



Traction Force Microscopy for Noninvasive Imaging of Cell Forces

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Abstract

The forces exerted by cells on their surroundings play an integral role in both physiological processes and disease progression. Traction force microscopy is a noninvasive technique that enables the *in vitro* imaging and quantification of cell forces. Utilizing expertise from a variety of disciplines, recent developments in traction force microscopy are enhancing the study of cell forces in physiologically relevant model systems, and hold promise for further advancing knowledge in mechanobiology. In

this chapter, we discuss the methods, capabilities, and limitations of modern approaches for traction force microscopy, and highlight ongoing efforts and challenges underlying future innovations.

Keywords

Traction force microscopy ·
Mechanobiology · Cell mechanics · Cell forces · Biophysical interactions ·
Quantitative imaging · Inverse problems ·
Collective behavior · Mechanical properties ·
Extracellular matrix · Continuum mechanics · Elasticity

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15.1 Introduction

The growing field of mechanobiology has resulted in a heightened understanding of how cells both shape and respond to mechanical properties and forces in their environment. Driving this understanding is a growing body of evidence, which has revealed that the biophysical interactions of cells with both the extracellular matrix (ECM) and neighboring cells play an integral role in the progression of

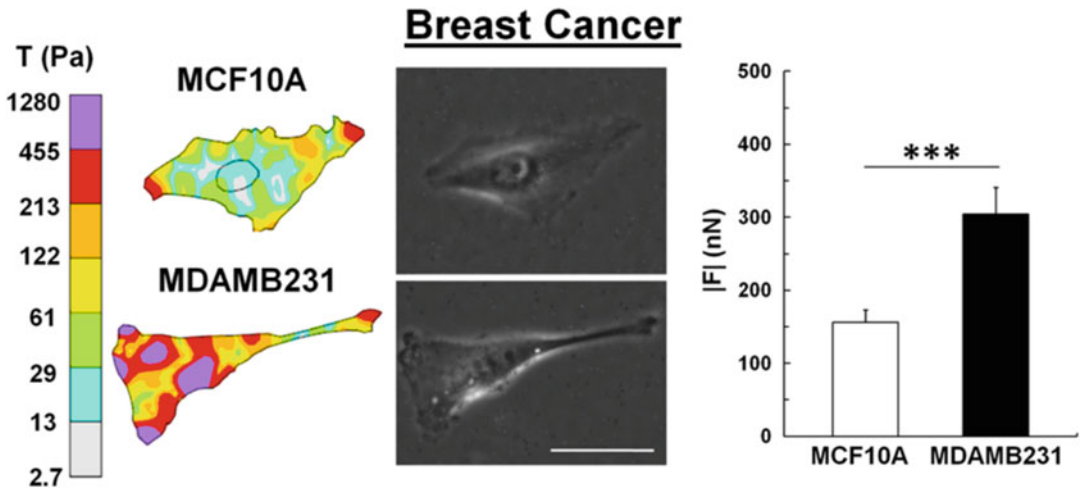


Fig. 15.1 Metastatic cancer cells exert greater forces than non-metastatic cells. Representative traction maps (left), corresponding phase images (middle), and magnitude of the overall net traction forces (right) exerted by non-metastatic mammary epithelial cells (MCF-10A) and highly metastatic (MDA-MB-231) cancer cells. Cells were cultured on polyacrylamide substrates with Young's

modulus (E) = 5 kPa, functionalized with type 1 collagen at a concentration of 0.1 mg/mL. Scale bar = 50 μ m. Mean + standard error of the mean; *** indicates $p < 0.001$. Traction force microscopy offers biophysical insights that can be used to both detect and study the metastatic potential of cancer cells, just one example of its value for mechanobiology research. Adapted from [22]

many physiological and pathological processes [1–8]. In tumor progression, for example, the ECM progressively stiffens due to increased cell-mediated collagen deposition and cross-linking [9, 10]. In turn, the increased stiffness influences cancer cell growth, angiogenesis, and metastasis [2, 10, 11]. Cells sense and respond to extracellular biophysical cues through molecular mechanotransduction mechanisms, such as integrin-based focal adhesion complex signaling and actin-myosin reorganization [12–14]. These biophysical interactions play a key role in the onset and progression of cancer [2, 3, 5, 6, 10, 15], stem cell differentiation [16–20], morphogenesis [21], and wound healing [19].

A central feature shared among these biophysical phenomena is cell force. Cell forces are well known to play critical roles in such processes as metastasis (Fig. 15.1) [22], angiogenesis [23, 24], and dynamic self-organization of cell aggregates [25]. It should therefore come as no surprise that the forces exerted by cells on their environment, and how cells respond to mechanical stress and strain, are of significant interest to researchers in the area of biophysics. As a result, there is an

ongoing demand in the field of mechanobiology to be able to quantify cell forces and their impact on biological systems and phenomena.

Among the techniques that have been developed to enable the study of cell forces, this chapter will focus on the methods that have collectively come to be known as traction force microscopy (TFM). TFM encompasses a family of techniques which enable the quantitative measurement of cell traction forces via noninvasive optical imaging of deformations induced within continuous elastic substrates. The term “traction force” initially referred to the shearing forces exerted by adherent cells cultured on flat 2D surfaces. However, TFM has since grown to enable the measurement of general forces in three dimensions, exerted by cells grown either on the surface of, or embedded within, a substrate. In brief, TFM enables the indirect assessment of cell traction forces by first imaging the deformations that traction forces induce in the ECM or other substrates. Cell forces are then computationally reconstructed using a suitable model that relates forces, deformations, and known substrate mechanical properties.

The origins of TFM lie in the experiments of Harris et al., who reported in 1980 that cells cultured on a thin membrane of silicone rubber exerted contractile forces which caused the membrane to buckle and wrinkle [26]. The amount of wrinkling could then be used to estimate the magnitude of cell traction forces. Although these experiments laid the initial foundations for the optical measurement of cell forces, they did not enable robust force quantification due to the highly nonlinear and chaotic nature of membrane wrinkling. In 1999, Dembo and Wang presented the seminal work which marked the beginning of true TFM, as it is known today [27]. Silicone membranes were replaced with slabs of polyacrylamide hydrogel, coated with ECM proteins. This change in material and geometry eliminated wrinkling behavior, necessitating the addition of fluorescent beads embedded in the substrate to be used as fiducial markers for measuring deformations. As the substrate underwent transverse deformations in response to cell traction forces, the embedded beads were dragged along with it. This enabled the measurement of local substrate deformations by imaging displacements of the beads. Traction forces were then computed from these displacements using a mechanical model of the substrate.

Since then, further developments have drawn upon various tools and advances in biology, materials science, imaging, signal processing, and computing, to make TFM the diverse and powerful tool that it is today. Alongside TFM, other technologies for measuring cell forces have emerged [28]. For example, to alleviate the difficulties of force reconstruction and substrate preparation in TFM, a new kind of substrate was developed, consisting of microfabricated arrays of silicone posts [29]. In response to cell forces, these posts act like deformable springs, with behavior that is both well-characterized and tunable by controlling post geometry. However, as cells may only adhere to the top surfaces of posts, such systems present a geometrical constraint that is not observed in typical flat, continuous substrates, raising concerns about physiological relevance. Another method has enabled the measurement

of molecular stretching under tension by making use of fluorescence resonance energy transfer (FRET) [30]. However, the difficulty of obtaining quantitative force measurements that account for cell environmental conditions currently limit this technology such that it may only be used to complement, rather than serve as a substitute for, TFM [31]. As a result, TFM remains at the leading edge for the quantitative measurement of forces exerted by single cells and cell collectives on their environment.

As a tool for research in mechanobiology, TFM is frequently applied to investigate the relationships between biochemical/biomechanical cues, signaling pathways, ECM mechanics, mechanotransduction, and subsequent cell behaviors [32–37]. Despite its broad use, there are limitations to common incarnations of TFM, and many opportunities exist for further innovation and application to novel biological questions. To address this issue, ongoing developments are enabling application of TFM to *in vitro* systems of ever greater complexity and physiological relevance.

The remainder of this chapter has been written with a focus on the principles and techniques behind these recent developments in TFM. We review the common methods and considerations which constitute the core of modern TFM techniques, with the intent of fostering an awareness and appreciation for the capabilities and limitations of common TFM methods. We also discuss potential areas of growth and innovation for TFM research in the near future. In doing so, we highlight various research achievements which have made critical steps toward developing TFM into a more powerful tool for the study of cell forces in physiologically relevant systems and for making contributions to the growing field of mechanobiology.

15.2 From Engineered Systems to Cell Forces

Although modern implementations of TFM are quite diverse, all methods follow the same basic workflow (Fig. 15.2). Depending on the biological question at hand and the system under study,

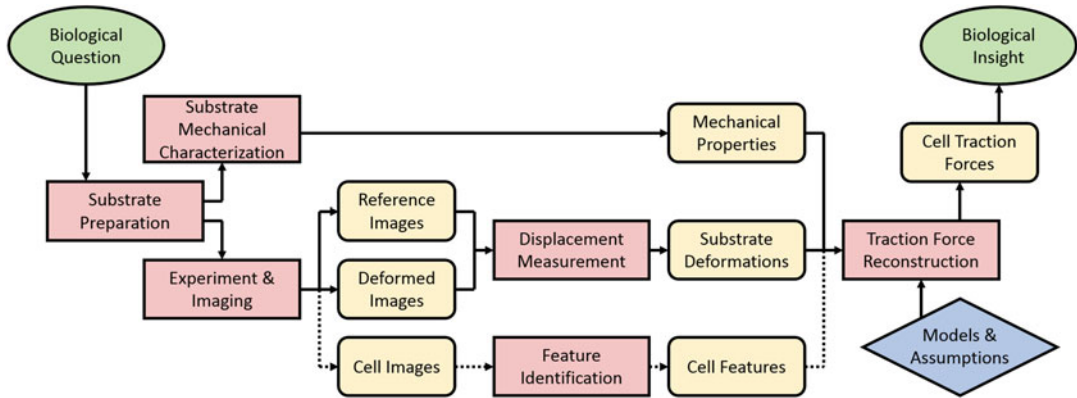


Fig. 15.2 High-level overview of the basic TFM workflow. Red (sharp rectangle) and yellow (rounded rectangle) panels indicate procedures and their output data, respectively. The models and assumptions used in TFM (depicted in the blue diamond) have direct bearing on the

type of traction force reconstruction methods that may be used. Dashed lines indicate experimental steps that may not be necessary, depending on the specific TFM methods chosen. For full details of the TFM workflow, please refer to the text

a substrate material is chosen. This material will deform when exposed to cell traction forces, and therefore must be mechanically characterized to enable the reconstruction of forces later on in the process. Fiducial markers (typically, fluorescent microbeads) are added to the surface of, or embedded within, the substrate. This adds optical contrast to the substrate, and allows traction force-induced deformations to be measured via the imaging of marker displacements.

Two or more images of the substrate are required. One image captures the non-deformed reference state, when there are no traction forces and the substrate is fully relaxed. The additional image/s capture the deformed state (at a single or multiple points in time), when adherent or embedded cells exert traction forces, causing marker agents to displace from their reference positions. The reference and deformed images are then used to generate measurements of the substrate deformations.

Once the traction force-induced substrate deformations are determined, this data is combined with the known (measured) mechanical properties of the substrate to reconstruct cell traction forces. Many force reconstruction methods exist to choose from, with the selection depending on the choice of mechanical model and any other relevant assumptions made for the study. Typical force reconstruction methods rely on the assumption that the substrate material is linear, elastic,

isotropic, and homogeneous and undergoes only small deformations/strains due to cell traction forces. However, as discussed in Sect. 15.3 of this chapter, recent advancements are beginning to reduce the need to rely on such assumptions [38–42]. Certain traction force reconstruction methods also rely on additional imaging data, typically in the form of cell structural information, such as a cell membrane outline, or the location of focal adhesion sites [27, 43–45]. (The fact that this information is only required by some TFM methods is indicated by the dashed lines in Fig. 15.2.) Once traction forces have been reconstructed, they may be used to yield insights which address the original biological question, or may even result in new unexpected discoveries.

