Title: The future of traction force microscopy

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Abstract

Animal cells continuously sense and respond to mechanical force. Quantifying these forces remains a major challenge in bioengineering; yet such measurements are essential for the understanding of cellular function. Traction force microscopy is one of the most successful and broadly-used force probing technologies, chosen for the simplicity of its implementation, flexibility to mimic cellular conditions, and well-established analysis pipe-line. Here, we review the accomplishments, and discuss the applicability and limitations of traction force microscopy. We explain fundamental shortcomings of the method, summarise latest improvements, and outline future pathways towards the impact of the method, especially considering latest developments in state-of-the-art super-resolution fluorescence imaging. In light of the increasing discovery of the importance of mechanobiology in cell physiology, we envisage traction force microscopy to remain a major player for quantifying mechanical forces in living cells.

Keywords: Traction force microscopy, mechanobiology, cell mechanics, actin cytoskeleton

Introduction

New perspective of mechanobiology is currently emerging across multiple disciplines in biomedical research. In contrast to conventional beliefs, recent evidence indicates that cells regulate their cell mechanics not only downstream of signalling events triggered by external stimuli or ligand–receptor binding, but that cells employ a diversity of feedback mechanisms enabling them to dynamically adjust their mechanics to meet physiological needs [1,2]. Consequently, this provides a previously unforeseen picture wherein cells actively exert and resist force to tune their material properties and thus facilitate their function, which is particularly important during physical interactions with other cells or with the extracellular environment [3,4]. Quantifying these cellular forces has therefore become an important mission across multiple disciplines at the interface of biophysics, cell-biology, and immunology [5–7].

Measuring these cellular forces is challenging but the methodology of traction force microscopy (TFM) is likely to remain the leading force probing technology. In addition to the complexity of the mechanical feedback mechanisms of cell mechanics, cellular force probing is itself inherently challenging because of the physics of mechanical force measurement. Because cells do not emit mechanical signals that could be detected and analysed in a contactless manner, such quantification demands direct engagement of the force probing technology with the cell. For example, the mechanical stiffness of cells in the form of the elastic modulus is determined by physically indenting their surface by a given force using e.g. atomic force microscopy [6,8]. In TFM experiments, cellular force production is quantified by monitoring surface tractions produced by cells onto an elastic substrate of a given elasticity [9–11]. To add further complexity to this picture, cell mechanical measurements also depend on how they are executed. Especially, cell rheology, time-dependent mechanical properties, vastly differs at different time- and length-scales. On short time-scales (milli-

seconds) and large length-scales (micro-meters) cells show poroelastic properties, and at long time-scales (~minutes) they exhibit a power law behaviour in response to application of external forces [12,13]. Hence, parameters such as displacements, cell tractions, and turnover rates must be monitored at a multitude of time- and length-scales in order to comprehensively characterise cell mechanical properties and force production.

Traction force microscopy

TFM is perhaps the most successful and broadly-used force probing technology, because it continues to offer the majority of the above discussed requirements for the quantification of force production in living cells [14,15]. Consequently, TFM is superior to other force quantification technologies due to its simplicity of implementation, flexibility to mimic cellular conditions, and well-established analysis pipe-line. In TFM experiments, cells interact with a thin (20–30 μm) elastic hydrogel by adhering to a protein functionalised surface [16,17]. Within the hydrogel, immobilised fluorescent beads serve as fiducial markers, and imaging of the bead positions over time in two or three dimensions (2D/3D) during the application of cellular tractions allows the 2D, 3D elastic displacement of the gel to be quantified. Combining the displacement measurements with knowledge about the mechanical properties of the hydrogel allows the forces applied by the cell to be recovered [14].

