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Measurement Systems for Cell Adhesive Forces

Cell adhesion to the extracellular matrix (ECM) involves integrin receptor–ligand binding and clustering to form focal adhesion (FA) complexes, which mechanically link the cell's cytoskeleton to the ECM and regulate fundamental cell signaling pathways. Although elucidation of the biochemical events in cell-matrix adhesive interactions is rapidly advancing, recent studies show that the forces underlying cell-matrix adhesive interactions are also critical to cell responses. Therefore, multiple measurement systems have been developed to quantify the spatial and temporal dynamics of cell adhesive forces, and these systems have identified how mechanical events influence cell phenotype and FA structure–function relationships under physiological and pathological settings. This review focuses on the development, methodology, and applications of measurement systems for probing (a) cell adhesion strength and (b) 2D and 3D cell traction forces. [DOI: 10.1115/1.4029210]

1 Introduction

Cell adhesion to ECM proteins is regulated by integrin receptors, which are composed of α and β subunits [1]. Following binding to ECM proteins, integrins cluster together to form FA complexes, which contain structural proteins that link the ECM to the cytoskeleton and signaling effectors that regulate cell proliferation, migration, and differentiation [2]. The importance of cell–ECM adhesion is underscored by the early stage lethality in mice that have genetic deletions or mutations for adhesion receptors, ligands, or associated components [3,4]. Cell–ECM adhesive interactions also regulate host-implant responses for medical devices and tissue-engineered constructs, further emphasizing the importance of cell adhesion to the ECM [5]. Moreover, recent studies have identified that aberrations in cell–ECM adhesion play a critical role in pathological conditions, such as atherosclerosis, blood clotting, and cancer metastasis [6–8].

FAs also provide anchorage for the cell, by mechanically linking ECM proteins to the cell's cytoskeleton [1,2], and transmitting adhesive forces that drive cell migration, signaling, and tissue morphogenesis [9–11]. Several systems have been developed to quantify the spatial and temporal dynamics of cell adhesive forces. These measurement systems have primarily focused on cell adhesion strength, which is defined as the amount of force required to detach the cell from the ECM [12], and cell traction forces, defined as the forces that cells exert on the ECM [9]. From the development of these measurement systems, our understanding of the forces underlying cell–ECM adhesion has increased significantly over the past decade.

2 Adhesion Strength Quantification

Cell adhesion strength measures cell–ECM adhesion and is strongly influenced by integrin-bond number and distribution, cell–ECM contact area and shape, and FA size and composition [13–15]. Generally, adhesion strength assays measure the ability of cells to remain attached when exposed to a detachment force (Table 1). The simplest adhesion strength assay involves seeding cells onto a substrate of interest, washing the cells with physiologic buffers, and counting the remaining cells afterward [16]. Although these “wash” assays have identified critical components and regulatory mechanisms of cell adhesion [16], they are severely limited by poor reproducibility and sensitivity, as the washes apply largely unknown and nonuniform detachment forces

[13]. Moreover, wash assays usually fail to provide sufficient detachment forces, even after short adhesion times (<60 min) for certain strongly adherent cells. Despite its simplicity, the wash assay is undermined by poor reproducibility and sensitivity. Consequently, more robust and quantitative assays have been developed to analyze cell adhesion strength.

Several quantitative assays have been developed to apply controlled detachment forces to adherent cells. These methods are generally divided into the categories of (1) micromanipulation, (2) centrifugation, and (3) hydrodynamic shear stress. Micromanipulation encompasses several techniques that apply normal or tangential forces with a micropipette, microprobe, atomic force microscopy (AFM) cantilever, or laser and magnetic tweezers [17–22]. These techniques provide highly sensitive (pico–nano range) real-time force–displacement relationships and are particularly useful for analyzing isolated or low-number receptor–ligand interactions. For example, Roca-Cusachs et al. used magnetic tweezers with FN-coated magnetic beads to show that clustering of FN domains within ~ 40 nm increased adhesion strength six-fold via $\alpha_5\beta_1$ integrin clustering, and this increase was independent of $\alpha_v\beta_3$ integrin or talin 1 and 2 [23]. Subsequently, they concluded that $\alpha_5\beta_1$ integrin clustering supports adhesive force generation, whereas $\alpha_v\beta_3$ integrin or talin 1 and 2 are implicated in mechanotransduction and reinforce integrin–cytoskeleton linkage via talin [23]. Furthermore, Sheetz and colleagues used optical tweezers to identify and quantify the strength of a molecular slip bond between integrins and a trimer of the fibronectin (FN) integrin-binding domain (FN III-7-10) [24]. They also showed that talin 1 is critical to the strength of this slip bond, suggesting that talin 1 is responsible for initiating this molecular slip bond between closely packed FN–integrin complexes [24]. Kong et al. used AFM and membrane force probes to demonstrate that cyclic application of 5–25 pN forces to FN–integrin $\alpha_5\beta_1$ bond significantly increases bond lifetime and identified a mechanism for cell adhesion strengthening by the application of cyclic forces [25]. Furthermore, they demonstrated with AFM that increasing applied force between 10 and 30 pN prolongs integrin $\alpha_5\beta_1$ –FN fragment bond lifetime, demonstrating catch bond behavior [26]. Although micromanipulation techniques are useful for analyzing isolated receptor–adhesive ligand interactions, they are typically not suitable for studying long-term cell adhesion strengthening responses, as the upper range of applied forces (~ 10 nN for AFM, ~ 100 – 1000 pN for optical and laser tweezers approximately) is usually too small [13]. Moreover, these micromanipulation techniques often require sophisticated equipment and expertise to operate, limiting accessibility for other users.