Although the description above is sufficient to understand the general principles behind TFM, further detail is required to appreciate the common experimental considerations, practical implementations, and limitations of TFM. The remainder of this section discusses the individual steps of TFM in greater detail. That said, the information provided below is still a very general overview. Many useful and extensive reviews exist on these topics, which the reader is encouraged to explore if seeking additional perspectives and discussion beyond that found here [31, 46–49].

15.2.1 Substrate Selection and Mechanical Characterization

Substrate selection is a critical choice in any TFM study. This is because substrate composition and geometry are fundamentally linked to what types of systems can be modeled, what behaviors cells will exhibit, what kinds of forces can be exerted, what imaging and data processing methods are required, and finally, how traction force reconstruction may be performed. The founding works of TFM provide an illustrative example of the importance of substrate design. The transition from silicone membranes to polyacrylamide slabs played a crucial role in enabling the first incarnation of modern quantitative TFM [27, 50]. The new polyacrylamide platform provided flexibility and convenience for the repeatable fabrication of substrates that could be tuned to match the stiffness observed in a variety of *in vivo* tissues [50]. ECM proteins covalently bonded to the substrate surface (to enable cell adhesion) provided cells with binding domains that more closely resembled those of native ECM/tissue and provided an extra degree of freedom in experimental design. (Collagen and fibronectin, which are among the most abundant ECM components found in tumors [51–53], are often used for this purpose.) Finally, the geometry of the substrate, in combination with its linear elastic, homogeneous, and isotropic properties, allowed the traction force reconstruction problem to be vastly simplified, making quantitative and reliable TFM feasible to implement. The development of these systems was so successful that cell culture experiments performed on the surface of polyacrylamide hydrogels have since become the gold standard for measuring cell forces with TFM.

With advancements in imaging, data processing, and computation, this classic platform has expanded capabilities. While 2D cell culture on the surface of polyacrylamide hydrogels has been traditionally used to study purely transverse deformations and forces, it has been shown that even cells grown on flat surfaces can exert three-dimensional forces, causing out-of-plane defor-

mations of the hydrogel substrate [54–56]. The measurement of 3D cell forces exerted in 2D cultures gave rise to what is referred to in the literature as either 2.5D- or 3D-TFM [54, 56]. (We will adopt the “2.5D” naming convention here to distinguish this method from 3D-TFM methods that quantify the 3D forces exerted by cells embedded within 3D environments.) These 2.5D-TFM methods can help to provide a more complete picture of traction force-mediated cellular activity than is offered by 2D-TFM methods [54]. Despite these advances, the polyacrylamide platform is limited in that it does not enable the measurement of 3D forces exerted by cells residing within fully 3D environments. As cell behavior can greatly differ in 2D versus 3D environments [1, 5, 8, 57], there is a need for substrate systems that enable TFM in 3D cell culture.

Approaches to obtain platforms compatible with 3D-TFM rely on either engineered polymers or the use of native ECM scaffold materials. Legant et al. performed 3D-TFM by making use of polyethylene glycol (PEG) hydrogels, incorporating domains that allowed for both adhesion (fibronectin RGD binding domain) and degradation (matrix metalloproteinase susceptible linkers) by embedded cells [58, 59]. Other studies in 3D settings have chosen to make use of materials that more closely approximate natural 3D tissue environments, such as fibrin [23], Matrigel [60], and collagen [41, 61]. As will be discussed in Sect. 15.3, the use of these biopolymer substrates enables TFM in fully 3D environments but can introduce complications such as nonlinearity, heterogeneity, and anisotropy. These factors complicate the characterization and modeling techniques required to accurately reconstruct traction forces. Nevertheless, the application of TFM to such systems that more closely approximate physiological environments is expected to be a major theme in future TFM research.

Once a substrate is constructed, its mechanical properties must be characterized, as these properties will inform how traction forces relate to observable deformations. As most TFM substrates are chosen/assumed to be linear, elastic, isotropic, and homogeneous, it is typical that

only macroscopic mechanical properties, like the Young's (elastic) modulus and Poisson's ratio, are sought [45]. As a result, mechanical characterization methods have historically been fairly simple. The most common techniques include bulk rheometry [38], indentation testing (such as by depressing a steel ball) [46], and atomic force microscopy (AFM) [39]. However, when biopolymers are used for constructing TFM substrates, the (typically heterogeneous) mechanical properties on the micro/nanoscale throughout the substrate volume are unknown/inaccessible to these methods. Possible future methods of characterization will be discussed in Sect. 15.3.8.

15.2.2 Obtaining a Reference State

In order to quantify the impact of cell traction forces on the environment, TFM requires measurements of the substrate in both reference (relaxed) and deformed (loaded) states. While the method for obtaining a reference state is rarely discussed at length in the literature, it is an important experimental design consideration. Some 2D culture-based methods image the substrate before the addition of any adherent cells to the sample [62]. This allows for a truly relaxed state to be obtained, with no risk of substrate alteration due to cellular activity. However, this arrangement can be problematic, as the act of adding cells to the sample may inadvertently disrupt the sample position and orientation relative to the imaging system. If not prevented or accounted for by hardware in the imaging setup, such misalignments complicate the measurement of traction force-induced deformations [62]. Specific experimental constraints such as long culture times, or the possibility that cells will migrate into/out of the field of view, can make obtaining the reference state first infeasible in some cases. This method is not used for 3D-TFM with embedded cultures, due to the fact that cells are added at the time of substrate fabrication, eliminating the opportunity to obtain a truly cell-free reference state. As one potential solution, samples may be imaged immediately after substrate polymerization, before cells have had ample time to apply significant

forces in the substrate [63]. However, other factors, such as swelling of the substrate over time when immersed in culture media, may hinder this approach.

Alternatively, the deformed state may be imaged first, after cells have been added to the system and have begun exerting traction forces. Cell forces may then be removed *in situ* via chemical treatment. The compounds applied may cause cell death, detachment, or inhibition of cell contractility. (In the last case, the effectiveness of traction force inhibition must be established to ensure complete relaxation is achieved.) Under the assumption that the substrate undergoes purely elastic (reversible) deformation, the removal of cell traction forces is sufficient to allow the substrate to return to its original relaxed state. However, this assumption is not necessarily valid when cells are capable of remodeling the substrate (such as in the case of 3D degradable ECM/hydrogels). In this scenario, measurements taken over a long period of time (several hours and longer) can be susceptible to alteration of the substrate geometry and mechanical properties by cell-induced remodeling. This would then have to be accounted for in the force reconstruction process [41]. Therefore, it is recommended that substrate recoverability is tested to ensure reliable traction force reconstructions when not using TFM models that account for remodeling.

Finally, fabrication techniques can assist in obtaining a reference state. For example, Polio et al. used an indirect micropatterning approach to bond fluorescently labeled fibronectin to the surface of a polyacrylamide gel [64]. The fibronectin was deposited in discrete dots, forming a rectangular grid with 5 μm spacing. These fibronectin dots were then used as fiducial markers to track substrate deformations resulting from cell traction forces. Because the fabricated pattern of markers was known *a priori*, deformations could be determined without imaging a reference state. As a result, a single prepared substrate could be used to image many separate cells across multiple fields of view, enabling high-throughput imaging for 2D-TFM experiments. As an added benefit, the fibronectin dots served as the only sites where cells could exert forces on the polyacrylamide

gel. Constraining the locations of cell tractions allows for simplified and robust traction force reconstruction procedures, as will be discussed in Sects. 15.2.5 and 15.3.1 [43, 45, 64, 65]. However, the artificial constraint on cell force locations imposed by this method may impact physiological relevance, similar to the micropillar arrays mentioned previously. In spite of this limitation, it should be noted that methods using micropatterned adhesion sites/markers do enable novel studies on the effects of different patterns and choices/combinations of ECM proteins on cell traction forces [66].

15.2.3 Noninvasive Imaging of Cell Force-Induced Deformations

TFM may be considered a noninvasive technique in that the measurements of substrate deformations are obtained through optical imaging, without disturbing the experimental system. As TFM frequently relies on the use of embedded fluorescent marker beads to track displacements within the substrate, widefield fluorescence and confocal fluorescence imaging are commonplace in many TFM procedures. When images of cellular structure are required for force reconstruction or visualization, phase-contrast imaging is also commonly used in 2D- and 2.5D-TFM settings. While these standard microscopy techniques have been in use for years, increasing demands for 3D imaging, speed, reduced photobleaching/phototoxicity, and higher resolution, among other factors, are driving the emergence of TFM conducted with alternative imaging methods, which will be discussed in Sect. 15.3.9. Regardless of the imaging technique used, there are three major factors that must be considered for imaging systems in TFM: field of view, acquisition speed, and resolution.

An imaging system must have a large field of view to make reliable measurements for TFM. In the context of cells cultured on a flat substrate, the field of view must be wide enough to capture regions far away from the cell/s under study. If this is not achieved, cells outside the field of view, but close to the cell/s of interest, may alter the

substrate deformations within the field of view. This can prevent the accurate reconstruction of traction forces exerted by the cell/s of interest. Moreover, if cell migration is expected, the field of view must be large enough to prevent the cell/s from exiting the field of view before the conclusion of the experiment. In the case of cells cultured in 3D environments, these field of view requirements must be extended to three dimensions. Therefore, the imaging system must also be able to capture images over a large depth range for the same reasons described above for the case of 2D systems.

Imaging speed is an important consideration when dynamic systems or photobleaching/phototoxicity are of concern. Cells can exert dynamic forces on timescales as short as minutes [60, 63]. Therefore, imaging speeds must be faster than these dynamic processes, or cell forces may change during acquisition. For 2D imaging systems, this is rarely an issue. However, it can become a major concern for 3D imaging systems, which can take several minutes to acquire a single volume. Moreover, longer imaging times can risk causing photodamage to cells (potentially altering cell behavior) and may result in photobleaching of fluorescent markers or labels (disabling them for use in measuring substrate deformations or cell structure).

Finally, imaging resolution is a vital component for TFM. As many TFM techniques rely on obtaining information about cell structure, the imaging resolution must be sufficient to capture these features. Failure to do so may result in inaccurate traction force reconstructions. Imaging resolution must also be high enough to distinguish fiducial markers and capture their displacements within the substrate. This is a concern particularly when dense marker concentrations are employed, a scenario which is discussed below in Sect. 15.2.4.

15.2.4 Measuring Cell Force-Induced Deformations

Substrate deformations are measured by tracking the displacement of attached/embedded markers

between the reference and deformed states of the sample. Each marker provides a unique measurement in space of the underlying deformations of the substrate. Therefore, the density of the markers (markers per volume) limits the spatial sampling frequency at which deformation data may be acquired. It is therefore crucial that marker densities are high enough to capture the spatial variations of the displacement field (i.e., to capture the variations with high enough resolution), while ensuring that the markers are small enough and the density is low enough that the presence of the markers does not appreciably alter the behavior of the system. Marker bead diameters typically lie within the range of tens of nanometers to micrometers [31], and typical mean particle spacings are in the range of a one to tens of micrometers [47]. As a general rule-of-thumb, bead spacings for high-resolution TFM applications are typically found to be on the order of ten times the bead diameter [58, 67, 68]. Those seeking very high resolution displacement field measurements often turn to novel methods, such as the use of beads of different colors and multiple imaging channels [49, 58, 69], or even super-resolution microscopy [70], to capture useful images in samples with very high bead concentrations. The tracking of markers is commonly performed using either of two paradigms: single-particle tracking or cross-correlation-based tracking.

Single-particle tracking involves tracking the position of individual markers. The primary challenge lies in uniquely identifying the same markers in both the reference and deformed state images [71]. Images must therefore be of high enough signal quality and imaging resolution that marker beads may be reliably tracked with minimal errors and noise artifacts. The resulting displacement field typically consists of measurements acquired at randomly distributed locations in space (resulting from the random positions of marker beads). When force reconstruction is performed, these random sampling locations may either be used directly, or may be interpolated onto a grid, depending on the force reconstruction method chosen.

