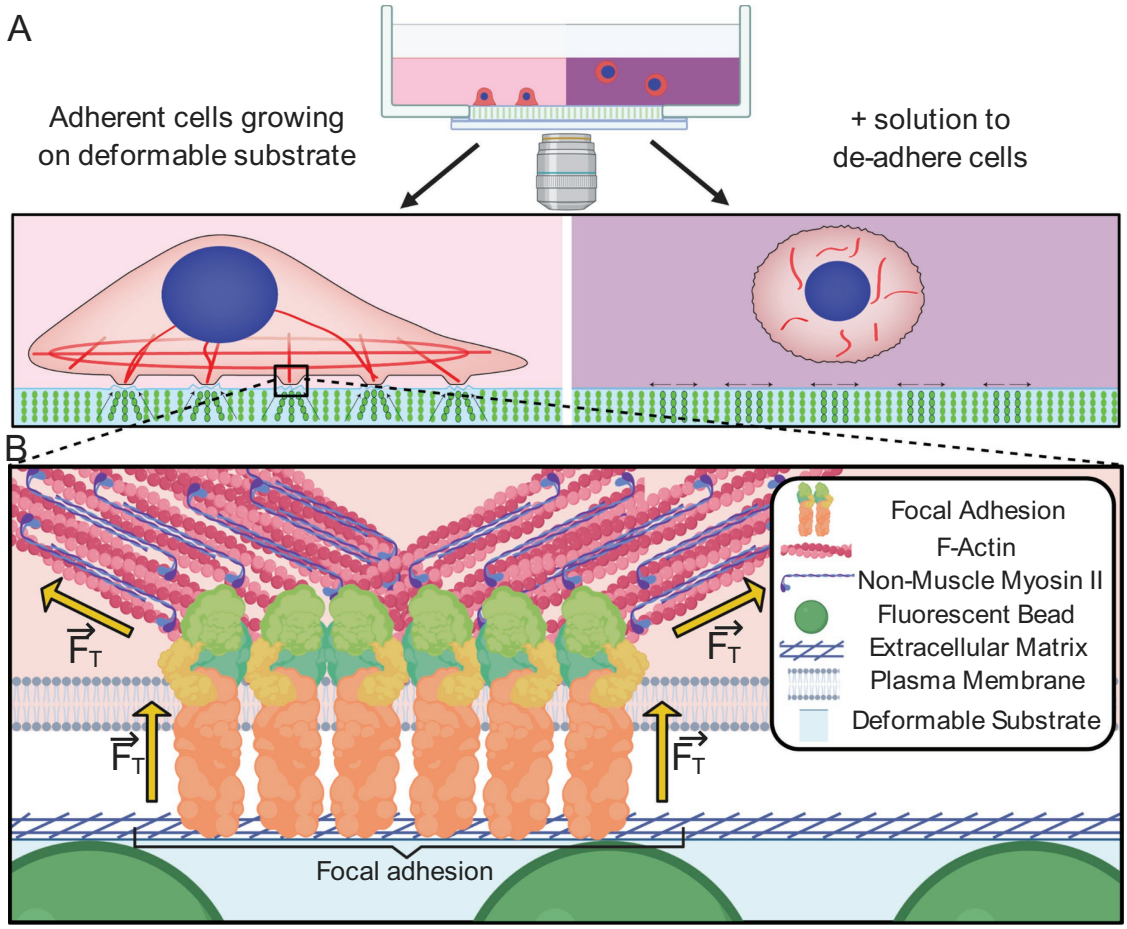

FIGURE 2: TFM allows quantification of cell–substrate force generation. (A) Cells are plated on a deformable substrate, which are embedded with fluorescent beads for tracing, or on deformable columns of varying stiffness. On the left, an adherent cell pulls on a deformable substrate and displaces fluorescent beads. Conversely, on the right, a treatment is applied to unadhered cells from the surface. In effect, the beads return to rest positions. Top created with BioRender.com. (B) A molecular scale view of the cell–substrate interface shows many adhesion complexes are present at the interface of attachment bound to ECM proteins on the surface of the substrate. Inside the cell, adhesion complexes are tethered to the actin cytoskeleton. Myosin II motors pull on the complex, generating traction forces that are measured by bead displacement. Created with Biorender.com.

specific differentiation were shown to vary dramatically. Inhibition of myosin II motor protein activity via blebbistatin reduced transcription of key lineage markers to levels seen in differentiated cells, again underscoring the importance of mechanical sensing in cell fate determination (Kovács *et al.*, 2004). Ultimately, it was an endorsement of the principles proposed at the turn of the 20th century, and definitive validation of hypotheses made by more recent work (Chen *et al.*, 1997; Pelham and Wang, 1997) connecting cell–substrate interactions and cell morphology to changes in cell fate. For the interested reader, we direct them to these excellent sources discussing in greater depth the methods for substrate engineering (Yu and Groves, 2010; Desai *et al.*, 2014; Enemchukwu and García, 2017; Faia-Torres *et al.*, 2017), impacts of treatments on a variety of cells (Cosson *et al.*, 2015; Vincent and Engler, 2017) and tissues in culture (Petrie *et al.*, 2017), and efforts to scale up these technologies to engineer transplantable tissue (Woodfield *et al.*, 2017).

Traction force microscopy (TFM)

An advantageous extension from studying cells on polyacrylamide gels was the ability to imbed fluorescent beads within the gels. The addition of these fluorescent fiducial markers to the variable stiffness substrate, and the necessary computational methods (Dembo *et al.*, 1996; Dembo and Wang, 1999; Munevar *et al.*, 2001b) for measuring cell tractions, allowed cell force to be quantified and correlated to locomotion and morphology (Figure 2). TFM leverages both transmitted light imaging as well as fluorescence to observe cells generating a displacement field of the embedded fiducial markers which can be compared with the rest position in a linear, uniform, and isotropic elastic material (Figure 2, A and B). Subsequently, assuming the substrate mechanical properties are unchanged by the inclusion of tracer beads and z-oriented traction forces are negligible, it is possible to back-calculate the force applied by the cell (Kraning-Rush *et al.*, 2012; Style *et al.*, 2014). This model can be further extended to small colonies or pairs of cells assuming a balance of cell–cell forces exerted on members of the cluster (Trepat *et al.*, 2009; Liu *et al.*, 2010; Maruthamuthu *et al.*, 2011). TFM can similarly track spatial changes in cellular forces during cell motility with both single cells and cell monolayers, although forces measured vary with cell type and distance from the edge of the cluster/monolayer (Dembo and Wang, 1999; Munevar *et al.*, 2001a; Trepat *et al.*, 2009). Additional details regarding the computation of traction forces and key assumptions and applications of the

technology can be found in Kraning-Rush *et al.* (2012) and Style *et al.* (2014).