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Table 1 Common measurement systems for cell adhesive forces

Assay	Methodology	Force Quantification	Advantages	Disadvantages	Ref.
Wash	Applies detachment force from washing with physiologic buffers	Unknown, Non-Uniform	(+) Simple/Convenient	(-) Poor reproducibility (-) Unknown forces (-) Short-Term Adhesion	13, 16
Centrifugation	Conventional centrifuge applies normal force to adherent cells	$F = VRD\omega^2$ V = Cell Volume R = radius; ω = Rotation Speed; D = Media density	(+) Simple/Convenient (+) Population Averages	(-) Single force applied each run (-) Restricted to short-term adhesion/Low applied forces (max $\sim 10^{-3}$ dynes/cell)	27, 28
Micromanipulation	Apply force with micropipette, AFM cantilever, laser tweezers, or microprobe.	Quantified from force transducer or calibrated deflections of micromanipulator	(+) Sensitive real-time force-displacement profiles (+) Analyzes individual receptor-ligand interactions	(-) Difficult and Time-Consuming (-) Maximum Force ~ 10 nN; restricted to receptor-ligand interactions	17-26
Hydrodynamic Flow	Shear forces generated by fluid flow over adherent cells	Proportional to applied fluid shear stress	(+) Highly tunable forces (+) Provides population average	(-) Requires special flow cells (-) Force depends on cell shape (-) Flow validation required
	<p>Radial Flow</p> <p>$\tau_w = 3\mu Q / \pi r h^2$ $\tau_{w \max} \sim 600$ dyn/cm² Q = Flow Rate; μ = viscosity; r = radial position; h = gap height</p>	$\tau_w = 3\mu Q / \pi r h^2$ $\tau_{w \max} \sim 600$ dyn/cm ² Q = Flow Rate; μ = viscosity; r = radial position; h = gap height	(+) Applied forces inversely proportional to radial position (+) Direct observation of the detachment process	(-) Flow profile validation required (-) Complex hydrodynamic conditions at impingement point	33
	<p>Parallel Plate</p> <p>$\tau_w = 6\mu Q / wh^2$ $(\tau_{w \max} \sim 120$ dyne/cm²) Q = Flow Rate; w = channel width; h = channel height; μ = viscosity</p>	$\tau_w = 6\mu Q / wh^2$ $(\tau_{w \max} \sim 120$ dyne/cm ²) Q = Flow Rate; w = channel width; h = channel height; μ = viscosity	(+) Direct observation of detachment process (+) Flow conditions typically laminar and easily validated	(-) Relatively low detachment forces / restricted to short-term adhesion (-) Single force applied per experiment without utilizing the variable height flow channel	30-32
	<p>Spinning Disk</p> <p>$\tau_w = 0.8 r (\rho \mu \omega^3)^{1/2}$ $\tau_{w \max} \sim 2500$ dyne/cm² r = radial position; ω = rotational speed; ρ = fluid density; μ = fluid viscosity</p>	$\tau_w = 0.8 r (\rho \mu \omega^3)^{1/2}$ $\tau_{w \max} \sim 2500$ dyne/cm ² r = radial position; ω = rotational speed; ρ = fluid density; μ = fluid viscosity	(+) High throughput; Large linear range of applied forces for each experiment (+) Uniform surface chemical properties	(-) Cell detachment cannot be visualized in real-time (-) Flow pattern validation required for high rotation speeds (~ 5500 RPM)	39-43

Centrifugation assays, in contrast, provide adhesion strength measurements for large cell populations [27,28]. In this configuration, a substrate containing adherent cells is spun at a specific speed to apply a controlled detachment force perpendicular to the cell adhesive area, and the number of cells before and after spinning is quantified. However, centrifugation applies relatively low detachment forces ($<10^{-3}$ dyn/cell) [13], limiting its applicability to short (<60 min) assays. Moreover, the assay is labor-intensive, as it requires many runs at different speeds to apply a wide range of forces. Alternatively, short-term cell adhesion can be analyzed as a function of ligand density at a fixed centrifugation speed. Afterward, a nonlinear profile with adherent cell fraction plotted against ligand density can be generated, and the ligand density for 50% adhesion strength can be a sensitive indicator of adhesion strength [27]. This strategy can be particularly useful for experiments involving multiple conditions, as it is significantly more high-throughput.