Cross-correlation-based tracking does not identify the motion of individual markers.

Instead, it captures the motion of local groups of markers. This is commonly done via digital image correlation (DIC) for two-dimensional systems or digital volume correlation (DVC) for three-dimensional systems. DIC and DVC track the bulk motion of windowed regions of the sample containing multiple markers. As displacements are computed wherever a window is constructed, correlation-based tracking allows for the measurement of the displacement field to take place on a rectangular grid, which can be convenient for later processing steps (such as Fourier transforms) during force reconstruction.

When implementing cross-correlation-based tracking, cross-correlation window design plays a critical role in computing the displacement field. Large window sizes help reduce noise in the displacement field measurements, but come at the cost of poorer resolution, degrading displacement features on the order of and smaller than the window size. In other words, the window acts as a low-pass filter over the displacement data. Window profiles modify the intensity across space within the windowed region and impact the spatial frequency response of the cross-correlation. Consequently, an improperly designed window may amplify or attenuate displacement features of differing sizes in a biased manner [72]. Correlation methods in TFM typically rely on the assumption of purely translational motion of marker clusters over small distances. Recent efforts in TFM have sought to mitigate this issue, enabling efficient correlation-based tracking of large deformations [72], as well as deformations which exhibit dilation/stretching [73].

Although various implementations of particle tracking and cross-correlation-based tracking are the most common tools employed by TFM researchers, it is worth noting that measuring deformations between images is a problem of ongoing interest and research in the field of computer vision. As such, a wide variety of algorithms are available for adaptation to specific TFM experimental settings and applications [74]. Optical flow algorithms are one example that has been explored for use in TFM [75]. Ultimately, the choice of tracking algorithm for a particular

study will be influenced by many factors, including experimental conditions, traction force reconstruction method, desired accuracy, and available time and computing resources.

15.2.5 Force Reconstruction

Of all the elements of TFM, the final reconstruction of cell traction forces is perhaps the most diverse. Various models and techniques have been introduced, with great potential for both refinement and innovation. Because force reconstruction is closely tied to both experimental design and ongoing developments in TFM, it is important to be aware of its various forms, requirements, capabilities, and limitations. What follows is an overview of common methods,

with large inspiration drawn from the review by Schwarz and Soiné [31], which the reader is encouraged to explore for further detail. A summary of the traction force reconstruction methods discussed here may be found illustrated in Fig. 15.3, with notable features outlined in Table 15.1.

15.2.5.1 Direct and Inverse Methods

One intuitive method available for the reconstruction of cell traction forces is what has been referred to as the “direct” TFM method [31]. By making use of measurements of the strain field within the substrate, stress can be computed “directly” via the stress-strain constitutive relation which characterizes the substrate material (such as Hooke’s law for linear elastic solids) [42, 47, 56, 76]. As a result, the stress field can be determined throughout the deformed substrate by

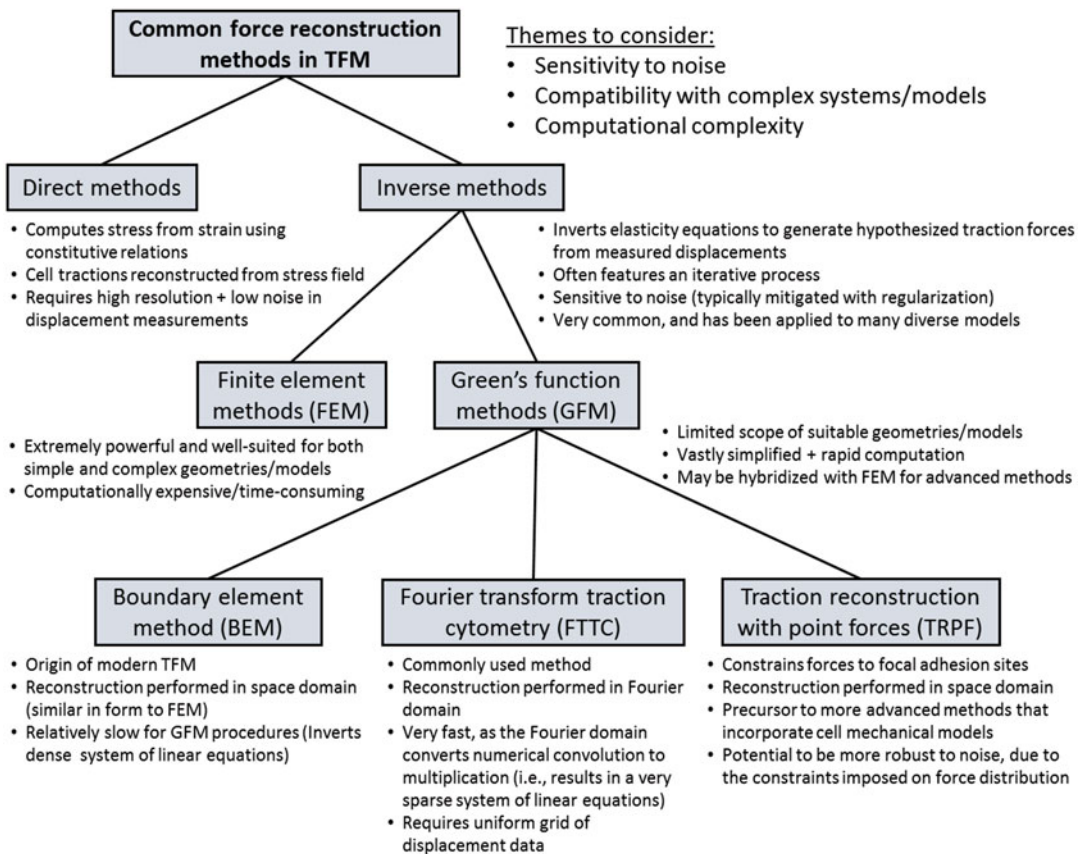


Fig. 15.3 Common traction force reconstruction methods in TFM, at a glance. Further details and discussion for each method can be found in the text

Table 15.1 Features of standard TFM reconstruction methods

TFM method	Sensitivity to noise	Typical noise mitigation	Computational cost	Compatible with complex geometries/materials?	Compatible dimensions
Direct TFM	High	Data smoothing	Low	Yes ^d	2D, 2.5D, 3D
FEM-TFM	High	Regularization	High	Yes	2D, 2.5D, 3D
BEM	High	Regularization	Medium	No	2D, 2.5D
FTTC	High	Data smoothing & Regularization ^b	Very Low	No	2D, 2.5D
TRPF	Low ^a	Regularization	Low ^c	No	2D, 2.5D

^aExtra information used by this technique helps stabilize force reconstruction

^bTypically uses cross-correlation-based tracking, which smooths data. Force reconstruction is often regularized

^cDetermined by number of focal adhesion sites

^dAssuming a suitable stress-strain constitutive relation is available

plugging the measured strain field into the constitutive relation. Cellular traction forces located at the cell membrane can then be computed from the stress field. This method relies on obtaining a reasonably accurate approximation of the strain field. In practice, the strain field is obtained by taking the spatial gradient of the measured displacement field data. As a consequence, the displacement field must be measured with high enough resolution to sufficiently capture its variability over small regions. Moreover, the measurements must have low noise, because gradient operations amplify noise artifacts, especially over short spatial scales. In the presence of sufficiently high noise, the gradient operation must often be accompanied by some form of filtering or regularization operation [56]. Direct TFM is a younger member in the family of traction force reconstruction methods. Used primarily in 2.5D-TFM settings, its emergence has been enabled by the growing availability of high-quality 3D imaging [31]. Though it is currently less prevalent than older methods, direct TFM has demonstrated promise for application in substrates that exhibit large deformations [42] or viscoelasticity [39], which many of the more common methods (e.g., Green's function methods, which will be discussed shortly) are not compatible with.

An alternative framework is the family of "inverse" TFM methods, which constitute the majority of methods reported in TFM studies. Inverse TFM does not compute stresses and tractions directly from the measured

displacement/strain data, as is done in direct TFM. Instead, a hypothesis is made about what distribution of cellular traction forces would be most likely to produce the measured displacement field, given the constraints of a suitable mechanical model. Depending on the specific technique chosen, this estimate may be arrived at either through direct computation or via iterative methods (though iteration is the dominant approach) [31, 44]. Iterative methods are constructed to minimize (typically in the least squares sense) the discrepancy between the measured displacements and the displacements that would result from the reconstructed (hypothesized) traction field. To mitigate the impact of noise and address the ill-posed nature of the inverse problem, this minimization procedure is often regularized [31, 45, 69]. That is, the possible traction reconstructions are constrained by the imposition of additional information and/or constraints beyond those directly underlying the mechanical model [43, 45]. Regularization for inverse TFM will be addressed in greater detail at the end of this section.

15.2.5.2 Finite Element and Green's Function Methods

In order to implement inverse TFM methods, one must be able to generate predictions of displacement fields that would result from hypothesized cell traction forces. There are two major approaches in the inverse TFM family for making

such predictions: finite element methods (FEM) and Green's function methods (GFM).

The details underlying finite element analysis are beyond the scope of this chapter. However, in brief, finite element methods operate by partitioning a model of the sample into a set of discrete subunits, or elements. The behavior of each element is governed by the fundamental (elasticity) equations of the system, with constraints imposed on each element by its neighbors and/or the boundary conditions of the substrate. This allows for the construction of a system of equations that may be solved through various methods. FEM has the advantage that it can be adapted to model complex geometries and governing equations. For this reason, FEM has found significant use in the area of 3D-TFM, where complex cell boundaries prevent the use of analytical solutions to the elasticity equations in the traction force reconstruction process [41, 58]. FEM is also suited to nonlinear material models and geometric nonlinearities resulting from large deformations [31, 38]. As a result of its broad capabilities, FEM has played a key role in many TFM studies and will likely continue to do so in emerging methods and future studies (although applications of FEM to biopolymer substrates will likely rely increasingly on novel mechanical characterization techniques in order to take advantage of more advanced mechanical models). Despite its clear advantages and future prospects, the power and flexibility of FEM come at substantial computational cost, which motivates the use of simpler models and computing methods to accelerate the process of traction force reconstruction.

One family of alternatives to FEM is Green's function methods. GFM models make use of several assumptions to enable efficient computation of traction forces. These include the ubiquitous assumptions which constrain the substrate to be composed of a linear, elastic, isotropic, homogeneous material (although these assumptions often do not apply in tissues). In addition, GFM models rely on the assumption of small strains (to avoid geometric nonlinearities from large deformations) and are often confined, in practice, to simple substrate geometries with traction forces

applied on a planar surface (though this is not always the case, as discussed in Sect. 15.3.1).