One major advance derived from TFM has been the development of the "clutch-motor" model by Chan and Odde (Chan and Odde, 2008). The Clutch-Motor Model suggests that large molecular complexes have a dynamic range of force generation which is related to the experienced strain. In their original work, Chan demonstrated that neuronal growth cones are able to generate tension on a substrate surface and induce actin polymerization immediately, increasing in magnitude proportional to a threshold substrate stiffness on which frictional slippage occurs. Others later showed the same clutch-motor phenomenon of force generation, failure, and slippage with focal adhesions (Plotnikov *et al.*, 2012), actin stress fibers (Owen *et al.*, 2017), and microtubules (Prahl *et al.*, 2018). Importantly, cellular force generation occurs in cycles at these structural locations, and the cyclic frequencies are a function of the substrate stiffness (Elosegui-Artola *et al.*, 2018). This model has been successful at predicting single cell migration as well as durotaxis (Sunyer *et al.*, 2016) and has helped explain how cell adhesions are mechanically integrated, albeit only for certain cell lines.

TFM involving micropillars and other deformable substrates have provided decades of invaluable data on cell force generation and mechanobiology using light microscopy. However, limitations exist that have necessitated parallel approaches by other technologies. TFM yields information about forces that cells exert externally, but explains little about internal force production. This becomes relevant when considering more than a single cell since cells redistribute forces from focal adhesions to cell–cell junctions (Maruthamuthu *et al.*, 2011; Sim *et al.*, 2015). Thus, in small cell clusters or in monolayers, limited information can be derived from TFM (Maruthamuthu *et al.*, 2011). Some modifications to TFM have included patterning of cell clusters to control cell–cell interactions (Liu *et al.*, 2010; Maruthamuthu *et al.*, 2011) and tracking the leading edge of a monolayer (Trepat *et al.*, 2009). Also, TFM analysis works well in two-dimensional (2D) culture conditions, but one-dimensional (1D) conditions (such as cells growing along fibers) or three–dimensional (3D) constructs are more difficult to experiment and analyze computationally. The imaging hardware to measure small deflections in 3D over time, as well as the postprocessing software to integrate forces over complex, time-dependent boundaries are both challenging. The 3D cell culture is arguably more relevant for tissue morphogenesis, wound healing, and cancer metastasis than 2D culture (Friedl *et al.*, 1998). But, single cell imaging of 3D TFM cell–matrix interactions conflicts with the calculated localization of traction stresses observed in 2D studies (Owen *et al.*, 2017) requiring further work to reconcile these approaches.

Groups around the world are actively working to develop new algorithms capable of accurately deconvolving 3D substrate deformation with cells in embedded matrices. Some approaches rely on a micropatterned substrate that exists as a series of columns for cells to grow on with embedded fluorescent dye molecules (Banda *et al.*, 2019). Compared with alternative microcolumn approaches (Schoen *et al.*, 2010), this novel technology allows for Z deformation to be observed, as unaltered pillars are the control reference for deconvolution of cellular deformations. Interestingly, this new technology has validated that cells not only can exert contractile forces but also can seemingly generate compressive forces, which has been indirectly shown through measurements with Förster resonance energy transfer (FRET) tension sensors (Rothenberg *et al.*, 2015). Another approach involves embedding cells within 3D hydrogels made of extracellular matrices like collagen (Cassereau *et al.*, 2015; Mekhdjian *et al.*, 2017) or fibrin (Owen *et al.*, 2017). Like 2D hydrogels, of which the elastic modulus can be modified by variable concentrations of crosslinker, 3D hydrogel elasticity can be modified by a combination of ECM protein concentration and stretch. These methods have demonstrated that matrix elasticity within 3D hydrogels can induce metastasis in tumorigenic cells, these cells are more contractile and exert higher integrin forces, and they are more efficient at crawling through the matrix (Mekhdjian *et al.*, 2017). Additionally, it was shown that unlike in 2D traction force measurements, cells exert strains that perturb the entire matrix around a cell protrusion from several regions along the surface of the cell (Owen *et al.*, 2017). Others are working to increase accessibility to 3D TFM by creating protocols and computational algorithms for epifluorescent 3D TFM (Hazlett *et al.*, 2020). Going forward, TFM methods, including multicellular interpretations and 3D protocols, are expected to be more accessible to researchers who do not specialize in these complicated technologies.

Particle tracking microrheology

In the early 2000s, physicists who had largely considered entanglements of semiflexible polymer networks became interested in cytoskeletal networks in cells. At first, purified cytoskeletal filaments were reconstituted and measured for their mechanical properties (Janmey *et al.*, 1991; Gardel *et al.*, 2003), but it was unclear whether ex vivo results could be extrapolated to intracellular conditions. Microrheology was one of the chief technologies developed that allowed the biophysical properties of the cell and molecular motors to be probed in situ. Rheological measurements inside the cell included particle tracking of phase dense or fluorescent fiducial markers, the frequency-dependent movement of which was a function of the viscoelastic properties of the cell (Tseng *et al.*, 2002; Valentine *et al.*, 2004). In other words, a tracer particle (described below) is tracked, and the motion of the particle is descriptive of the material within which it is embedded. Cellular rheology has shown that the material properties of the cell are best modeled by a weak power law, as was demonstrated through combining theory with many frequency domains of data (Fabry *et al.*, 2001). Importantly, it was discovered that the primary driving force of cellular fluctuations comes from molecular motor proteins, and that these fluctuations exist on different imaging frequencies than thermal motion (Lau *et al.*, 2003; Van Citters *et al.*, 2006). Thus, although particle tracking microrheology was developed to measure cell mechanics, in many ways it measures a "noise" above the thermal floor via force generation provided by molecular motors (Lau *et al.*, 2003; Hwang *et al.*, 2016).