Hydrodynamic shear assays utilize specialized flow setups to apply a wide range of detachment forces to a large cell population, and generally provide the most robust and sensitive quantitative measurements of long-term cell adhesion strength. After shear stress exposure, cell adhesion strength is typically defined as the shear stress (τ_{50}) that produces 50% cell detachment from the ECM. For hydrodynamic shear assays, however, the net detachment force applied to an individual cell depends on the cell's area, shape, and FA size and position [13]. Therefore, variations in cell spreading and FA geometry must be accounted for in these assays, as τ_{50} does not accurately represent the applied detachment force if these variations are present between conditions.

Common hydrodynamic shear configurations include flow between parallel plates, radial flow between two parallel disks, and spinning or rotating disks [29–33]. With the exception of the spinning disk setup, these configurations can be combined with a microscope, which allows for the real-time monitoring of cell attachment–detachment and flow profile validation and

quantification. However, the above flow configurations often provide insufficient detachment forces for well-spread or strongly adherent cells under laminar flow. Alternatively, rotating disk setups, such as the small-gap parallel disk viscometer [34] or the single spinning disk [15,35], apply shear stresses that vary linearly with radial distance, and allow users to apply a wide range of forces to a cell population in a single experiment. Other flow setups that apply a range of detachment forces within an individual experiment include the Hele-Shaw chamber [36], jet-impingement device [37], and variable height flow channel [38]. Amongst all of these systems, however, only the spinning disk can apply a linear range of detachment forces under an individual experiment with uniform chemical conditions across the surface. For example, García and colleagues developed a robust spinning disk device that applies a wide range of detachment forces to an adherent cell population and provides highly sensitive measurements of cell adhesion strength [35,39]. In a typical experiment, cells are first seeded onto a circular coverslip coated with an ECM protein of interest [12]. ECM proteins can also be patterned onto the coverslip if significant differences in cell spreading are expected, as spreading differences can alter the detachment force applied to each cell between conditions [12]. Subsequently, the cells are spun at a fixed speed, and adherent cells are counted at different radial distances, each corresponding to known shear stress values [12]. The fraction of adherent cells decreases nonlinearly with respect to applied shear stress, and the adhesion strength is typically defined as the shear stress which produces 50% cell detachment [12]. At high rotation speeds, however, flow inside the spinning disk device becomes turbulent and requires experimental validation of the flow patterns [12,13]. To mitigate this potential issue, viscosity enhancers such as dextran can be used to increase the applied detachment force, while maintaining low rotation speeds to avoid turbulent flow.

Overall, the spinning disk system produces highly robust, sensitive, and reproducible measurements of cell adhesion strength and

has been utilized for a diverse array of applications. For example, the spinning disk system has been used to characterize the dynamics of initial cell–ECM attachment, spreading, and adhesion strengthening [12,35,39,40]. Furthermore, Friedland et al. utilized the spinning disk system to show that $\alpha_5\beta_1$ integrin activation via myosin II-generated cytoskeletal force strengthens $\alpha_5\beta_1$ -FN bond strength, and activates subsequent signaling pathways via phosphorylation of FA kinase (FAK) [41]. The spinning disk device has also been used to identify how different FA protein mutants affect cell adhesion strength, which is indicative of FA function [3]. The spinning disk assay has also been utilized for 3D cultures, showing that fibroblasts cultured in 3D fibrillar FN matrices have significantly lower adhesion strength than fibroblasts cultured on 2D FN-coated coverslips [42]. Microscopy subsequently revealed holes inside the matrix after spinning, suggesting that cells can detach via fibril breakage in 3D, instead of integrin–matrix bond breakage in 2D [42]. Furthermore, nanopatterning has been combined with the spinning disk assay to dissect how FA geometry and size influence cell adhesion strengthening. For example, Coyer et al. applied subtractive contact printing to generate nanopattern “islands” of different sizes and geometries [14]. They subsequently showed that above an individual island area of $0.11\ \mu\text{m}^2$, individual nanoisland area significantly influences cell adhesion strength, whereas the geometric arrangement of the islands (island spacing, number of islands, and total area of the islands) has minimal effect on cell adhesion strength [14]. Recently, Singh et al. also used the spinning disk system to identify unique adhesion strength values for human induced pluripotent stem cells (hiPSCs) [43]. These adhesion strength values were then used to design a flow-driven microfluidic device that purifies hiPSCs, by detaching them from an initially heterogeneous cell population. By altering the applied fluid shear stress to match the hiPSC’s unique adhesion strength, users can isolate hiPSCs from the other cells, which have significantly higher adhesion strengths than the hiPSCs [43].

3 Two-Dimensional Traction-Adhesive Force Quantification

While adhesion strength measurement systems have identified key adhesion components and how they participate in the cell adhesion strengthening process, cell–ECM traction forces also significantly influence cell phenotype. It is becoming increasingly evident that cell–ECM traction forces regulate cell fate, tissue formation, and even malignant transformation [8,9]. Traction forces also regulate FA composition and intracellular signaling, further underscoring the importance of cell traction forces [3].