Although using these assumptions and constraints can limit the accuracy and physiological relevance of TFM studies, they vastly simplify the computation required for traction force reconstruction. For GFM in particular, these assumptions allow for the substrate to be regarded as a linear space-invariant (LSI) system which takes cellular traction forces as the input and yields substrate deformations as the output. The response of such a system to a point-like cell traction force (as might approximately occur at a focal adhesion site [43, 45, 64]) is described by a Green's function, which is determined by the properties and geometry of the system. Due to the linearity of the substrate, the solution to the elasticity equations that relate traction forces to substrate displacements may be written as a weighted sum of these Green's functions. Specifically, the relationship between the substrate deformations and the applied traction forces is described by a convolution relation [69].

$$\mathbf{u}(\mathbf{r}) = \int \mathbf{G}(\mathbf{r} - \mathbf{r}') \mathbf{f}(\mathbf{r}') d\mathbf{r}' \quad (15.1)$$

where $\mathbf{u}(\mathbf{r})$ denotes displacement of the substrate at the location $\mathbf{r} = (x, y, z)$, $\mathbf{f}(\mathbf{r}')$ denotes the cell traction force applied at the location $\mathbf{r}' = (x', y', z')$, and $\mathbf{G}(\mathbf{r} - \mathbf{r}')$ denotes the (spatially invariant) Green's function of the system. The integration over \mathbf{r}' signifies a summation of contributions from all the traction forces exerted throughout the sample. In other words, the substrate displacement at any one location is a net effect of all traction forces exerted throughout the sample. The dimensionality of the system under study will determine the number of components/elements in $\mathbf{u}(\mathbf{r})$, $\mathbf{f}(\mathbf{r}')$, and $\mathbf{G}(\mathbf{r} - \mathbf{r}')$. As a simple example of how Green's functions relate traction forces to substrate displacements, consider a 2D-TFM system which assumes only transverse forces and displacements (as is common throughout early and many modern TFM works). The displacement of the substrate \mathbf{u} (at \mathbf{r}) in response to a single-point force \mathbf{f} (at \mathbf{r}') can be expressed using Cartesian coordinates (x, y) by

$$\begin{bmatrix} u_x \\ u_y \end{bmatrix} = \begin{bmatrix} G_{xx} & G_{xy} \\ G_{yx} & G_{yy} \end{bmatrix} \begin{bmatrix} f_x \\ f_y \end{bmatrix} \quad (15.2)$$

where u_i and f_j denote the components of displacement and force, respectively, and G_{ij} denotes an element of the system's Green's function, which describes the contribution of the j -component of force (at \mathbf{r}') to the i -component of displacement (at \mathbf{r}). Figure 15.4 provides an illustration of this example. As can be seen with this notation, it is important to note that a force in one direction can contribute to displacements in any direction.

As the relation of force to displacement is given by a convolution, the objective of GFM is then to perform deconvolution, using the known Green's function and displacement data to invert the relation in Eq. (15.1) and reconstruct the cell traction forces. There are various methods by which this deconvolution is achieved in the field of TFM, which will be detailed below. Currently, GFM has been applied to 2D- and 2.5D-TFM systems [27, 44, 45, 77, 78], with cells adhered to an elastic substrate with a flat surface geometry. Green's functions have been determined and used for models of the substrate as an elastic half-space [44] and as a slab of finite thickness [77]. A variation of GFM hybridized with FEM has also been applied to 3D-TFM (detailed in Sect. 15.3.1).

One major theme to keep in mind throughout the following sections is the issue of experimental noise. Green's functions in TFM act as low-pass filters, attenuating features that span short spatial scales. Upon measurement of substrate displacements, noise corrupts the true displacement signal. As traction force reconstruction involves inverting the low-pass effects of Green's functions, noise artifacts are amplified over short spatial scales and can have a severe impact on the quality and accuracy of reconstructed traction forces [69]. This motivates the use of regularization, which is detailed at the end of this section.

15.2.5.3 Common Variations of Green's Function Methods

There are three primary techniques used in TFM to reconstruct forces using Green's functions.

These are the boundary element method (BEM) [27], Fourier transform traction cytometry (FTTC) [44], and traction reconstruction with point forces (TRPF) [45]. Application and implementation of these methods involves several important considerations, which are discussed in the primary literature. The basic concepts are outlined below.

BEM was the first method to emerge among modern TFM techniques that enable accurate quantitative traction force reconstructions [27]. BEM requires, in addition to the displacement data, a tracing of the cell boundary. Once this boundary is established, the surface region of the cell that is in contact with the substrate may be approximated by a discretized mesh. It is assumed that traction forces may originate only from within this surface (Fig. 15.5, panel 3). The discrete set of locations where cell tractions may originate is combined with the discrete displacement data to convert Eq. (15.1) into a linear system of equations, which may be solved using standard methods. (This makes BEM similar in form to FEM but performed with simplified equations and without generating a mesh of the surrounding substrate.) In practice, due to noise constraints, the system is usually inverted with a variation of regularized least squares. In summary, this method solves the inverse problem in the space domain but depends upon reliable cell tracing and can be sensitive to the chosen meshing procedure [27, 69]. Because the linear systems of equations solved by BEM are often very large and dense/non-sparse, BEM can take longer to execute than other GFM techniques.

In contrast, FTTC solves the inverse problem in the Fourier domain, where the relation described in Eq. 15.1 takes the form

$$\tilde{\mathbf{u}}(\mathbf{k}) = \tilde{\mathbf{G}}(\mathbf{k}) \tilde{\mathbf{f}}(\mathbf{k}) \quad (15.3)$$

where $\tilde{\mathbf{u}}(\mathbf{k})$, $\tilde{\mathbf{G}}(\mathbf{k})$, and $\tilde{\mathbf{f}}(\mathbf{k})$ denote the Fourier transforms of the displacement field, Green's function, and traction field, respectively, and \mathbf{k} denotes the spatial frequency coordinate [31, 44, 69]. As convolution (Eq. 15.1) is converted to multiplication in the Fourier domain (Eq. 15.3), the reconstruction of traction forces is reduced to

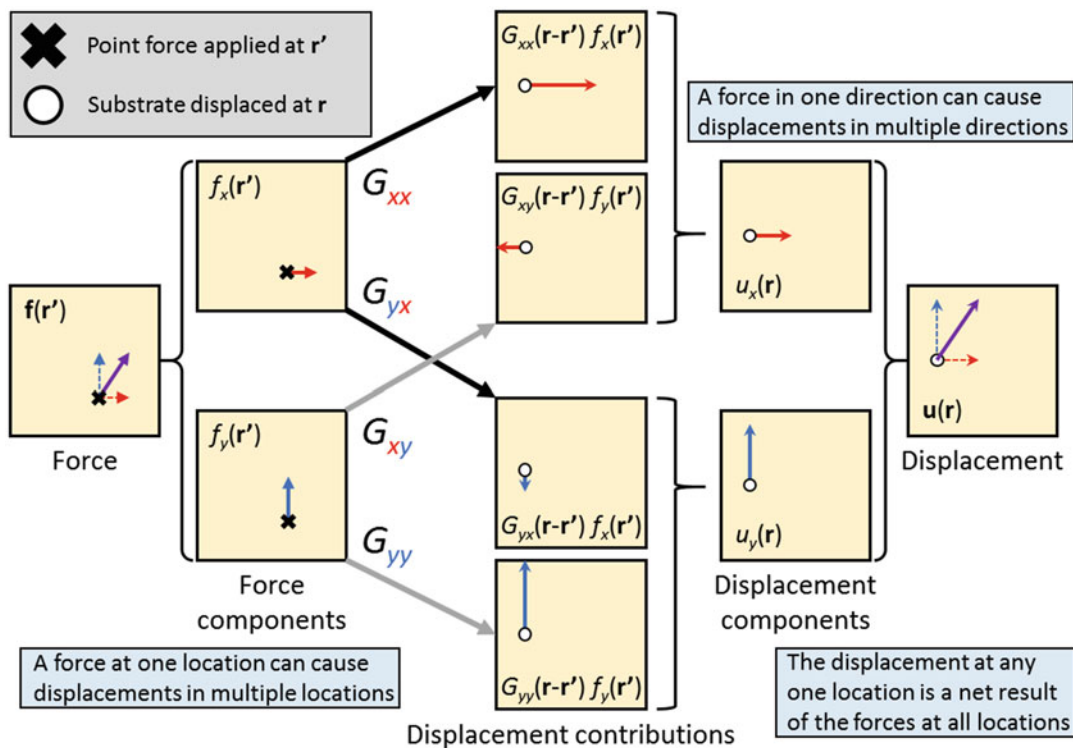


Fig. 15.4 How Green’s functions relate traction forces to substrate displacements. The above diagram serves as a depiction of how a 2D system governed by Eqs. (15.1) and (15.2) responds to localized traction forces. In principle, Eq. (15.1) allows displacements throughout the substrate to be computed from any general distribution

of cell traction forces, so long as a Green’s function for the system can be determined. The goal of GFM-based force reconstruction is to invert the above process (i.e., to generate a distribution of traction forces from the known Green’s function and measured substrate displacement data)

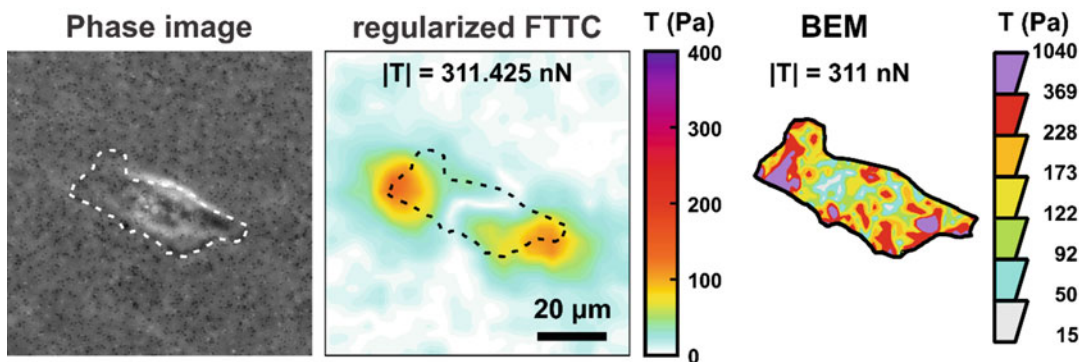


Fig. 15.5 Comparison of traction force reconstruction with FTTC versus BEM. Phase contrast image of an MDA-MB-231 cell (left) and associated traction force reconstructions using FTTC (middle) and BEM (right). The substrate consisted of a collagen-coated polyacrylamide gel with Young’s modulus of 5 kPa, with embedded Alexa fluor 488 polystyrene beads (diameter 0.5 μm). FTTC was regularized with Tikhonov regularization. Note that

the tractions reconstructed using FTTC do not necessarily correspond to the true cell surface. The reconstructed traction forces are also very smooth, due to a combination of regularization and low fluorescent bead density. The tractions reconstructed with BEM, on the other hand, are confined exclusively to the cell surface but possess a more irregular distribution of forces. This feature is likely to be an artifact of noise and insufficient regularization

$\tilde{\mathbf{f}}(\mathbf{k}) = \tilde{\mathbf{G}}(\mathbf{k})^{-1}\tilde{\mathbf{u}}(\mathbf{k})$, which is simply a multiplication of the Fourier domain displacement data with the inverse of the Fourier domain Green's function. However, this procedure is sensitive to the presence of noise or other errors in the displacement data, and therefore is typically modified with a regularization procedure (which will be discussed in the next subsection). Following the inversion process, the reconstructed traction forces are obtained by taking the inverse Fourier transform of $\tilde{\mathbf{f}}(\mathbf{k})$. Note that in order to make efficient use of Fourier transforms, displacement data must be provided at locations on a uniform rectangular grid (either through interpolation or the use of correlation-based displacement tracking). In general, FTTC is very fast compared to space domain methods, as the Fourier transforms and element-wise multiplications used by FTTC have lesser computational complexity than space domain operations like convolution and matrix inversion. As a result, FTTC methods are very common in the literature due to both their simplicity and efficiency. One drawback of FTTC is that it does not make use of any information about the cell structure and as a result is vulnerable to predicting the presence of traction forces originating outside the cell boundary (Fig. 15.5, panel 2). FTTC may be modified to mitigate this concern, though such procedures are not frequently reported in the literature [44].

TRPF, as its name suggests, seeks to reconstruct a force distribution consisting of point-like forces, unlike the smoother/continuous distributions generated by BEM and FTTC [45]. TRPF assumes that cell traction forces are localized to focal adhesion sites. By imaging the locations of these sites in any given cell with appropriate fluorescent labeling (assuming these additional imaging capabilities are available), a set of acceptable locations where traction forces may originate is established. Similar to BEM, this set of locations is used in conjunction with the displacement data to allow Eq. 15.1 to be converted to a linear system of equations. Due to the sparsity of locations where traction forces may be reconstructed, TRPF can mitigate the effects of noise (and the associated need for regulariza-

tion) by constraining the possible traction force solutions, although this potential benefit degrades with increasing numbers/density of point forces [45, 69].