Particle tracking microrheology approaches rely on measuring quantitative displacements of intracellular tracers that either exist endogenously or are introduced into the cell (Tseng *et al.*, 2002; Wirtz, 2009). There are many factors to consider when choosing tracers to determine rheological properties. For example, to consider rheological properties of actin networks, tracers should be bound to the actin matrix or should be sufficiently large to fill a void pocket of the actin network. Otherwise, the tracer could diffuse within the void fraction or "cage" of the actin network, and particle tracking would yield the diffusion coefficient of cytosol. So long as the particle is approximately the correct size to fit the matrix void of whatever intracellular material is being probed, the measurements attained are independent of the probe's size or shape.

Additionally, the biological impact of the tracer should be considered. For example, large beads can be microinjected or introduced to cells by endocytosis and have the advantage of phase-contrast differences or magnetic manipulation. However, microinjection is a low-throughput approach and can be highly

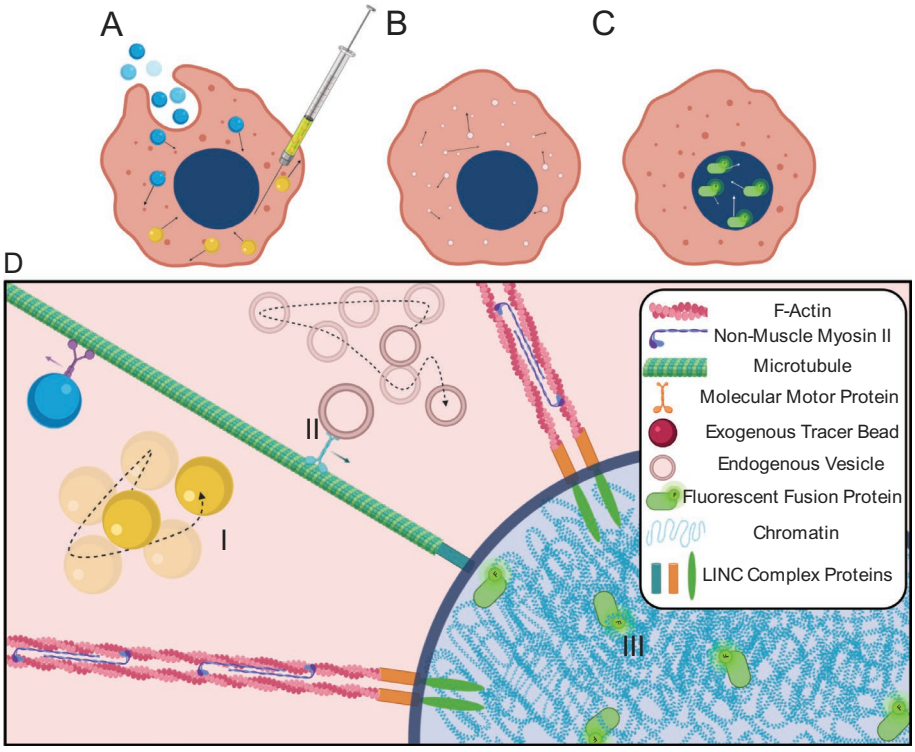

FIGURE 3: Particle tracking microrheology allows for rheological studies of various cell compartments. (A) Either fluorescent (yellow) or nonfluorescent (blue) microbeads can be artificially introduced via endocytosis or microinjection for particle tracking. (B) Endogenous lipidoids or vesicles can also be used as fiducial markers for tracking. (C) Fluorescently expressed proteins can be used to target to specific regions of the cells, including nuclear proteins for the SINK methodology. (D) A variety of phenomenon that can be studied with different particle tracking approaches. (I) Brownian motion of the cytosol or any intracellular medium can be evaluated by microrheology, performed on both exogenous microspheres and endogenous vesicles. (II) Motor protein activity or other forms of directed motion within the cell can be tracked by visualizing cargo. (III) A mix of both molecular motor activity as well as Brownian motion, such as that sampled by the SINK approach. Created with BioRender.com.

cytotoxic and may disrupt endogenous structures, while endocytosis relies on cellular behavior which takes control out of the researchers' hands (or can be countered by exocytosis of the tracer as well). Furthermore, microinjection is a slow process, and endocytosed materials may show properties of the endosome rather than the cellular compartment of interest. Tracking of endogenous tracers such as lipidoids or vesicles can be relatively easy to implement and has the potential to be high throughput, but researchers lose the ability to probe subcellular structures to which the tracer is not endogenous. Lastly, fluorescent tags have allowed particle tracking of compartment-specific regions and organelles, and these proteins are often bound to the matrix of interest simplifying experimental concerns. Unfortunately, overexpression of genetically encoded tracers may alter the subcellular structure probed, and relevant controls may be necessary. Furthermore, photobleaching or expression levels may severely limit data collection, making this approach more challenging to optimize. Recently developed, bright, photostable fluorescent proteins may alleviate this problem, and creation of stable cell lines may help balance out inhomogeneous expression typical to transfection-based technologies. Figure 3 summarizes each of these individual approaches (Figure 3, A–C) as well as the biophysical phenomena that can be sampled through particle tracking microrheology (Figure 3D, I–III).