Accordingly, numerous measurement systems have been developed to assess how forces, both sensed and exerted at FAs, regulate FA structure–function and cell phenotype. The majority of these measurement systems rely on linearly elastic deformable substrates, where the forces exerted by individual cells can be measured from the deformations induced in the substrate (Table 2) [9,44,45].

Harris et al. initiated the use of deformable substrates by flame-exposing silicone fluid to polymerize a thin “skin”-like layer on the surface, while the un-cross-linked fluid beneath serves as a lubricant [45]. These silicone film substrates are compliant to the extent that adherent cells were able to induce wrinklelike deformations on the film’s surface as they applied contractile forces. This system offers its advantages from its simplicity, and that its mechanical properties can be tuned by altering the heat exposure time and silicone fluid viscosity. Although these substrates are not widely used today due to their qualitative nature, they helped inspire the next-generation measurement systems for cell traction forces.

Consequently, more advanced 2D traction force measurement techniques have emerged. These systems typically measure substrate deformations induced by individual cells, while utilizing engineered materials whose mechanical and chemical properties can be precisely tuned over a wide range. Amongst these measurement systems, polyacrylamide (PA) gel based traction force microscopy (TFM) and microfabricated elastomeric post array deflectors (mPADs) are currently, the most widely used, and typically provide the most sensitive and reliable measurements of 2D cell traction forces.

Wang and Dembo developed PA gel TFM, in which the PA gel is first functionalized with cell adhesive ligands, and fluorescent beads (approximately 200–500 nm in diameter) are embedded near the gel surface [44]. Cells cultured on these substrates generate traction forces that deform the gel’s surface plane, which can be quantified by tracking the displacements of the fluorescent beads. This is typically done by imaging the bead fields before and after cell detachment with trypsin so that the bead positions in both “stressed” and “unstressed” states are known [44]. As the gel deformations are generally significantly smaller than the gel thickness, the PA gel can be treated as a semi-infinitely thick, incompressible, and linearly elastic material during traction stress computation [44].

Subsequently, the traction stress field $T_\beta(r)$ and displacement field d_{pz} are related by the Fredholm integral equation of the first kind

$$d_{pz} = \iint_{\beta z}^g (\mathbf{m}_p - \mathbf{r}) T_\beta(r) dr_1 dr_2 \quad (1)$$

Table 2 Common measurement systems for cell traction forces

Schematic	Methodology	Advantages	Limitations
Elastic gels: Microscopy tracks bead (green) displacements to quantify cell traction forces (44).	(+) Simple/Convenient (+) Flexibility over matrix chemistry/mechanics	(-) Potentially unreliable traction force maps
Micropost Array Deflectors: Micropost first functionalized with ECM protein (red) and cells seeded on top. Micropost deflections are used to quantify cell traction forces (46).	(+) Reliable traction force quantification (+) Flexibility over substrate chemistry and mechanics	(-) Discrete nature of substrate (-) Requires special expertise and equipment to fabricate
(Not drawn to scale)	3D hydrogels: Cells embedded into 3D matrix (gray) that is functionalized with adhesive peptides and crosslinked. High resolution microscopy tracks bead (green) displacements to estimate cell traction forces (91, 92).	(+) More physiologically relevant environment for certain cell types (+) Flexibility over matrix chemistry, mechanics, and topography	(-) Potentially unreliable traction force maps (-) Highly computationally intensive; requires expensive high-resolution microscopy

where g represents a green's tensor function and gives the displacement of the p th marker " m ." Traction stresses are exerted by a cell through its contacts on the substratum surface at position r . Given the bead displacements d_{pz} , the integral must be inverted to solve for the traction stress field, $T_\beta(r)$ [9,44]. However, this inversion is highly computationally intensive and requires careful selection of mathematical constraints, such as penalty terms for higher numbers of force vectors or restriction of traction forces to the cell's edge [9,44,46]. Because these constraints can be subjective, an individual bead displacement field can lead to multiple different traction force fields, making the method vulnerable to user bias and error [9,46]. However, recent experimental and computational advances have helped improve the reliability and resolution of PA gel TFM. Experimentally, the use of two different fluorescent bead markers inside the gel allows users to increase bead density and obtain a richer sampling of the displacement field, which ultimately helps improve traction force resolution [47,48]. Improved algorithms, such as the boundary element method, Fourier-transform traction cytometry, and point force traction reconstruction, can also improve traction force resolution and reliability [47]. By combining these recent experimental and computational advances, Sabass et al. managed to reconstruct fibroblast traction force maps with a $\sim 1\ \mu\text{m}$ spatial resolution, which is a 5–10 fold improvement over earlier methods [47]. Although this is a significant improvement, this approach usually requires a high-end confocal microscope and tends to have difficulties computing traction forces at smaller adhesion sites [47].