15.2.5.4 Regularization

The reconstruction of cell traction forces from measured displacements via inverse methods is an ill-posed problem. That is, when the true substrate displacements are not precisely known due to uncertainties from noise or errors in the data, cell traction forces cannot be precisely reconstructed (i.e., the reconstruction process does not produce unique solutions). In addition, small changes in the displacement data can result in large changes in the reconstructed traction field (i.e., the reconstruction process is sensitive to noise) [45]. As a result, the presence of noise can have a severe impact on the accuracy and quality of traction force reconstructions. To address this issue, the inverse problem may be regularized.

Regularization incorporates additional a priori information into the inverse problem, beyond that which is already contained in the displacement data and mechanical model used during force reconstruction. This information helps constrain and stabilize the possible traction force solutions to the ill-posed inverse problem presented by TFM [45]. In other words, regularization assumes that certain types of reconstructed force distributions are not valid solutions to the inverse problem. The specific regularization procedure determines what types of solutions are suppressed and what trade-offs may result. Although regularization can be formulated to impose many types of constraints, most forms of regularization employed in TFM are specifically designed to suppress the effects of noise artifacts in the reconstruction process.

FTTC can provide some intuition as to why noise is such a prevalent concern. Following from Eq. 15.3, unregularized FTTC reconstructs traction forces as a product of the inverse Green's function and the displacement data: $\tilde{\mathbf{f}}(\mathbf{k}) = \tilde{\mathbf{G}}(\mathbf{k})^{-1}\tilde{\mathbf{u}}(\mathbf{k})$. However, Green's functions in TFM typically act as low-pass filters. In other words, the Green's function may have singular values that approach zero

at higher spatial frequencies (i.e., when the magnitude of \mathbf{k} is large). When singular values are small, multiplying the displacement data by $\tilde{\mathbf{G}}(\mathbf{k})^{-1}$ is akin to performing division by very small numbers. When this occurs, values in the displacement data are strongly amplified during force reconstruction. This is a problem because any noise in the displacement data is also subject to these amplification effects. Regularization in TFM seeks to mitigate this effect.

The most common form of regularization in TFM for mitigating noise is zero-order Tikhonov regularization, which penalizes large values in the reconstructed traction force data [31, 69]. That is, force reconstructions that contain very large forces are assumed to be undesirable solutions to the inverse problem. Regularization suppresses these solutions by modifying the functions that relate traction forces and displacement data. For example, zero-order Tikhonov regularization applied to FTTC modifies the inversion process to take the form [31, 69]

$$\tilde{\mathbf{f}}(\mathbf{k}) = \left(\tilde{\mathbf{G}}(\mathbf{k})^T \tilde{\mathbf{G}}(\mathbf{k}) + \lambda^2 \mathbf{I} \right)^{-1} \tilde{\mathbf{G}}(\mathbf{k})^T \tilde{\mathbf{u}}(\mathbf{k}) \quad (15.4)$$

where \mathbf{I} denotes the identity matrix and λ is a scalar value, referred to as the regularization parameter, which determines the strength of the regularization procedure. In the case where $\lambda = 0$, Eq. 15.4 is equivalent to the original unregularized FTTC formulation, $\tilde{\mathbf{f}}(\mathbf{k}) = \tilde{\mathbf{G}}(\mathbf{k})^{-1} \tilde{\mathbf{u}}(\mathbf{k})$. The effect of this new formulation is to alter the Fourier domain Green's function such that singular values close to zero have their magnitudes increased, while large singular values are left relatively unchanged. Specifically, a singular value with magnitude σ is modified by the regularization procedure to obtain a new magnitude $(\sigma^2 + \lambda^2)/\sigma$. This reduces the amplification of noise where the inversion process is most vulnerable (i.e., when the values of σ are close to zero). The trade-off of this regularization procedure in TFM is that reconstructed traction fields may be smoother than the true traction field and may underestimate the maximum traction values. Although FTTC was highlighted in the

above example, the same principles apply to zero-order Tikhonov regularization in other force reconstruction techniques.

Selection of the regularization parameter λ involves making a trade-off between suppressing noise artifacts and over-smoothing the reconstructed traction field and must be taken into account when interpreting results. Selection of a parameter often involves solving the inverse problem several times until an optimal value can be determined. Because the optimal value may vary between datasets, this iterative optimization procedure must often be repeated between datasets, meaning that regularization parameter selection can add significant computational cost.

Although zero-order Tikhonov regularization is prevalent in TFM, its inherent suppression of large forces and tendency to smooth out the reconstructed traction field may be undesirable for a given study. Alternative regularization schemes may be sought to better meet experimental demands. For example, first-order Tikhonov regularization suppresses rapid spatial fluctuations in the traction field (by penalizing the gradient of the traction field, instead of the traction field itself). Although this regularization scheme would not directly suppress large forces, it would still act to smooth out the final reconstructed traction field, in exchange for suppressing noise artifacts [69]. In applications where cells exhibit strong localized forces, such as those seen at focal adhesion sites in 2D/2.5D settings, L_1 -regularization may be an appropriate choice [79, 80]. This scheme imposes the assumption that the true traction field is sparse and so attempts to reconstruct a small number of regions containing strong localized tractions. In this manner, the mechanism by which L_1 -regularization constrains the inverse problem is reminiscent of the TRPF method. However, this method requires longer computation times than other common forms of regularization. In general, this is because the solution to the L_1 -regularized problem cannot be expressed in closed form and must be determined through an iterative process. From another perspective, unlike TRPF, which takes focal adhesion sites as an input and reconstructs force, L_1 -regularization must determine both the location and strength

of cell traction forces simultaneously. Further discussion and details regarding other possible methods for regularization may be found in the literature of inverse problems [81].

It should be noted that regularization is not strictly necessary to address sensitivity to noise. For example, the use of cross-correlation-based displacement tracking (as is often done for FTTC) reduces noise artifacts through the filtering effects of the cross-correlation windowing functions. Noise is therefore reduced in the displacement data before it is input into the inverse problem. Of course, accurate reconstructions rely on raw images and correlation windows that support the spatial resolution (and corresponding spatial bandwidth) necessary to preserve all the relevant displacement data across the filtering operation. If this condition is not met, noise may be suppressed, but the reconstructed traction field may also become over-smoothed, similar to what happens in the case of over-regularized traction reconstructions.

15.3 The Future of TFM

Much remains to be achieved in order to extend TFM to the study of more complex, physiologically relevant, biological systems. In addition, expanding the use of TFM within mechanobiology will require advances that make new methods more accessible to researchers from diverse areas of study. This will be important to enable discoveries made through fundamental mechanobiology and biomechanics research that may be later translated into new clinical diagnosis and treatment paradigms. Future improvements, such as addressing nonlinearity, heterogeneity, ECM remodeling, etc., have often been relegated to a few cursory statements in the discussion sections of primary literature. This is not unreasonable, as the work involved to experimentally address these concerns is far from simple. However, ongoing research efforts have nevertheless persisted and sought to advance TFM to address new levels of system complexity. In this section, we review the motivations and recent advancements made to

address several factors that may be critical to future research efforts in TFM and cell mechanics.

15.3.1 Advanced Force Reconstruction Methods

The development of novel force reconstruction methods largely relies on two primary resources. First is the development of new mechanical models that incorporate additional information about the mechanical properties and features of both substrates and cells. Second is the development of reconstruction methods that minimize computational complexity, in order to make efficient use of available time and computing resources. New methods that help better address these needs hold strong promise for advancing TFM and mechanobiology at large.

For example, the incorporation of additional cell structural data may enable the development of more robust and useful TFM models and results. Soiné et al. presented what has been termed “model-based TFM” (MB-TFM) [43]. MB-TFM uses images of both focal adhesion sites and stress fibers to construct an approximate mechanical model of the cell under study (Fig. 15.6). Presently demonstrated in a 2D setting, MB-TFM models stress fibers and the actin network together as a cable network that distributes internal cellular tension and uses focal adhesion sites as anchor points that exert traction forces on the substrate. An FEM-based algorithm determines what distribution of fiber tensions will produce the necessary forces at the focal adhesion sites to generate the measured displacement field. This method has been shown to be robust under measurement noise, without the need for regularization. In essence, the constraints imposed by the cable network mechanical model provide the necessary stabilizing information that would otherwise be provided by regularization in other TFM methods. The reason for this becomes clear when observing that the traction forces at individual focal adhesions are related to one another, as mediated by the communication of forces over the stress fiber + actin cable network. MB-TFM is thus more strongly constraining than

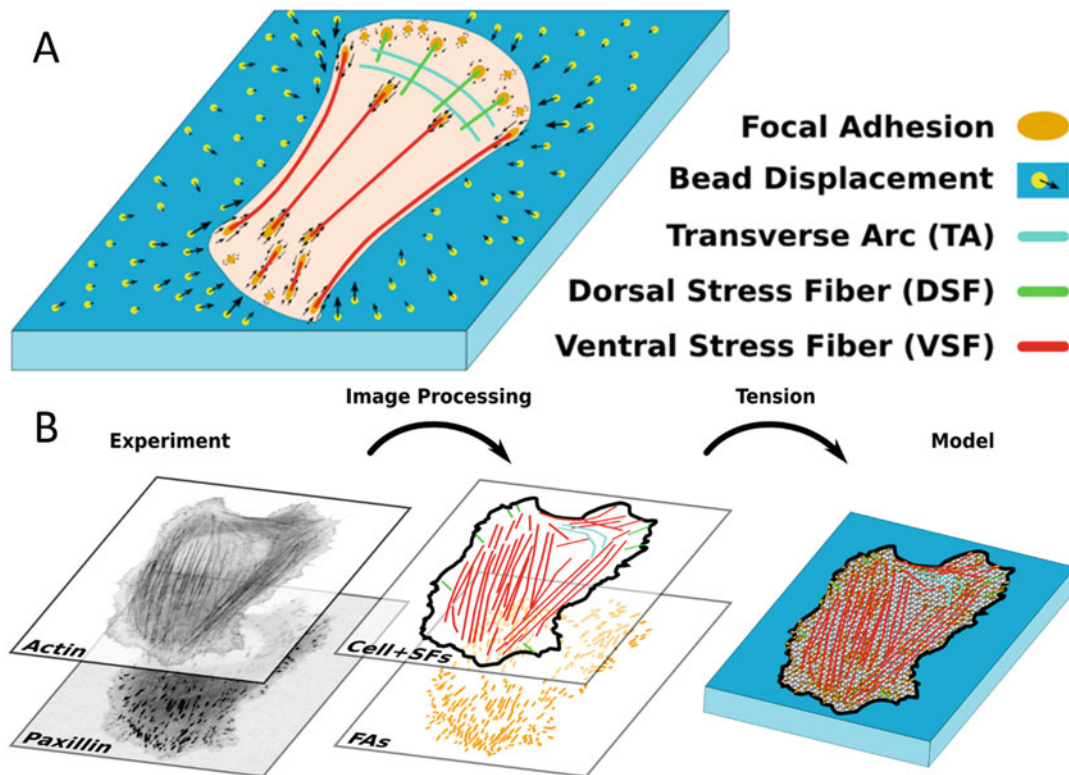


Fig. 15.6 Model-based TFM. (a) Schematics of a cell cultured on a soft elastic substrate with embedded fluorescent marker beads. Stress fibers and the actin network transmit forces to the substrate via focal adhesions. (b) Imaging, feature identification, and construction of a whole-cell mechanical model. MB-TFM assigns tensions

to each fiber and finds the tension distribution most likely to produce the measured displacement data. MB-TFM stands out for being robust to noise without a need for regularization and for generating information about internal cell stress distributions (in addition to the usual traction forces). Adapted from [43]

the TRPF method discussed previously, which did not assume a mechanical correlation between forces at different focal adhesion sites. Finally, in addition to the reconstructed traction forces, the network tensions yield information about the internal distribution of forces within the cell, offering potential insight into the mechanisms of force transmission and mechanosensing beyond what could be achieved with prior TFM methods. MB-TFM still has its limitations, however. As described in the original work, MB-TFM cannot be universally applied to all cell types/scenarios. In addition, MB-TFM relies on high-quality imaging of cellular features, which is not always readily available, and can be negatively impacted by chemical reagents that modulate cell contractility behavior [43].