While introducing tracer particles into cells has become increasingly easier, the processing and interpretation of the displacement data—similar to TFM—requires significant postprocessing. Theoretically, movement in a viscoelastic continuum is incoherent, and the net displacement of the tracers should be statistically zero, consistent with a random walk (Berg, 1993). Measurements of tracer movements are therefore presented as the mean square of displacement (MSD) and averaged over the imaging range of frequencies or times (Wirtz, 2009). Based on the sampling time, T, either the inherent thermal fluctuations of the cell can be measured (10^{-6} s < T < 10^{-3} s) related to the material properties of the cell or the effects from molecular motors can be observed (10^{-2} s < T < 10^{1} s) (Van Citters *et al.*, 2006). Furthermore, it is possible to expand this approach by correlating the movements of two particles within a matrix, which yields additional insights. Two particle microrheology can distinguish localized activity from global modifications to the polymer network, making it possible to measure a tracer being actively carried by motor proteins versus modifications to the cytoskeleton such as by depolymerization (Lau *et al.*, 2003; Crocker and Hoffman, 2007). Particle tracking microrheology has shown mechanically distinctive regimes of the cortical cytoskeleton, the lamellipod, and the cytoskeleton within the body of the cell (Hoffman *et al.*, 2006). Fluctuation in the cytoskeleton can be measured directly by changes in the structure of the network itself or by changes in structurally associated organelles. Given these two techniques, rheological measurements reveal information solely about the polymer network but do not imply what biological phenomena caused the state of the network. In other words, whether actin is depolymerized via capping of free ends with cytochalasins or inhibition of g-actin dimerization via latrunculins cannot be determined from a rheological measurement. For rheological approaches, specific mechanistic predictions or conclusions about the cell or biochemical pathways is challenging in the absence of a priori biological knowledge.

Our group has worked actively to develop an approach that can estimate relative changes in rheological properties of the actin-myosin network of the cell by measuring fluctuations within the cell nucleus (Booth-Gauthier *et al.*, 2012; Spagnol and Dahl, 2014) (Figure 3, C and DIII). The nucleus is interconnected with the cytoskeleton and does not fluctuate greatly within the interphase cell compared with actin, which actively remodels (Dahl *et al.*, 2006; Kirby and Lammerding, 2018). Energetic fluctuations in the cytoskeleton propagate into the nucleus and can be observed in intranuclear tracer motion. For example, reduced myosin motor activity on the actin cytoskeleton can be seen in a decrease in the MSDs of fluorescent tracers in the nucleus (Spagnol and Dahl, 2014). Subsequent studies have been able to demonstrate for the first time that changes to the external environment of the cell, such as substrate compliance or cell confluence, can actively affect movement

of the nuclear interior (Armiger *et al.*, 2018). The interconnectivity of regions of the cell as well as cell–cell connections can be measured by tracking fluctuations inside the nucleus (Spagnol *et al.*, 2016).

Thus, the unique contributions of motor proteins and a heterogeneous cytoskeleton to cellular rheology complicate measurements and theory both spatially and with respect to time (or frequency). Generally, the rheological model of the cell is widely accepted to be a viscoelastic solid; the exact parameters of the viscoelasticity appear to be "scale-free" similar to that of a soft-glassy material (Fabry *et al.*, 2003). While there are times and frequencies associated with motor movements, actin polymerization, and leading edge "waves" (Masters *et al.*, 2016; Zhan *et al.*, 2020), there are no characteristic timescales associated with mechanics or relaxation states. This model has been used to fit many types of experimental data, has proven useful in characterizing cell behavior, and has predictive power for cellular deformability under unique conditions (Mandadapu *et al.*, 2008). However, within a model that has nearly infinite relaxation states comes the problem of molecular anonymity: specific proteins or molecular pathway factors cannot be easily ascribed to specific changes in mechanics the way that the stiffness of a spring could be modulated in a simple spring-dashpot model. Thus, mechanical changes often appear convoluted beyond the other confounding effects on the structure and biology of the cell.

FRET tension sensors

TFM and particle tracking rheology both integrate large numbers of motors throughout the cell: traction force measures displacement of the cell's substrate and particle tracking measures primarily fluctuations of an entangled cytoskeleton from motors. However, measuring discrete forces and displacements within the cell has been limited. Developed within the past 11 years, intramolecular tension sensors based on FRET have allowed for spatially relevant measurements of nm displacements and pN forces. This highly adaptable technology has been used to probe mechanical function of molecular motors (Yildiz *et al.*, 2008) and structural proteins (Smith *et al.*, 2007), protein–protein interactions (Meng *et al.*, 2008; Grashoff *et al.*, 2010; Kumar *et al.*, 2016), DNA binding and interactions (Long *et al.*, 2016), cell–substrate (Smith *et al.*, 2007; Grashoff *et al.*, 2010; Kubow *et al.*, 2015; Kumar *et al.*, 2016) and cell–cell linkages (Conway *et al.*, 2013; Baddam *et al.*, 2018; Narayanan *et al.*, 2020). Unlike either of the previously discussed microscopy techniques, variants of molecular tension sensors have been applied in vitro using single molecule approaches, in single cells (vinculin, Grashoff *et al.*, 2010; talin, Kumar *et al.*, 2016) or small groups of cells, whole monolayers (cadherin, Borghi *et al.*, 2012), and even in vivo (B-spectrin in *Caenorhabditis elegans*, Krieg *et al.*, 2014; E-Cadherin in *Drosophila* and zebrafish, Cai *et al.*, 2014; Lagendijk *et al.*, 2017).

Tension sensor technologies have undergone many design iterations before arriving at the currently existing TSMod-based sensors. In FRET, two fluorescent tags (blue and red blocks in Figure 4) on either side of the linker (spring in-between red and blue blocks in Figure 4) are a FRET pair, which allows for tracking displacement in mechanotransducers with nm precision. The energy transfer occurs when three physical conditions that are met are true: molecules in the pair are within the critical distance of 1–10 nm (Figure 5B), the emission spectra of the donor fluorochrome must overlap with the excitation spectra of the acceptor fluorochrome, and both fluorophores must be aligned in the proper orientation. The FRET phenomenon exponentially decays with increasing distance and is very difficult to detect over inherent noise (beyond 10 nm with current instrumentation). Principally, all FRET tension sensors depend on a molecular spring that deforms with a range of pulling forces; therefore, as a Hookean spring the force applied is directly proportional to the displacement. Originally, tension force probes were generated from human macromolecules including the ECM protein fibronectin (Baneyx *et al.*, 2002), the spring-like protein spectrin (Meng *et al.*, 2008), or have relied on DNA constructs of various lengths (Janissen *et al.*, 2014). The dynamic range of tension forces and cooperativity of repetitive segments sampled by these proteins (Law *et al.*, 2003) as well as their large size make them poor candidates to use for protein engineering approaches. Conversely, DNA molecules have primarily been introduced into protein constructs in vitro experiments as linker integrity is compromised *in cellulo* by enzymes capable of degrading the DNA (Blanchard and Salaita, 2019). Crucially, early studies with FRET tension sensors demonstrated that cells exert nonlinear forces on their environment via the ECM, and that these forces play a role in cell–cell communication (Smith *et al.*, 2007).