Nevertheless, PA gel TFM offers numerous advantages. For example, PA is an inexpensive and convenient material, making this technique easily adaptable to many laboratories. Moreover, the mechanical and chemical properties of PA gels can be precisely tuned over a wide range [6,49–51], making these gels adaptable to a large variety of cell types and experimental conditions. This flexibility is particularly advantageous, as substrate stiffness and ECM composition both significantly influence critical cell behaviors, such as mesenchymal stem cell differentiation [52], vascular smooth muscle cell durotaxis [53], skeletal muscle stem cell self-renewal [54], and cancer cell migration [8]. Therefore, PA gel TFM remains a widely used experimental tool and has uncovered multiple insights on cell traction force generation under physiological and pathological settings. For example, Huynh et al. discovered that increased substrate stiffness induces greater endothelial cell traction forces, which destabilizes cell–cell junctions, leading to increased vascular permeability to inflammatory agents during atherosclerosis progression [6]. Subsequently, Huynh et al. used PA gel TFM to characterize how increased matrix stiffness promotes smooth muscle cell prestress, traction force generation, and circular dorsal ruffle formation, thus facilitating smooth muscle cell invasion into the arterial intima layer [55]. Moreover, using PA gel TFM, Califano and Reinhart-King characterized how matrix mechanics and chemistry synergistically regulate traction force generation and cell migration during angiogenesis [49,56]. Furthermore, Chowdhury et al. used PA gels to demonstrate that mechanical properties of stem cells regulate stress-induced spreading, suggesting that local small forces play a critical role in early development of soft embryos [57]. During stem cell differentiation, traction forces also play a critical role in cell fate, and recent studies have characterized how changes in ECM orientation and chemistry can alter stem cell traction forces, and ultimately cell differentiation [58–60]. Finally, PA gel TFM has been used to characterize how cell traction forces drive cancer cell metastasis and malignancy. For example, Weaver and colleagues demonstrated that increased matrix stiffness drives cancer cell migration by modulating integrins and cell traction stresses via Rho and extracellular signal-regulated kinase (ERK) signaling [61]. Furthermore, Kraning-Rush et al. demonstrated that traction forces for metastatic human breast, lung, and prostate cancer cell lines were significantly increased compared to nonmetastatic counterparts, suggesting that

contractility plays a critical role in cancer cell invasion and migration [8]. Subsequently, the authors demonstrated that traction forces between metastatic and nonmetastatic cell lines were independent of cell area, suggesting that cell morphology does not necessarily dictate risk of contractility-mediated metastatic behavior [8].

PA gel TFM has also been used to better characterize basic cell adhesion and migration. Since the mechanical properties of these gels can be precisely tuned over a wide range by varying the acrylamide to bisacrylamide ratio, numerous studies have shown that substrate stiffness typically induces greater cell spreading, FA formation, migration, and traction force generation [51,62–65]. Overall, despite their limitations, PA gel TFM has been an invaluable tool for mechanobiology research. By allowing researchers to modulate the gel's mechanical and chemical properties over a wide range, PA gels have been utilized for a diverse array of research applications. Moreover, the strategy underlying PA gel TFM has been extended to inspire additional traction force measurement systems that utilize linearly deformable substrates.

Alternatively, methods utilizing microfabricated systems have been developed to quantify cell traction forces at discrete locations on the cell–substrate interface. Galbraith and Sheetz initiated the use of these discrete substrates, by developing a silicon array of microcantilevers each tipped with a $4\text{--}25\ \mu\text{m}^2$ pad coated with ECM adhesive protein [66]. By monitoring the cantilever deflections as the cell migrates across the pad, the traction force exerted by the cell at a discrete adhesion site can be quantified using basic beam theory [66]. However, the high fabrication cost required of this system is a significant limitation [66]. Moreover, the traction forces can only be measured at positions where the cell forms an adhesion, whereas continuum approaches allow for traction force measurements at virtually any point of the cell–substrate interface.

A more robust form of this approach was pioneered by Chen and involves the use of mPADs, which consist of silicone polydimethylsiloxane substrates with arrays of microposts ($D \sim 3\ \mu\text{m}$, $L \sim 8\text{--}12\ \mu\text{m}$) fabricated using soft lithography [9,46,67,68]. The tips of the microposts are functionalized with ECM adhesive protein, and the remaining regions are passivated to prevent cell adhesion between the posts [9]. As cells apply traction to the tips of the microposts, the microposts deflect [68]. The resulting forces, F , are calculated using Euler–Bernoulli beam theory, in which E , D , L , and δ are the Young's modulus, post diameter, height, and deflection, respectively [9,67]

$$F = \delta \frac{3\pi E D^4}{64 L^3} \quad (2)$$

Although this makes force quantification significantly easier and more reliable, mPADs have several limitations. The discrete nature of the substrate can potentially alter cell behavior and only allows force quantification where cells form adhesions to the microposts. mPADs also require sophisticated expertise and equipment to fabricate, which limits end-user accessibility. Moreover, the sensitivity of the microposts to the particular cell type may need to be optimized. If the micropost is too soft, the post deflections become very large and extend toward the nonlinear range, thus obscuring force quantification. If the microposts are too stiff, the micropost deflections will be too small to visualize, thus reducing the system's sensitivity. Therefore, optimization of the micropost dimensions may be necessary for probing traction forces for different cell types. In addition, due to the discrete nature of the substrate, ligand density quantification is more challenging. Furthermore, mPADs currently rely on the passive adsorption of large ECM proteins onto the micropost tips via microcontact printing [9]. Consequently, smaller adhesive peptides cannot be used with the current mPADs system, thus limiting the system's versatility. This is not an issue with PA gel TFM, however, as users have previously tethered small adhesive peptides onto the gel surface [50,51].