Other advances have led to progress in the area of computation time. While GFM-based traction force reconstructions have allowed for the accelerated development of TFM as a tool for studying cell mechanics, future advances will likely rely on more computationally expensive methods like FEM to analyze increasingly complex models of cells and surrounding environments. As such, novel computing methods that maximize the ability of researchers to study complex systems while minimizing computational cost/time are of high value to the field of TFM. One prime example is the method presented by Legant et al. for 3D-TFM [58]. In brief, the complicated 3D geometry of the cell-substrate interface made standard GFM impossible, as the system's Green's function was no longer spatially invariant, a funda-

mental characteristic leveraged by standard GFM models. In other words, the function $\mathbf{G}(\mathbf{r} - \mathbf{r}')$ in Eq. (15.1) became a more general $\mathbf{G}(\mathbf{r}, \mathbf{r}')$. In addition, an analytical formula to describe the new spatially varying Green's function would be incredibly complex, which implies that FEM would be a superior computing option. However, iterative execution of FEM solvers to reconstruct the traction field would be extremely computationally expensive. A hybrid solution was therefore developed. First, the cell-substrate interface was approximated with a discretized mesh. FEM was then used to compute the displacements induced by a unit traction applied at a single facet of the discretized mesh. By repeating this process for each facet, the spatially varying Green's function was computed (Fig. 15.7). This numerically derived Green's function was then used to accelerate the reconstruction of traction forces beyond what could be achieved with FEM alone. This method and future hybridized methods like it, which use FEM to do the "hard work" of faster algorithms, may pave the way to making TFM a more efficient and readily accessible tool in the arsenal of cell mechanics researchers working on difficult and computationally intensive problems like 3D-TFM.

15.3.2 3D Forces and Environments

It has been established that cell behavior can greatly differ in 2D versus 3D environments [1, 5, 8, 57]. Although 2D cell culture is convenient to study, many cell systems of interest natively develop and interact with 3D tissue environments, where they are influenced by ECM mechanical properties and 3D forces [11]. As such, the measurement of cellular forces in 3D settings may be critical to future research in morphogenesis, cancer, and other processes. For years, TFM was largely restricted to 2D environments and 2D cell traction reconstructions. However, even cells cultured on 2D surfaces can exhibit significant forces with components in all three dimensions [54, 56, 78], motivating the development of "2.5D-TFM" techniques that capture these 3D forces. In recent years, 3D-TFM

research has expanded the field to the study of 3D forces exerted by cells cultured within fully 3D environments. As discussed in the previous section, the methods presented by Legant, et al. [58], which made use of a hybridized FEM-GFM approach for traction force reconstruction, are a predominant work in the growing collection of 3D-TFM research [41, 47, 60, 61]. Although 3D-TFM is not necessarily a "new" technique and has already been the subject of substantial research efforts, many outstanding challenges remain to be addressed in order to realize its full potential for revealing the roles of cell forces in 3D environments and behaviors.

Major challenges to 3D-TFM span the full spectrum of stages in the TFM workflow, including sample fabrication, imaging, mechanical characterization, modeling, and traction force reconstruction. Fabricated 3D substrates must present a physiologically relevant environment that enables normal cellular activity (and therefore requires the use of engineered polymers or natural biopolymers). The 3D imaging required to capture cell features and substrate displacements can be time-consuming (a problem if cell behaviors and forces are dynamic) and can be impeded by optical challenges like scattering, absorption, and photobleaching/phototoxicity. Many 3D substrate materials (such as ECM proteins) can be highly nonlinear, heterogeneous, and anisotropic and are difficult or impossible to characterize on the micro- to nanoscale with conventional methods. Finally, modeling and force reconstruction (implemented using FEM) can be difficult to implement and incur substantial computational costs. Despite these challenges, the need to understand the role of forces in 3D tissue models will promote a continued growth of 3D-TFM methods in the future.

15.3.3 Collective Behaviors

Many questions of interest in the field of mechanobiology involve not just single cells, but entire cell collectives which contribute to emergent features and behaviors [7, 62, 82–

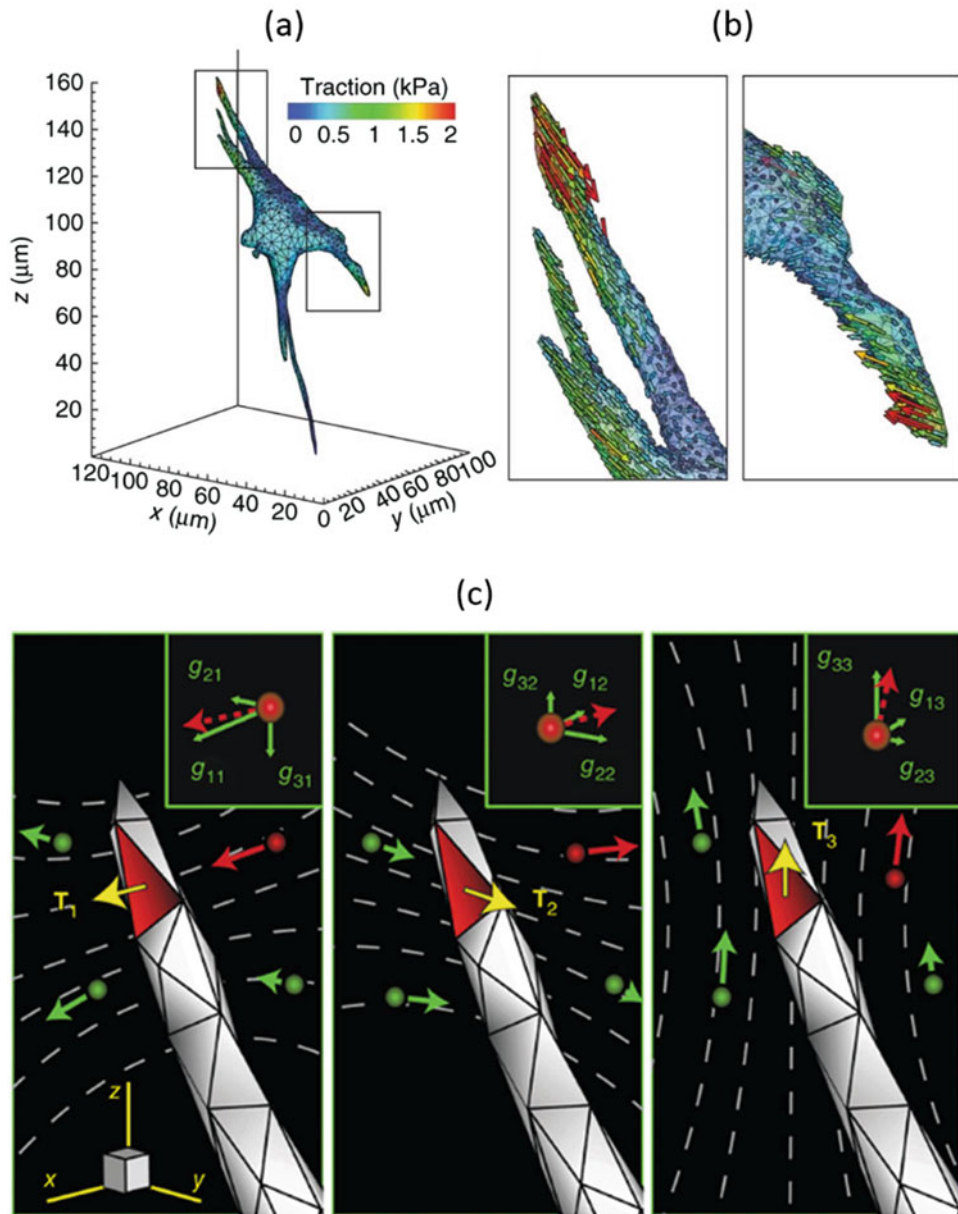


Fig. 15.7 3D-TFM with a hybrid computation scheme, combining aspects of FEM and GFM. **(a)** Contour plot of the tractions (magnitude) exerted by a live NIH-3T3 fibroblast embedded in a PEG hydrogel. **(b)** Magnification of the regions outlined in **(a)** showing the individual traction vectors on each facet of the meshed cell surface. **(c)** Schematic outlining the use of the finite element method to reconstruct a spatially varying Green's function. A surface traction (\mathbf{T}) applied to the highlighted facet induces displacements in the surrounding beads (g_{ij} , inset). When repeated over all

facets and beads, these relationships describe a discretized Green's function that can be used to calculate the tractions applied by the cell. The subscript indices of \mathbf{T} and g represent Cartesian components of the bead displacement in direction i in response to an applied surface traction in the direction j . This method allows for accelerated computation compared with FEM alone, while enabling the analysis of 3D systems too complex for GFM alone, expanding the capabilities of TFM for rapid analysis of traction forces in 3D environments. Adapted from [58]

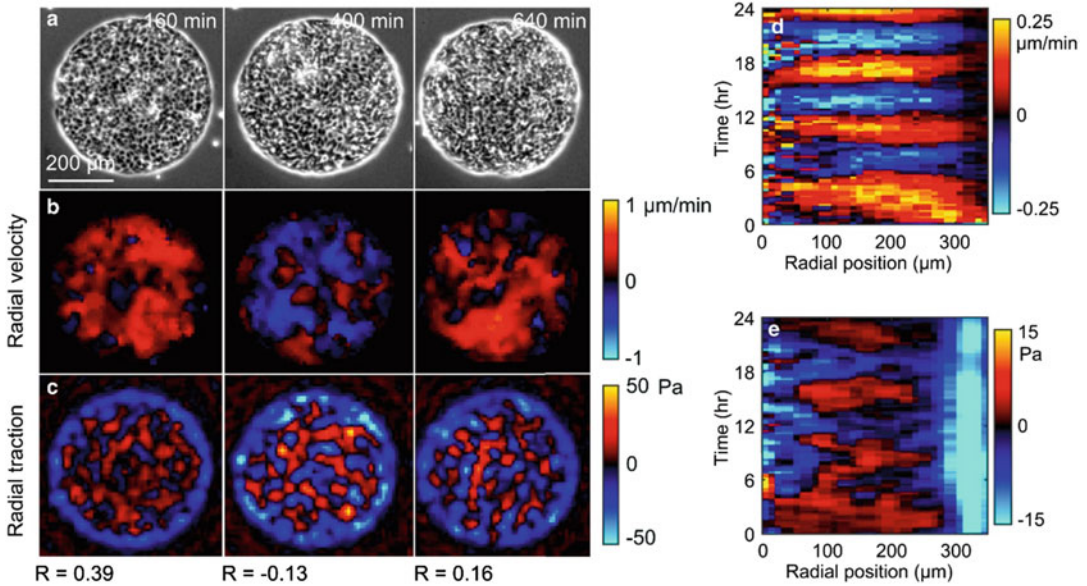


Fig. 15.8 TFM captures dynamic collective cell behaviors. (a) Phase-contrast images of a confined cell monolayer. (b) Radial component of cell velocity. (c) Radial component of cell traction. Both velocity and traction are dynamic, as shown in each column (acquired at 160, 400, and 640 min, respectively). Dynamics over a span of 24 h are shown in kymographs of the velocity (d)

and traction (e), where time zero corresponds to the first image acquired. These results are just one example of how TFM enables the study of how collective cell/tissue systems evolve over time. Extensions of these studies to 3D environments will likely face challenges related to imaging, substrate/ECM remodeling, and accounting for force transmission between individual cells. Adapted from [82]

95]. An increasing interest in the collective mechanical behavior of cells has arisen from experimental evidence in both normal organism development [96] and pathological data [84]. Collective cell behaviors have already been the subject of extensive study in 2D settings, while recent research has begun to yield insights into collective behaviors in 3D environments. These investigations have enabled the observation of such phenomena as cell jamming [7, 84, 93], collective polarization and migration (Fig. 15.8) [82], wave-like propagation of cell velocities and tractions [97], emergent compressive stresses in the ECM, and mechanical interaction of separated cell clusters [41]. TFM has contributed to several studies of traction forces exerted by cellular collectives in both 2D and 3D settings [41, 62, 82, 91, 97], with expanded application likely in the future. Measuring the traction forces of cell collectives presents several imaging challenges. Observation of collectives requires

a large imaging field-of-view (in two or three dimensions) while maintaining a sufficiently high resolution to capture cell features and substrate deformation data at length scales appropriate to the phenomena being studied. Moreover, imaging may need to take place over a wide range of time scales, from minutes, to hours, to several days for standard model systems, or even weeks in future studies of tumor formation or development. Finally, as cell collectives can substantially modify the ECM and exert strong forces, TFM with cell collectives will likely have to address several of the other challenges discussed in the rest of this section, such as heterogeneity, remodeling, and nonlinearity.