Transgenic versions of FRET tension sensors have widely been developed with a highly reproducible and Hookean-spring linker known as TSMod. The flexible linker used in TSMod is the flagelliform protein (Becker *et al.*, 2003), a 40 amino acid-long peptide that functions as a molecular nanospring with a resolution of 1–5 pN that can stretch and return to a resting conformation without permanent deformation or significant hysteresis (Grashoff *et al.*, 2010). A generic FRET construct illustrating the TSMod insert is shown in Figure 4. In intramolecular FRET, loss of FRET is typically the result of separation of the pair of fluorescent molecules rather than changes in their relative orientations (Shrestha *et al.*, 2015). TSMod leverages this phenomenon to constrain the distances and geometries between fluorescent pairs and increase the likelihood of observing a signal that is independent of physical properties that naturally reduce FRET.

The TSMod construct has allowed new insights into how specific cellular loci transmit forces as well as how cells adapt to mechanically changing environments. Originally, Grashoff *et al.* demonstrated temporal, force-dependent assembly of focal adhesions with recruitment of vinculin (Grashoff *et al.*, 2010). As adhesions grow and stabilize, the average vinculin molecule experiences average forces of approximately 2.5 pN in endothelial cells. When the adhesion begins disassembly, vinculin is disconnected from the actin cytoskeleton resulting in a time-dependent loss of force on the protein before the disappearance of the retracting focal adhesion. Both RNAi-mediated knockdown of the myosin II or inhibition by Y-27632 dramatically reduce tension forces across vinculin relative to control cells (Grashoff *et al.*, 2010) and inhibit migration (Vicente-Manzanares *et al.*, 2009). Additionally, Borghi *et al.* (2012) integrated the same TSMod construct into E-Cadherin, showing that adherens junctions are under a constitutive tension of approximately 1.7 pN (Borghi *et al.*, 2012; Arsenovic *et al.*, 2017). Further, disruption of the actin cytoskeleton or myosin II motors reduced tension across Cadherin-TS (CadTS) measured between cells. Figure 5A illustrates the localization of signal of CadTS and Figure 5B shows a schematic representation of how CadTS works on a molecular level.

Although FRET tension sensors have allowed for quantification of biologically relevant in situ molecular forces, there are limitations that are difficult to recognize without experience. Compression of the sensor resulting in a loss of separation between the donor and acceptor fluorescent proteins will artificially increase the FRET efficiency measured from a single voxel. As force is extrapolated from FRET efficiency, an artificial increase in FRET efficiency may result in a diminished force calculation (Ham *et al.*, 2019). For some proteins, like cadherins, surface density may alter the perceived signal and subsequently underestimate average force on a single junction. It is

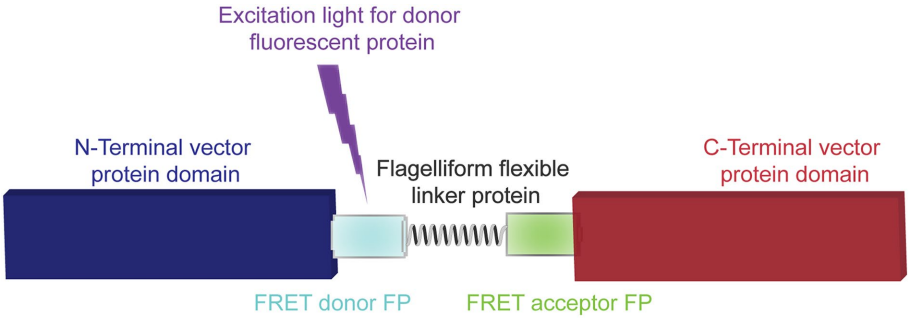

FIGURE 4: Design of the current iteration of modern tension sensor constructs based on TSMod. Seen internally, TSMod is a combination of a FRET pair (shown in teal and green above), separated by a flexible linker (40 amino acid repeat $GPGGA_8$). Flanking the TSMod insert on either side are the N- and C-termini of the protein being modified (when generating a fully functioning construct). FRET occurs when the teal-colored donor fluorescent protein is close enough to the green-colored acceptor, with simultaneous excitation of the donor. A detectable emission from the acceptor can then be observed, and the amount emission of the acceptor is related to the distance separating both molecules.

possible that high levels of expression or local packing of proteins may also introduce either intermolecular FRET or quenching of fluorescence from donor molecules. Moreover, given these challenges in cellular systems, some have leveled valid criticisms regarding the scalability of the technology and the potential to use it in complex organisms (Eder *et al.*, 2017). Furthermore, there are likely other uncharacterized phenomena that impact measured FRET and there is still some debate about the validity of using in vitro tension calibrations to extrapolate *in cellulo* forces (LaCroix *et al.*, 2018). Last, FRET data collection and processing are both cumbersome and computationally intensive, as spectral comparisons must be resolved pixel by pixel (or voxel by voxel) requiring sophisticated microscopy and data analytics software.

Fortunately, tension sensor microscopy is still evolving, providing researchers the means to test these ambiguous questions and resolve challenging problems. Researchers have been steadily working to generate new methodologies for improving sensor reliability and quality, both on the biological level and on the computational level (LaCroix *et al.*, 2018; Gates *et al.*, 2019). To deal with the difficulty of generating FRET measurements, we have developed a new approach for measuring tension sensor FRET and a corresponding Python package for analyzing force data (Arsenovic *et al.*, 2017). Similarly, several different groups now have been successful at implementing tension sensor proteins across various organisms and cellular milieu (Kumar *et al.*, 2016; Meng *et al.*, 2008; Lagendijk *et al.*, 2017; Grashoff *et al.*, 2010; Borghi *et al.*, 2012; Conway *et al.*, 2013), validating the *in cellulo* measurements.