mPADs, however, offer multiple advantages over PA gels. For example, mPADs rely on basic elastic beam theory, which makes force quantification easier and more reliable, as there is only one traction force field for each micropost displacement map. Moreover, mPAD stiffness is modulated geometrically instead of chemically [9,69]. By simply changing the height or diameter of the posts, the bulk substrate stiffness can be tuned over a wide range [70]. Moreover, there is no need for recording the unstressed state of the substrate, as the posts are uniformly and precisely spaced after fabrication. Since the system is microfabricated, mPADs give users excellent precision over the surface chemical properties on the micrometer scale [9,10,71]. For example, users have generated highly intricate ECM surface patterns to study how cell–cell contact or cell shape regulates cell phenotype and traction force generation [10,71]. Other studies have uncovered important insights on how substrate stiffness affects stem cell traction force generation, and differentiation into adipocytes or osteoblasts [9]. At the molecular level, recent studies have characterized the role of FA protein mutations on traction force generation [3] and examined how the cytoskeleton affects traction force generation and rigidity sensing [72]. Overall, mPADs offer users easier and more reliable force quantification compared to PA gel TFM and give researchers substantial flexibility over matrix mechanics and chemistry to suit their experimental application.

Finally, PA gel TFM and mPADs have been used to characterize traction forces for collections of cells, ranging from two cells-in-contact to entire cell monolayers and sheets. Under the assumption that the net traction force is zero for both individual cells and the cell collection [10], multicell TFM has been utilized for a diverse array of applications. For example, Ting et al. used mPADs to demonstrate that laminar atheroprotective fluid shear stress induces increases in traction force generation and endothelial cell alignment compared to endothelial cells exposed to low-oscillatory fluid shear stress, which is associated with inflammation and atherosclerosis progression [69]. Furthermore, Califano et al. used PA gel TFM on individual endothelial cells and cells-in-contact to identify that cell area and matrix stiffness are sensitive indicators of endothelial cell traction forces, which play an important role in atherosclerosis progression and endothelium function [6,49]. Ladoux and colleagues have also characterized the forces exerted by neutrophils during transmigration across endothelial cell–cell junctions using a laminar fluid shear stress model, where a confluent endothelial monolayer was first grown on an mPADs substrate [73]. Subsequently, the authors demonstrate that the neutrophil transmigration process results in a rapid and significant increase in endothelial cell traction force, as the neutrophil disrupts cell–cell junctions while it migrates across the endothelium [73]. Finally, Chen and colleagues have developed micropatterned mPADs to create “bowtie” configurations for two cells-in-contact to adhere to, which can then be used to quantify tension applied across the cell–cell junction, based on the traction forces outside of the junction [10].

Besides endothelial cell monolayers, many recent studies have focused on traction forces generated by epithelial cell sheets. For example, Ladoux and colleagues utilized mPADs to quantify the distribution of traction forces for epithelial sheets and demonstrated that traction forces are most prominent at the edges of the sheet, although there are smaller traction forces that exist internally within the cell sheet [74]. Subsequently, using micropatterned substrates, the authors demonstrate that cell growth is aligned with the stiffest direction along with the applied traction stresses [74]. Treppe and colleagues have also recently utilized mPADs to characterize how traction forces drive wound healing. By monitoring epithelial cell migration, FA activity and traction force generation after laser ablation of an epithelial sheet region [75], the authors demonstrate that epithelial cells cooperatively compress the underlying substrate to close the wounded area through close integration of cytoskeleton structural elements and traction force generation [75].