15.3.4 Beyond Linear Elasticity

While linear elastic environments are easy to fabricate and study, they do not capture many of the

complex properties of biological tissue, such as viscoelasticity or nonlinearity, which may be crucial for understanding cell behavior. For example, it has been shown that stem cell differentiation and behavior are altered by the viscous/relaxation properties of their surroundings [98, 99]. However, whether viscoelasticity has a corresponding effect on cell traction forces remains to be characterized. Studying cell traction forces in such environments requires new models to connect traction forces to substrate deformations. Toyjanova et al. have demonstrated one framework for TFM which incorporates viscoelastic properties, opening new avenues for studying systems beyond what current quasi-static/purely elastic models can accommodate [39].

Nonlinearity is a potentially rich area for exploration with TFM. Indeed, most biological materials in which cells reside exhibit nonlinear mechanical behavior. It is therefore not surprising that cells are able to respond to these nonlinear properties. For example, fibrous networks such as collagen support long-range force transmission over small collections of fibers, a highly nonlinear process that can enable long-range mechanical communication between cells [23, 38, 68, 100, 101]. Steinwachs et al. recently demonstrated 3D-TFM of cells cultured in a collagen environment, making use of a nonlinear model [38]. In this work, collagen was modeled as having three regimes of mechanical behavior, corresponding to the buckling, straightening, and stretching of collagen fibers. The FEM-based nonlinear 3D-TFM framework was used to study cell traction forces and migration dynamics, as well as responses to varying collagen concentrations. Hall et al. used another approach [68], wherein the 3D collagen network surrounding the cell was modeled as containing both regions of isotropically oriented fibers and regions of (anisotropically) aligned fibers. A fiber network model was used to study how cell-induced strain may create regions of aligned collagen fibers from initially isotropic orientations and how such alignment alters the local ECM mechanical properties [102]. This network model was then used to yield a nonlinear continuum model for FEM-based cell force reconstruction, allowing insight

into mechanical feedback interactions between cells and the surrounding collagen ECM [68, 102]. Future TFM studies incorporating nonlinearity will likely face significant challenges in achieving reliable mechanical characterization of samples. Inverse TFM methods will also face a need for computationally intensive FEM-based models to enable traction force reconstruction in nonlinear systems. Nevertheless, progress will continue, as further extensions of TFM for nonlinear systems stand to greatly enhance understanding of the diverse physical interactions of cells with physiological ECM environments.

15.3.5 Heterogeneity

While homogeneity has been a convenient assumption for the field of TFM, the environments presented by tissues are often highly heterogeneous. Notably, as revealed by *in situ* observations, the stroma becomes increasingly heterogeneous as collagen is deposited during tumor progression [9]. Heterogeneities in tissue can take many forms, including changes in density, stiffness, architecture, pore size, and levels of cross-linking, all of which can have bearing on cellular behaviors [11, 103, 104]. It is therefore likely that future TFM studies will need to address the effects of heterogeneities on cell force. For example, cells cultured on micropillar arrays with spatially varying stiffness have been shown to exhibit a preference for stiffer substrates, where they exert greater force [105–107]. Some initial work of TFM in the area of heterogeneity has investigated the effects of stiffness gradients [106], barriers to cell migration [108], and cell-induced mechanical heterogeneities [41]. Heterogeneities not only affect cell behavior, but cell activity induces heterogeneity on many length scales [41, 63, 109]. Cell-induced heterogeneity can also negatively impact cell traction force reconstruction, if not properly accounted for [41]. Future work will require both novel substrate fabrication techniques as well as new mechanical characterization methods and improved computational models to better understand the impact of heterogeneity on cell forces and behavior.

15.3.6 Anisotropy

It has been demonstrated that anisotropy significantly impacts cell behavior [63, 110]. For example, substrates with oriented nano/microtopographies have been shown to influence cell alignment [111] and the differentiation of adult neural stem cells [112]. Cells will also preferentially align and migrate in the direction of greatest rigidity [107]. In addition, remodeling of the ECM by both single cells [63] and cell collectives [113] tends to result in anisotropic fiber alignments (Fig. 15.9). That is, cells not only react to anisotropic environments, but they actively create them as well. Anisotropy is therefore a potentially rich area of application for future TFM research. Though uncommon, some work has been done to apply TFM to anisotropic settings. For example, FEM-based TFM has been conducted to reconstruct the 3D forces exerted by cells grown on a non-planar, “wavy” surface (i.e., with topographical, as opposed to mechanical, anisotropy) [40]. Anisotropic systems pose challenges for both mechanical characterization and computational reconstruction of traction forces. In tissues, anisotropy is often accompanied by heterogeneity and nonlinearity, adding further complications to traction force reconstruction. Depending on the system under study, anisotropic samples may result in imaging consequences, such as a spatially varying optical point spread function, which can impact the tracking of embedded bead displacements [40]. Future TFM methods that address anisotropy and its associated challenges will likely be crucial to the future study of accurate tissue models and cell forces.

15.3.7 Remodeling and Dynamics

ECM remodeling and dynamics are essential features of many cellular processes and behaviors [114]. For example, the migration of highly invasive cancer cells is facilitated through remodeling of the ECM, resulting in the formation of tumor-associated collagen signatures (TACS), such as increased collagen density, the presence of straightened (taut) collagen fibers, and radially aligned collagen fibers that facilitate invasion [9]. Radially aligned fibers oriented away from a tumor, sometimes referred to as “collagen highways,” are associated with the most invasive phenotypes of cancer and have been observed in vitro [83], in animal models [9], and in clinical cases [115]. Cells can modulate the mechanical properties of the ECM with traction forces via strain-hardening, and through degradation of the ECM with matrix metalloproteinases [109]. Moreover, cells can exert forces on the timescale of minutes [63] and can induce significant ECM remodeling on the timescale of hours [41, 83]. Not only are dynamics and ECM remodeling of interest to biomechanics research, but their effects can severely impact traction force reconstructions (such as through the formation of heterogeneities, anisotropy, and nonlinear effects) [38, 41]. As a result, TFM techniques that capture and accommodate ECM remodeling and cell dynamics are crucial to generating a complete picture of biophysical phenomena.

Some works in TFM have already begun to investigate the relationship of remodeling and dynamics with cell traction forces. Gjorevski and Nelson investigated the forces exerted by microfabricated mouse mammary epithelial tissues embedded in collagen gels [41]. It

Fig. 15.9 (continued) **(b)** Extension and maintenance of actin-rich cellular protrusion along radially aligned matrix fiber at the cell periphery (arrowhead) that supports protrusion persistence. Scale bar = 5 μm . **(c)** Confocal reflectance images of collagen matrix structure (left) around an embedded MDA-MB-231 cell (projected area shown as “c”) as well as heat maps illustrating collagen fiber density (middle) and orientation (right) immediately after matrix polymerization (top row) and following 24 h of culture (bottom row). Arrows indicate anisotropy of ECM

structure. Scale bar = 20 μm . These results highlight several important considerations for increasing the physiological relevance of TFM. The short timescales of cellular protrusion dynamics imply that rapid imaging methods are required to accurately capture the contractile states of cells. The dependence of protrusions on matrix fibers is a nonlinear interaction. Cell remodeling creates anisotropic conditions, which current standard TFM models do not address. Adapted from [63]

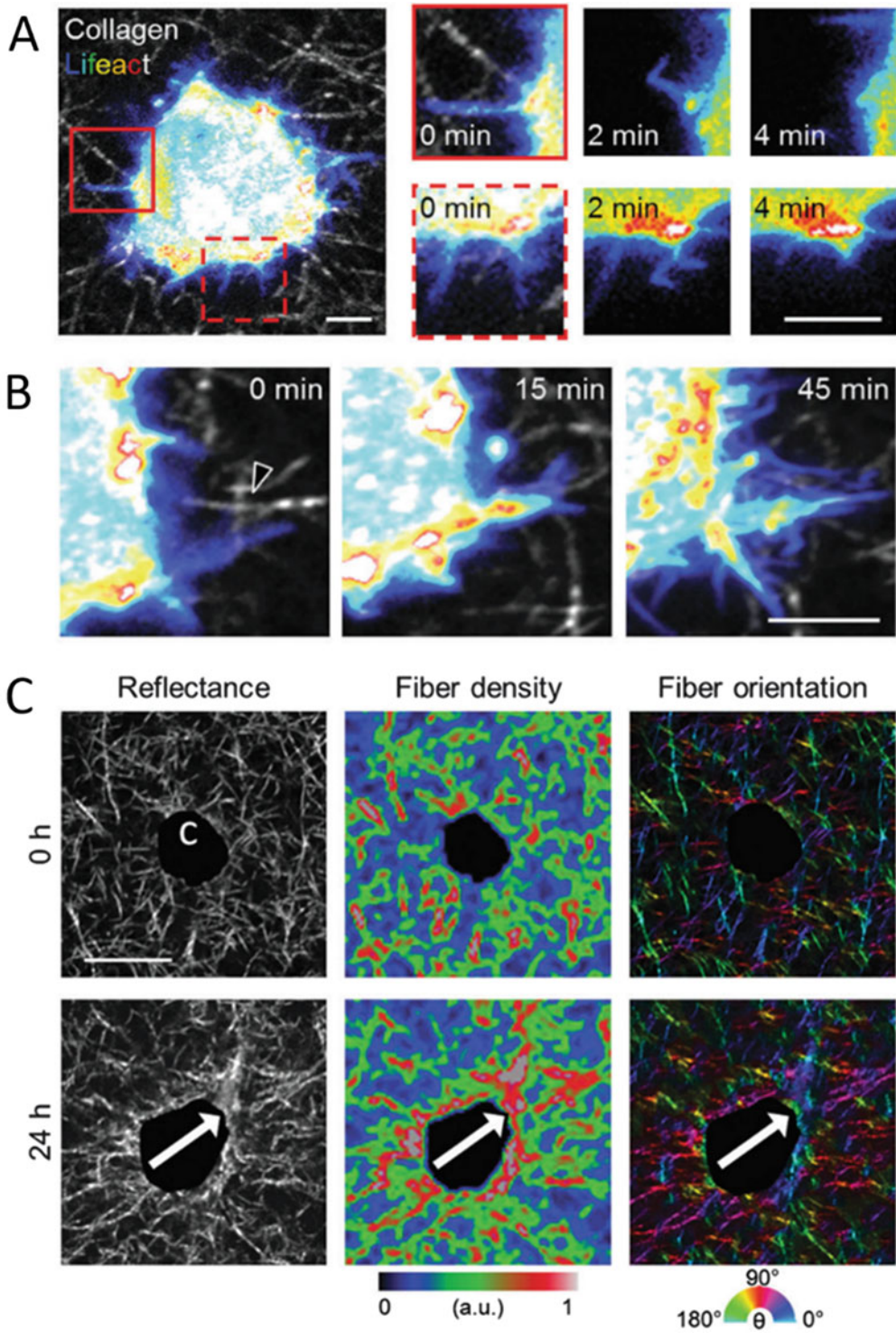


Fig. 15.9 Dynamic biophysical interactions during cell migration and ECM remodeling span a wide range of timescales. (a) Lifeact-GFP-transfected MDA-MB-231

cell spreading in collagen matrix immediately after polymerization. Insets highlight the rapid dynamics of transient cellular protrusions. Scale bars = 5 μm .

was determined through imaging and AFM that cellular activity introduced significant mechanical heterogeneity into the collagen ECM. Incorporating this heterogeneity into the traction force reconstruction process suggested that failing to account for these cell-induced ECM modifications may result in severe underestimation of cellular traction forces. Other works in TFM have explored the role of traction forces in many scenarios involving 2D collective cell migration and dynamics. For example, Notbohm et al. investigated the traction forces and migration dynamics of confined monolayers of canine kidney cells. The monolayers exhibited collective traction forces and motions that oscillated in time (as depicted in Fig. 15.8). Serra-Picamal et al. reported the presence of “waves” of traction forces, intercellular stresses, and cell velocities propagating through a cell monolayer [97]. These waves are not the result of passive phenomena (as are everyday waves like sound, light, or vibrations). Instead, these waves are hypothesized to be an active spatiotemporal phenomenon governed by dynamic cellular responses to mechanical communication from neighboring cells. Future works that explore cellular remodeling and dynamics with TFM may lead to further novel observations of cellular behaviors and their effects on the ECM environment.