Others instead work to show the dynamic capabilities of tensions sensors at consistently measuring a wide range of physiological forces. Work from Alex Dunn (Chang *et al.*, 2016; Morimatsu *et al.*, 2013) showed a bimodal distribution of forces across integrins, most in a "low force" state (<3 pN) with a small fraction in a "high force" state (>7 pN). This helped to reconcile previous work that demonstrated low average force across vinculin (~2.5 pN) (Grashoff *et al.*, 2010) versus significantly higher peak forces (~40 pN) across integrins using tension gauge tethers (Wang and Ha, 2013) (not unexpected since talin, another focal adhesion protein, can bind up to 11 individual vinculin molecules; Gingras *et al.*, 2005). Further supporting the validity of pN force estimates using TSmod, different combinations of fluorophores than Grashoff *et al.* (2010), Morimatsu measured remarkably similar tension forces exerted by the extracellular integrin complex on the substrate-bound tension sensors (Morimatsu *et al.*, 2013). Alternatively, switching the flexible linker entirely as Tan (Tan *et al.*, 2020) showed still derived similar forces to both Morimatsu and Grashoff, though with a larger dynamic range. Thus, though all the factors that influence fluorescent protein-based tension sensors have not been elucidated, there is evidence to suggest that changing the fluorescent molecule may not invalidate measurements made by intracellular tension sensors. Further studies using alternative peptide sensors (Evers *et al.*, 2006; LaCroix *et al.*, 2018) or DNA hairpins (Wang and Ha, 2013; Zhang *et al.*, 2014; Liu *et al.*, 2016) have continued to validate the initial measurements made by Grashoff, further underscoring the accuracy and reproducibility of tension sensor approaches. Tension sensor microscopy is helping resolve how mechanotransduction occurs at the molecular level, even at single-molecule resolution, as Dunn's group has shown with TSmod (Chang *et al.*, 2016). As new data come forward, it will be interesting to see how existing mechanical models of the cell are adapted to properly reflect the state of the cell.

A BRIEF LOOK FORWARD

Over the past few decades, significant work has gone into expanding knowledge and understanding of the mechanical composition of the cell. The role of the mechanical environment is now widely appreciated in having a role in development (Mammoto *et al.*, 2013; Vining and Mooney, 2017), differentiation (Engler *et al.*, 2006), regeneration (Ananthanarayanan and Kumar, 2010; Vining and Mooney, 2017), aging (Phillip *et al.*, 2015), and disease progression (Ingber, 2003; Phillip *et al.*, 2015; Cho *et al.*, 2017). Despite the significant research that has developed the understanding of mechanobiologically relevant pathways, cellular targets that can be modulated to alter disease state or regeneration have yet to be identified within the space of mechanotransduction. Broadly, we propose that molecular regulators and actuators of mechanotransduction can be considered in four categories: structural proteins (actin, microtubules, and intermediate filaments), force-sensitive junctional elements (cadherins, vinculin, and talin in focal adhesion, filamin, tropomyosin, etc.), motor proteins (myosin II, kinesin, dynein, etc.), and regulatory elements (focal adhesion kinase, Rho-activated kinase, Ena/VASP, etc.). Although many of these proteins have been considered for therapeutic drug targets, few have become successfully employed in a clinical setting—the most obvious being microtubule-altering elements to stop cell division in cancers (Dumontet and Jordan, 2010). The main concern is that maintenance of baseline mechano-homeostasis is ubiquitous for all cell types in the body.

Determining causation versus correlation remains a frustration in the field of mechanobiology. Unlike chemical factors, which can be added or removed, it is difficult to perturb mechanical elements in the absence of chemical factors. For example, it is possible to directly measure forces of molecular interactions in force-sensitive adhesion elements, which are integrated into the pathway and directly responsible for propagating the physical signal. However, determining whether propagation of force is primary or secondary to biochemical signaling is a difficult