In addition to traction forces at the ECM surface, the tension applied across the ECM-FA-cytoskeleton interface can also significantly influence cell behavior [76,77]. Schwartz and colleagues pioneered the development of molecular sensors to quantify these forces [77,78]. For example, a vinculin tension sensor was engineered to contain the mTFP1-Venus fluorophore pair between the vinculin head and tail domains [77]. The tension (~ 2.5 pN/FA average) applied across the sensor influences the spacing between the two fluorophores, which changes the sensor’s fluorescence emission due to Förster resonance energy transfer (FRET) [77]. Subsequently, the change in fluorescence can be used to quantify the tension across the sensor with pN sensitivity after sensor calibration and validation [77]. Using the vinculin tension sensor, this group showed that vinculin tension is highest during FA assembly and enlargement, and lowest during FA disassembly or sliding [77]. Schwartz and colleagues also developed similar FRET tension sensors for endothelial cell VE-cadherin and PECAM-1 junctions [78], which play a critical role in vascular endothelial cell signaling and cytoskeletal alignment to laminar arterial-level (>10 dyn/cm²) fluid shear stress [6,78,79]. Subsequently, this group showed that laminar fluid shear stress at 15 dyn/cm² triggers a rapid (<30 s) decrease in junctional VE-cadherin tension and a simultaneous increase in junctional PECAM-1 tension (~ 2.0 pN/molecule average) that was dependent on vimentin [78]. Taken together, their results suggest that fluid shear stress initiates cytoskeletal reorganization, which then alters forces across cell–cell junction receptors [78]. Overall, these FRET tension sensors allow for the real-time monitoring of dynamic changes in molecular tension with pN sensitivity. However, these FRET sensors may not be adaptable to all cell types and proteins, depending on the cell type and sensor structure and sensitivity. Additionally, converting FRET change to molecular force usually requires the use of fluorescence lifetime imaging microscopy, which is not accessible to most laboratories.

To further characterize how molecular forces regulate integrin and notch signaling and initial cell–ECM adhesion, Wang and Ha developed a “tension gauge tether” (TGT) approach, where the ligand is immobilized onto a surface through a rupturable deoxyribonucleic acid (DNA)-based tether [80]. The TGT rupture strength was tuned over the 12–56 pN range to restrict the maximal tension underlying the receptor–ligand interaction over several different values. Using this system, the authors showed that a peak tension of ~ 40 pN is critical to single integrin–ligand bonds during initial cell–ECM adhesion, although cells require significantly less force (<12 pN) to activate notch receptors [80]. Therefore, TGT not only measures forces across individual integrin–ligand receptors but also provides molecular mechanical cues to regulate important cell behaviors, such as cell spreading, FA formation, and signaling [80]. Recently, another fluorescent sensor was developed to measure tension between integrins and adhesive ligands for cells cultured on 2D substrates [76]. Liu et al. developed a springlike polyethylene glycol (PEG) chain (MW ~ 3400) coupled to a gold nanoparticle anchor on one end and both a fluorescent dye and an adhesive peptide on the other end. Under relaxed conditions, the fluorophore is close to the nanoparticle and the fluorescence is quenched from nanometal surface energy transfer [76]. However, if the PEG chain is under tension, the fluorophore is pulled 1–20 nm away from the gold nanoparticle, and the quenching effect is abrogated to produce a significant change in detected fluorescence on the order of $1/r^4$ [76]. This change in fluorescence can be used to quantify molecular tension between the integrin receptors and adhesive peptide. Subsequently, they utilize the sensor to quantify tensions on the order of 1–15 nN across the integrin–ECM interface. Moreover, this system offers high spatial (<1 μ m) and temporal (100 ms) resolution [76]. In fact, the authors report a sensitivity 3 orders of magnitude greater than PA gel TFM or mPADs [76]. The system also allows users to incorporate different adhesive ligands onto the PEG chain, increasing the flexibility of the system, and allowing users to probe tension across specific integrin receptors. However, the system currently requires specialized

reagents and sophisticated expertise to operate, limiting its accessibility to other users.

4 Three-Dimensional Traction Force Quantification

Although significant progress has been made on understanding 2D cell traction forces, little is known about how cells generate traction forces in three-dimensional (3D) environments. Although 2D culture can be appropriate for certain cell types, such as endothelial cells and epithelial sheets, most cell types behave very differently in 3D culture, which may be more appropriate for certain cell types [81,82]. For example, cancer cells, stem cells, and fibroblasts exist physiologically in 3D and exhibit significantly different cytoskeletal and FA structure [61,83–85] and migration mechanisms in 3D compared to 2D [81,82,86,87]. From a pathological context, certain cancer cells in 3D exhibit significantly different gene expression, drug resistance, and metastatic behavior compared to cells cultured in 2D [88,89], further underscoring the importance of moving to 3D environments for certain cell types. From a regenerative medicine perspective, recent studies suggest mechanistic differences in stem cell differentiation between 2D and 3D, further underscoring the importance of 3D culture for certain cells [85]. Therefore, measurement systems for quantifying the spatial and temporal nature of 3D cell traction forces are paramount to attain a more physiologically relevant understanding of how mechanical events drive cell function for certain types of cells (Table 2).