15.3.8 Mechanical Characterization of Substrates

The use of synthetic substrates with established fabrication protocols has made the task of substrate mechanical characterization a relatively simple one, when compared to the biological and computational components of typical TFM experiments. Mechanical characterization has often been performed using bulk rheometry, indentation testing, or atomic force microscopy. However, as TFM applications move increasingly toward the use of natural biopolymers which exhibit complex mechanical behaviors, these established methods will become less applicable. The study of three-dimensional systems and ECM remodeling will further compound this problem.

As a result, future TFM efforts will rely on the use of novel techniques for mechanical characterization in 3D ECM and tissue environments. Several emerging techniques have made significant strides toward addressing these needs. These include Brillouin microscopy (BM) [116–119], optical coherence elastography (OCE) [120–122], and optical tweezers-based active microrheology (AMR) [109, 123]. These emerging methods enable the noninvasive measurement of mechanical properties in 3D substrates. Although the ability of these methods to provide reliable quantitative mechanical properties relevant to TFM are currently limited, future research into these and related techniques may enable the development of novel imaging platforms capable of noninvasively capturing the 3D distributions and dynamics of cell structures, traction forces, and ECM properties, an ambitious endeavor with great potential for accelerating cell biophysics research.

15.3.9 Novel Imaging Platforms

To date, the imaging platforms of choice for TFM have been fluorescence microscopy and confocal fluorescence microscopy. However, with a growing diversity of TFM methods and application spaces, TFM research stands to benefit from the use of novel imaging platforms to expand its capabilities. For example, TFM has been recently performed using stimulated emission depletion (STED) microscopy [70]. STED and other super-resolution methods may allow substrate deformations to be imaged with higher resolution (by allowing for higher bead concentrations) and can provide detailed information about protein structures in and around cells. Confocal reflectance microscopy was recently employed for 3D-TFM [124], eliminating the need for either fluorescent labels or marker beads. Instead, deformations were measured by directly tracking the motion of collagen fibers. Optical coherence microscopy (OCM) was recently proposed as a means to enable 3D-TFM in highly scattering media with rapid volume acquisition speeds (Fig. 15.10) [60]. Another advantage of an OCM-based system would be the opportunity to merge

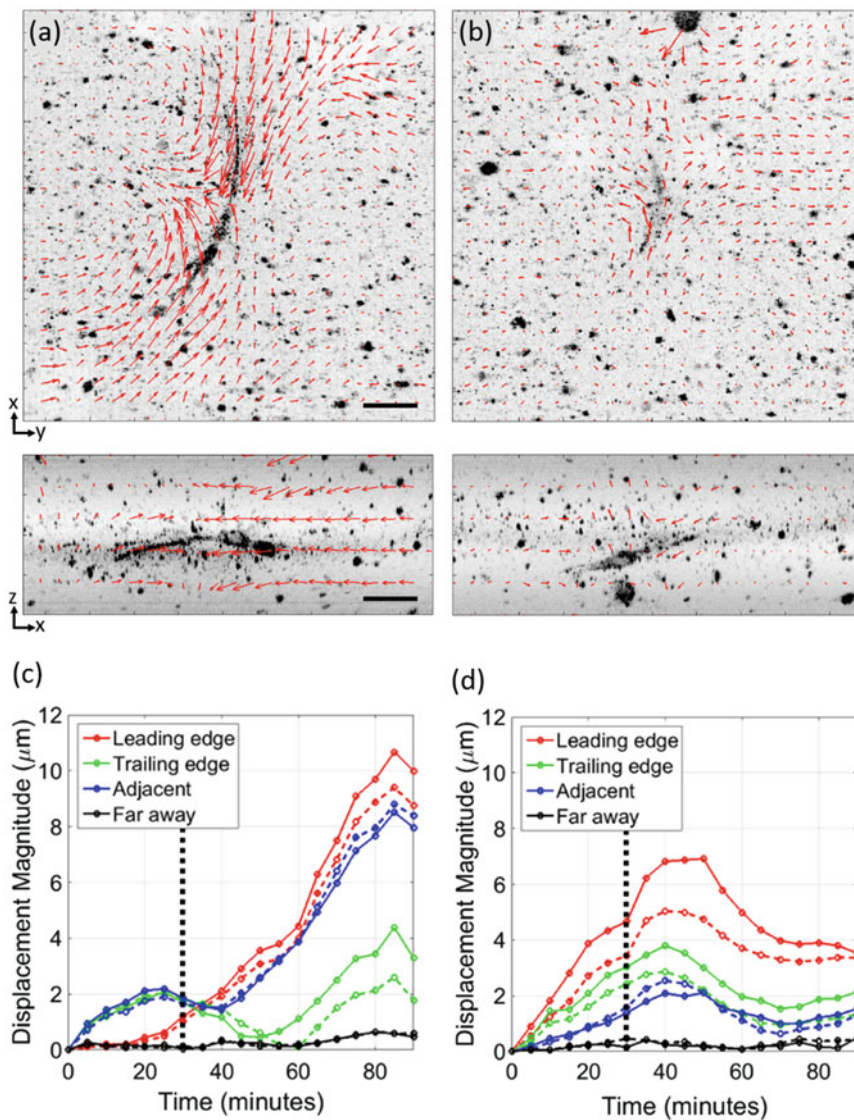


Fig. 15.10 Initial work toward the development of TFM using optical coherence microscopy. NIH-3T3 fibroblasts were embedded in a 3D Matrigel environment. Images were acquired every 5 min for 90 min in total. Cells were allowed to interact with their surroundings freely for the first 30 min of imaging, after which they were exposed to either a control reagent (DMSO) or a contractility inhibitor (cytochalasin D) and imaged for the remaining 60 min. (a) and (b) depict the total substrate displacements accumulated from time $t = 0$ to time $t = 90$ min for the DMSO and cytochalasin D cases, respectively,

with displacements computed in the xy - (top) and xz - (bottom) planes. (c) and (d) depict the dynamics of measured displacements at various points of interest around the cell in the DMSO and cytochalasin D experiments, respectively. The curves demonstrate the dynamics of cell traction forces after exposure to the control and contractility inhibiting reagents. Optical coherence microscopy has the potential to enable 3D, label-free imaging for TFM experiments that capture cell force dynamics on the minute timescale. Adapted from [60]

TFM and optical coherence elastography in a single imaging system, enabling measurement of both substrate deformations and changes in

substrate mechanical properties due to remodeling and/or strain-hardening. Other rapid volumetric imaging modalities, such as light sheet

microscopy or swept confocally aligned planar excitation (SCAPE) microscopy [125], may be well-suited for volumetric TFM studies in substrates with lower optical scattering.

15.3.10 Advancing Mechanobiology

Although great strides have been made in TFM to increase resolution, accuracy, and compatibility with new environments, the fact remains that implementing TFM, from experimental design, to imaging, to data processing and force reconstruction, is not a trivial task. This is because the development and implementation of new techniques relies on expertise from across a wide range of academic disciplines. Ultimately, researchers in the field must often make trade-offs. For example, incorrect assumptions or approximations can severely corrupt traction force reconstructions. However, the data obtained may still yield insight toward answering the biological questions at hand, and the “inaccurate” method may turn out to be less time-consuming or easier to implement. The question that must be answered is, what TFM protocols and performance levels are sufficient for a given experiment? Alternatively, in the context of Fig. 15.2, what methods are sufficient to provide the necessary insights that address the biological question? In some contexts, forces may not even be strictly necessary, so long as information related to forces and cell energy expenditure are available. Koch et al. presented methods for quantifying ECM strain energy, circumventing the reconstruction of traction forces exerted by cells embedded in 3D environments [61]. Similarly, Stout et al. devised the use of “mean deformation mechanics” as a substitute for cell force reconstructions when the mechanical properties of the substrate are not well-characterized [126]. As TFM methods advance, those who wish to make use of TFM as a tool for mechanobiology research will have to carefully consider the quantitative needs of their research questions and what the various TFM methods have to offer.

As new techniques in TFM migrate from development to widespread application, methods to

make the new tools compatible with the high-throughput needs of biophysics and mechanobiology researchers will be a necessity. Even if a method is imperfect, its ability to perform rapid and repeated experimentation will be crucial to moving research efforts forward. As one example, Park et al. have presented a high-throughput cell traction force screening platform based on FTTC to enable rapid testing of how drug compounds impact cell forces [127]. With the development of such platforms, TFM can begin to make broader impacts and help further transform biophysics and mechanobiology research into a standard practice in biomedicine.

15.4 Conclusion

We have reviewed the central techniques and principles of traction force microscopy, including substrate choice, mechanical characterization, imaging, measurement of substrate deformations, and traction force reconstruction. Building on and moving beyond these principles, we have highlighted several areas of active research and potential future innovation, which may fuel the growth of TFM toward application in the study of more realistic/physiological engineered tissue and tumor-like microenvironments which manifest traits such as nonlinearity, heterogeneity, and temporal variations due to cell-induced remodeling. TFM relies on a strong foundation of carefully engineered techniques, as demonstrated by ongoing research efforts taking place at every step of the process.

Underlying many of the ongoing technical innovations in TFM are two major themes: (1) the utilization of new constraints and information to form more complete mechanical models of cell biophysics and behavior and (2) the creation of novel force reconstruction methods that address both the challenges of speed and compatibility with physiologically relevant sample properties and geometries. Accompanying both of these themes are a few critical challenges. The development of high-throughput experimental methods and the minimization of computational complexity will play key roles in accelerating the

investigation of new biological questions with TFM. And in order to make effective use of new models that are compatible with physiologically relevant environments, TFM will rely on the continued development of technologies that enable high-resolution mechanical characterization of tissue and ECM environments. Although advances made in TFM and other fields will enable research under increasingly diverse conditions, managing trade-offs in accuracy versus throughput will likely remain a common theme in the near future.

Overall, TFM is more than a rapidly growing tool for the noninvasive measurement of cell forces. TFM has already played a leading role in many seminal works of mechanobiology, revealing the influence of physical properties and forces on cell behavior and exposing intrinsic differences between normal and cancerous cells [2, 22, 128, 129]. As an area of research, it has merged expertise from a wide range of academic disciplines and is fostering close collaborations between physical scientists, biological scientists, and clinicians. With future application in more physiologically relevant environments, TFM holds the potential to offer insights into the biophysical behaviors of both single cells and collectives over multiple length scales, spanning processes over minutes to days. Whether used to investigate the processes of how cancer develops and progresses, how wounds heal, or how cells go about their “normal everyday functions” such as growth and morphogenesis, TFM remains and will continue to be a central tool to help understand cellular forces and their role in mechanobiology.

Acknowledgments Recent studies and results presented here were funded in part by a grant from the US National Institutes of Health (R21EB022927, Adie) and a Cornell Discovery and Innovation Research Seed Award (Adie). Additional information can be found at <http://adie.research.engineering.cornell.edu>.

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