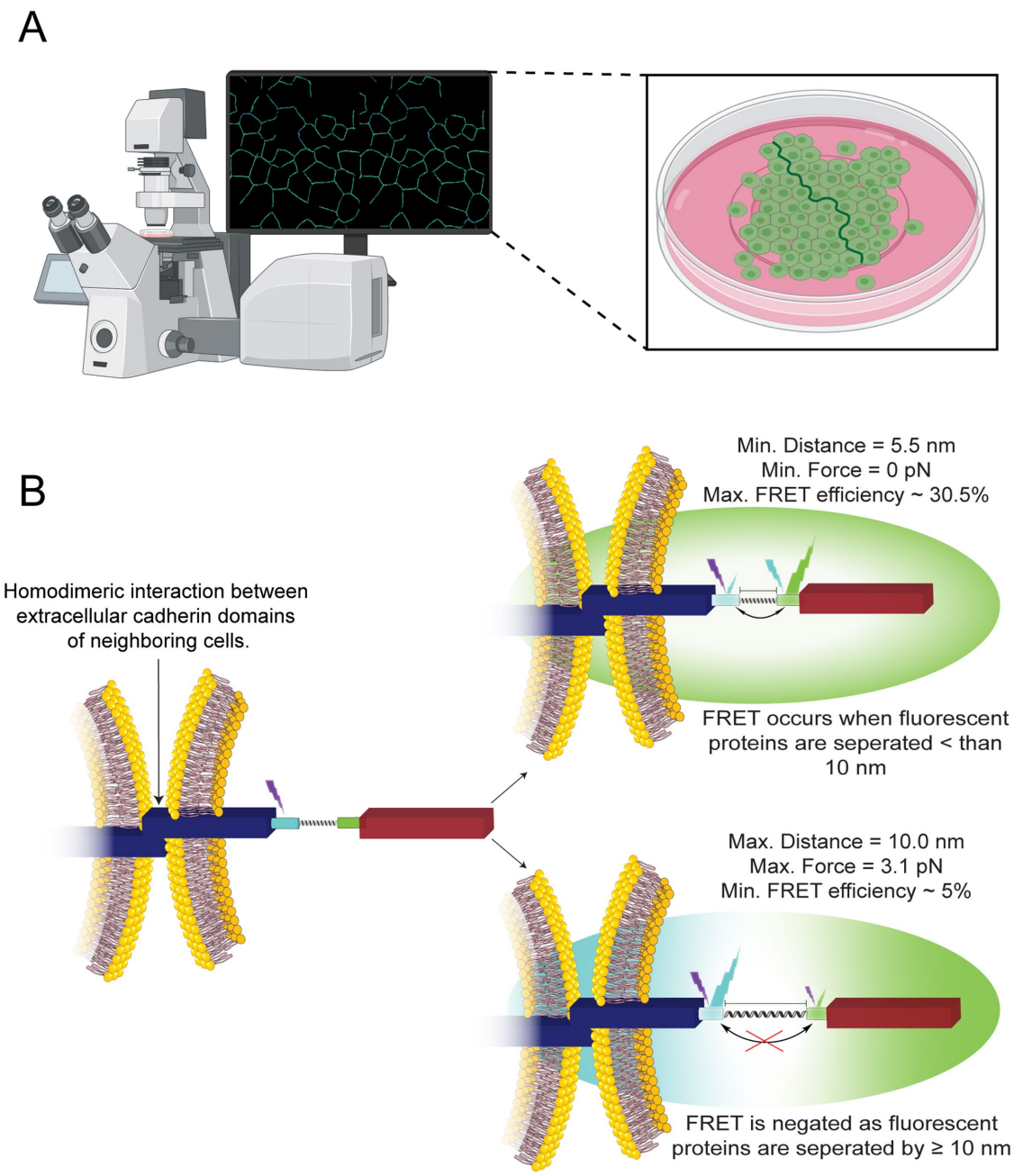

FIGURE 5: FRET-tensions sensors provide molecular force information when expressed in cells. (A) An example tension sensor experiment allows for imaging of intercellular junctions, where the signal is being collected from between cells in a cluster or monolayer. Created with BioRender.com. (B) CadTS functions by measuring tension between adjoining cells: neighboring cells will form homodimeric bonds with adjoining cadherin molecules. Subsequently, cells exert tension on one another which is transmitted intracellularly through a connection to the actin cytoskeleton. Tension generated either internally or externally will increase the separation between the fluorescent protein pair, reducing FRET (B, right, lower). Relaxation of the cytoskeleton increases FRET between pairs (mTFP–eYFP) in CadTS (B, upper). As the necessary force to stretch TSMod is known, an average force of tension per molecule of CadTS can be calculated for every pixel seen in A.

question to answer. Neither phenomenon can be probed in isolation, and the conclusions drawn generally depend on the context of the study. Furthermore, it is likely that mechanosensitivity in cells depends on the level of differentiation of the cell type in question as well as lineage specificity (Engler *et al.*, 2006). Finally, the cross-talk between biochemical and mechanical pathways complicates target therapies or development of druggable molecules for medical applications.

One potential exception is a relatively recent pair of tightly regulated transcription factors: YAP and TAZ, signaling proteins in the Hippo pathway that are associated with cell proliferation, development, regeneration, and oncogenesis. Specifically of interest is the mechanical control exerted on YAP/TAZ signaling (Dupont *et al.*, 2011). In healthy, confluent cells, YAP/TAZ are sequestered at the plasma membrane by association with α-catenin in the cadherin junctions. When cadherin is absent from the membrane (in the case

of growing cells), YAP/TAZ are released from the plasma membrane and relocate to the nucleus to bind with nuclear factors, promoting transcription of proliferative factors. Similarly, mechanical perturbation of the monolayer in the presence of cadherin promotes nuclear translocation of YAP/TAZ (Aragona *et al.*, 2013; Benham-Pyle *et al.*, 2015). In addition, release of YAP/TAZ from the plasma membrane will activate β-catenin signaling in the Wnt pathway as well, further increasing proliferation (Azzolin *et al.*, 2014). YAP/TAZ-mediated transcriptional activity is similarly modified by cellular geometries (Dupont *et al.*, 2011; Wada *et al.*, 2011), substrate stiffnesses (Dupont *et al.*, 2011; Aragona *et al.*, 2013), and hyperactivation/relaxation of the actin cytoskeleton (Dupont *et al.*, 2011; Aragona *et al.*, 2013). Even translocation of YAP/TAZ through the nuclear pore is in part dependent on mechanical strains applied to the nucleus (Elosegui-Artola *et al.*, 2017), underscoring yet another level of mechanobiological control on the YAP/TAZ signaling pathway. Furthermore, implications of mechanobiological control on nuclear import and export would be of paramount importance, necessitating further study as this process is fundamental to survival of eukaryotic cells. These are perhaps some of the best-studied mechanosensitive proteins, and they directly link biochemical signaling to many of the biomechanical phenomena observed over the past three decades. Researchers are actively looking for ways to modify YAP/TAZ activity (Elisi *et al.*, 2018) to control its role in oncogenesis (Zanconato *et al.*, 2016) or exploit its proliferative properties (Han *et al.*, 2015).

A previously unexplored route for mechanosensitive imaging technologies would be to examine the temporal impacts on cellular structure of small molecule drugs. In our recent work, we demonstrated that derivatives of piperazine, one of the earliest therapeutics for treating helminth infections (Page, 2008), actively induce contractility in the actin cytoskeleton (Zheng *et al.*, 2020). Along with other cellular effects, actomyosin contractility resulted in disassembly of cadherin junctions and the appearance of pores in the monolayer (Zheng *et al.*, 2020). In the context of oral drug delivery, inducing slight disassembly of cadherin junctions and cell monolayers may prove extremely useful for efficacious drug delivery, as intestinal permeabilization is typically judged on the ability of compound to disassemble tight junctions. Thus, an approach that leverages mechanostimulation as well as targeting of ZO junctions may be more successful. As the search for drugs that can effectively and safely permeabilize the intestine continues, one potential benefit of the above-discussed methodologies could result in a novel assay to track cytoskeletal contractions or nuclear particle fluctuations for estimating permeabilization. Looking forward, as measuring mechanical signals becomes more routine, cell mechanics studies may yet find a role in rational drug discovery.