3D TFM was first initiated through the use of 3D gels made from biological polymers, such as collagen or fibrin. For example, Koch et al. embedded carcinoma cells and 1 μm beads inside a 3D collagen matrix, and subsequently used the bead displacements to generate a strain energy map of the cell [89]. Although precise measurement of traction force is not possible due to the nonlinearity of the matrix material, these strain energies were used to approximate cell traction stresses. Using this measurement technique, the authors ultimately demonstrated that directionality of cell tractions may be more significant than traction force magnitude for cancer cell malignancy in 3D. Nevertheless, these biological matrices are still used today, as a qualitative measurement of cell traction force generation can sometimes be sufficient. For example, Kraning-Rush et al. showed that contractility inhibition abrogates traction force generation in both 2D and 3D, demonstrating that cytoskeletal contractility is similarly important for traction force generation in 2D and 3D [90]. Other users have modified this strategy to quantify forces underlying tissue morphogenesis, where fluorescent deformable microdroplets are functionalized with adhesive ligands and embedded inside embryonic tissue matrices [91]. Cells subsequently adhere to the droplets, and the droplet deformations can be used to estimate local stresses inside the tissue [91]. Using this strategy, the authors show that cells within mammary epithelial 3D aggregates generate stresses on the order of 3.4 $\text{nN}/\mu\text{m}^2$, which is nearly twofold higher than the stresses generated by cells inside the embryonic tooth mesenchyme, further underscoring the importance of forces during development [91].

Alternatively, the use of 3D PEG gels allows for more reliable cell traction force quantification, as the material is linearly elastic [84,92]. Chen and colleagues first demonstrated this technique by tethering adhesive peptides onto the PEG macromer and crosslinking the functionalized PEG macromers with protease-sensitive linkers to encapsulate the cells [84]. This ultimately creates a dense hydrogel network with pores on the nanometer scale [84,92]. The adhesive peptides allow cells to adhere, while the protease-sensitive linkers allow cells to remodel the matrix to invade, migrate, and spread via protease secretion [84]. As the pore sizes are approximately an order of magnitude smaller than the beads, the beads are physically entrapped inside the hydrogel, which allows users to track bead displacements to estimate cell traction stresses [84]. Just like 2D PA gel TFM, the bead fields are

imaged before and after cell detachment or contractility inhibition so that the bead positions in both the stressed and unstressed state are known [84]. Using the finite element method, a strain map is subsequently generated to estimate the cell traction stresses [84]. Although the geometry is different, the mathematical derivation of the cell's traction stresses is very similar to 2D PA gel TFM, although inversion of the green's function integral is much more computationally intensive. 3D gel TFM similarly requires regularization constraints, such as penalty terms for higher numbers of force vectors, restriction of traction forces to cell edges, or requiring that the net traction force is close to zero [84,92]. Because these constraints can be subjective, an individual bead displacement field can lead to multiple different traction force maps, making the method susceptible to user bias and error. Imaging can also be a significant challenge for 3D hydrogels. Typically, high-magnification objectives are required to visualize beads and individual cells, but usually have significant working distance limitations into the gels. Recent studies also suggest the possibility of different hydrogel structural and mechanical properties as a function above the glass coverslip between 0 and 200 μm , which causes cells to be encapsulated in "2.5D" near the coverslip, and further compound the working distance problem [93]. Moreover, this method suffers from uncertainties in tracking the bead positions and displacements, although uncertainty analysis can be performed to estimate these potential inaccuracies [84,92]. Furthermore, 3D PEG gel TFM usually requires the use of a high-end confocal microscope to eliminate out-of-focus light, making the system less accessible to others.

However, 3D PEG gel TFM does offer numerous advantages. For example, the gel's mechanical properties can be adjusted, by modulating gel composition and crosslinking. However, this can potentially alter the gel's architecture, which can influence cell behavior [94,95]. Likewise, the chemical properties can be adjusted to incorporate different adhesive ligands and other bioactive molecules, such as growth factors [96]. Therefore, 3D PEG hydrogels are a versatile traction force measurement system and adaptable to a wide variety of different cell types and experimental conditions. Moreover, by allowing cells to be cultured in 3D, this technique allows us to examine cell mechanics in a significantly more physiologically relevant context for certain cell types.

Although most studies still utilize 2D force measurement systems, 3D PEG gel traction force measurements are rapidly gaining in popularity. 3D gel TFM has also been used already to better characterize stem cell differentiation and cancer cell migration. For example, recent studies identify how stem cells form protrusions inside the matrix to generate force and migrate during differentiation [85]. Furthermore, several studies have uncovered insights between traction force generation and risk of cancer metastasis [89]. Although it is a relatively new experimental tool, 3D gel TFM is a rapidly growing system that will identify important insights on 3D cell-matrix mechanosensory interactions in the coming future.

5 Conclusion

Cell adhesion to the ECM is a highly critical physiological process, and measurement systems for cell adhesion strength and traction force generation have identified invaluable insights on how adhesive forces regulate cell behavior. However, it is evident that there is no "ideal" adhesive force assay in terms of simplicity, reproducibility, and sensitivity. Selection of an appropriate traction force assay depends significantly on the experimental conditions and frequently requires compromise between simplicity and robustness. As the differences between 2D and 3D culture become more evident, there is increasing movement toward 3D traction force quantification. Although it is a relatively new technique, 3D TFM is an extremely exciting and rapidly growing tool that will likely identify significant insights on how adhesive forces drive cell phenotype within a more physiologically relevant context.

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