Unified theory of cell mechanics

We have covered three major imaging techniques for quantifying cell mechanics: 1) TFM that measures how well the cell generates forces on the environment; 2) particle tracking rheology, which measures mechanical properties and energetic landscapes within the cell; and 3) FRET tension sensors, which look at tension on individual molecules. An overview of the pros and cons of the techniques is also provided in Table 1. These techniques rely exclusively on microscopy as a means of reporting endogenous cellular forces compared with traditional biophysical approaches that rely on physical manipulation of the cell (atomic force microscopy, Newton *et al.*, 2017; Xu and Siedlecki, 2017; magnetic twisting cytometry, Zhang *et al.*, 2017; and micropipette aspiration, Hochmuth, 2000). All of the listed technologies provide complementary information on different length scales, timescales, and spatial regimes, giving researchers the flexibility to choose a single approach, or pair multiple approaches that are relevant to the questions being investigated.

Given the explosion of data generated using different mechanical methodologies that are analyzed using different models of cellular mechanics, logical questions arise, such as: is there a unified model of cell mechanics? Are all methods incorrect in some respect or do they show distinct but unique aspects of the same cell structure? Cells and cellular structures—the plasma membrane, the cytoskeleton, and organelles—all have nonlinear viscoelastic mechanical complexity of their own and combined present spatial and temporal intricacy. Beyond this, relatively simple models like tensegrity or even soft glassy materials, lacking molecular resolution, are neither able to provide a consistent mechanical understanding of the enormous cellular diversity in humans nor able to describe any particular cell type in great depth.

However, mechanobiologists are not starting from scratch. If looking from the top down, the soft-glassy model of the cell cytoskeleton and chromatin describes the rheological deformation of the cell, but lacks molecular-level predictive power. From the bottom up, the Clutch-Motor models can accurately represent cellular force generation at specific loci within the cell, but lack a connection to the broader structures. Going forward, tension sensor microscopy has the potential to reconcile both models—material properties from rheology (stress vs. strain) and force generation from Clutch-Motor Model (stress)—by providing molecular insight into local deformation (strain). There is still much that is unknown about the cell and whether cellular measurements may translate to higher-order structures generated by cells, like monolayers, tissues with ECM, and organs. However, the ability to multiplex the above-listed techniques will allow for a more comprehensive study that can simultaneously provide information of cell–substrate and cell–cell forces, cytoskeletal tension, and quantification of nuclear chromatin states in single cells and higher-order cellular structures. The ultimate end goal would be a model that can accurately predict measured force at the molecular scale at any point throughout the cell for tissues and organs, although a more attainable goal in the short term would explain how rheological measurements predicting cytoskeletal tension are related to measured forces of cellular mechanotransducers continuously distributed throughout the cell. Beneficially, as the above-listed approaches do not always measure the same direct physical phenomena, they make predictions that are verifiable by each listed approach. A unified model of cell mechanics will be able to reconcile measurements made on several orders of length scales, from molecular interactions, to multimeric complexes or organelles, and the cell as a whole. Thus, to uncover the mechanism of mechanotransduction and downstream biological effects, we need an integrative model of the cell that accurately accounts for each major mechanical component.

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Technique	Traction force microscopy	Particle tracking microrheology	Tension sensor microscopy
Pros	No genetic manipulation necessary.	Can sample a variety of subcellular structures (Tseng *et al.*, 2002, 2004).	Utilized in 2D (Hwang *et al.*, 2016) and 3D (Wirtz, 2009) cell culture.
	Many protocols exist significantly simplifying substrate engineering (PA, [Kraning-Rush *et al.*, 2012; Lampi *et al.*, 2016] PDMS, [Palchesko *et al.*, 2012; Ochsner *et al.*, 2007], and micropillars [Enemchukwu *et al.*, 2017]).	Samples many frequency regimes (Wirtz, 2009).	Applicable with any cell type.
	Photobleaching nonexistent with commercial microspheres on relevant timescales.	Can distinguish bulk material properties and local activity by motor proteins (Lau *et al.*, 2003).	Single-nanometer spatial resolution.
	Applicable to virtually any adherent cell type.	SINK can track forces internalized within the nucleus (Booth-Gauthier *et al.*, 2012; Spagnol *et al.*, 2014).	Can yield either relative or absolute force measurements (Borghi *et al.*, 2012; Arsenovic *et al.*, 2017).
	Imaging protocol relatively straightforward compared with the other approaches.	Can be done on most fluorescent microscopes (for fluorescent methodologies). No expensive fluorescent components for transmitted light approaches.	More efficient FRET pairs extend sensitivity range (LaCroix *et al.*, 2018).
	Measures absolute forces generated by the cell.	Fluorescent (Tseng *et al.*, 2002; Booth-Gauthier *et al.*, 2012) or non-fluorescent trackers (Crocker *et al.*, 2007), both manufactured, endogenous, or genetically engineered and expressed.	Can be used on confluent monolayers, cell clusters, and single cells.
	Can be done on any confocal microscope.	Can establish constitutively expressing cells using new genetic approaches.	Has been using in living organisms (Krieg *et al.*, 2014).
Cons	Preparation of substrate reduces window for experimentation.	Requires complicated algorithms to accurately track particle movement.	Spectral detector significantly simplifies data analysis. Challenging without one.
	Resolution limited in Young's moduli greater than 30 [kPa].	Information can be construct dependent or biased by delivery method.	Limited library of constructs.
	Resolution decreases as cell cluster size increases.	Genetic tagging results in cell–cell heterogeneities.	Novel constructs require significant controlling to validate biological function.
	Limited insight within the cell.	Traditionally low throughput. High magnification necessary for accurate tracking of vesicles/lipidoids limits data collection to single cells.	FRET signals can change depending on local environment.
	Image analysis requires complex track analysis algorithms.	SINK currently only provides relative force measurement.	Analysis algorithms are difficult to apply and can be applied improperly.
	3D analysis softwares limited.	SINK works best in confluent, flat monolayers rather than single cells or small cell clusters.	Autofluorescence complicates blue-green construct imaging.
	No information about nonadherent cells.	Imaging of suspended cells complicated by drift, compounded during long imaging times.	Transient or stable overexpression result in mosaic expression of endogenous protein and tension sensor construct.

All techniques are compatible and can be multiplexed in a single experiment. However, carefully consider the selection of fluorescent molecules and proteins to avoid spectral overlap and eliminate spectral mixing that requires additional postprocessing.

TABLE 1: Comparison and capabilities of each imaging modality.

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