Implementation of conventional TFM experiments can be achieved at any confocal or epifluorescence microscope without additional optical components [15]. TFM elastic substrates, such as the often used polyacrylamide (PAA) hydrogels, can be fabricated with elasticities in a range extending from <1 kPa to a few hundreds of kPa, allowing TFM measurements the flexibility to imitate a multitude of different cell surface stiffnesses and tissue environments [14]. In addition, nano-topological features, such as the gel mesh-size can be straight-forwardly altered by shifting the balance of monomers and crosslinkers within the PAA gel, without changing the gel stiffness [18]. Alternative materials such as silicon, collagen, and polydimethylsiloxane, known as PDMS, exhibit similar optical and elastic properties, and are considered promising candidates for force probing using TFM-like experiments [19–21]. The refractive index of silicon hydrogels matches the index of the sample coverglass and therefore allows total internal reflection fluorescence (TIRF)-based TFM, which is naturally limited to 2D tractions due to the TIRF imaging [20]. Collagen-based 3D TFM promises the possibility to suspend cells within physiological 3D microenvironments, but its analysis still demands complex algorithms due to its non-linear mechanical properties. Specifically, analysis frameworks necessitate in-depth mathematical knowledge and are practically not available to most biologists or biophysicists [22,23]. In contrast, in TFM, the recovery of mechanical forces from the acquired tractions is in most cases well-established and open-source software solutions are widely available also to non-experienced users. Moreover, the hydrogel's top surface can be covalently functionalised with lipids and proteins mimicking physiological conditions with which the cells interact. Together, these properties allow to quantify the production of cellular tractions by a variety of cells at multiple mechanical conditions.

Limitations of TFM

TFM has been successful in quantifying cellular force production but precludes the characterisation of mechanical properties, which naturally limits the method to only one of the two branches of cell mechanics. TFM does not allow active interrogation of cells through e.g. indentations for the quantifications of mechanical properties such as the deformability, viscosity, or stiffness of cells. Interpreting 2D TFM experiments are also fundamentally limited to examination of force at the ventral membrane of cells. 2D TFM can only provide insights into force production of cell compartments that generate tractions on the apical flat surface of the hydrogel. Efforts to extend TFM to 3D hydrogels have shown promising results but at low throughput and hence limited statistical-relevant output, and further necessitate specialised analysis such as finite element simulations to recover the forces from 3D traction data sets.

One important hardware factor for the precision of TFM is the scanning frequency of the fluorescence bead detection. TFM precision is affected by the acquisition duration of the fiducial marker positions. Only sufficiently high acquisition frequencies allow accurate tracking of the bead positions over time. Similarly, at any given experiment the sampling of the displacement field must

meet the Nyquist criteria i.e the spatial sampling frequency of the displacement field must be twice that of any feature that may be resolved in the displacement field [24]. This restricts the density of fiducial markers that must be high enough to reflect the complexity of the traction field that is applied by the cell. If the bead density is too low, areas of the gel will move without being reported by any bead movement, and the traction information will be lost. Conversely, if the bead density is too high, information will also be lost as a result of the point spread functions (PSFs) of each individual bead overlapping with those nearby, making their relative displacements hard to recover accurately. Further, the maximal bead density within the gel must also be balanced with the ability to track the beads and the fluorescence light sensitivity of the biological specimen. Systematic bead localisations could improve the bead density which can be achieved through pattern printing [25]. Consequently, the quality and robustness of TFM experiments could be improved by better preparation of the gels with precise, regular, and pre-determined bead concentrations using e.g. substrate printing systems. Consequently, the greatest shortcoming of classical TFM is its limited sensitivity due to the finite density at which the displacement field can be sampled within the gel, which must be high enough to reflect the complexity of the traction field that is applied by the cell.

Advances in technology and analysis

To overcome these challenges, we recently improved the spatial resolution and accuracy of TFM using optical super-resolution stimulated emission depletion (STED) microscopy [15,24]. The increased spatial resolution of STED-TFM (STFM) allows a greater than five-fold higher sampling of the forces generated by the cell than conventional TFM, leading to more accurate quantification of cellular tractions. This must be balanced with other optical factors, such as maximal bead density within the gel, the ability to track the beads and the fluorescence light sensitivity of the biological specimen. Earlier attempts to overcome this limitation involved the use of two different colours of marker beads, which proved that the recovery of micron-sized tractions is feasible [14]. However, because of its reliance on the spectral separation of the beads, this technique is ultimately limited by the spectral range of the microscope.

Theoretical aspects of TFM are equally important in optimising the accuracy of force reconstruction. Critical improvements of STFM can be expected from the image analysis, and there are a number of methods by which bead displacement can be quantified. These methods can be broadly divided into those that depend on tracking each individual bead, single-particle tracking techniques, and those that depend on correlating displacements within regions of an image, such as particle image velocimetry. The choice of method depends on the expected nature of the forces and on the density of beads within the image; thus, it is important to consider at the outset which analysis is more suited to the particular experiment. Optical flow tracking has for instance recently been shown to improve the effective resolution of TFM, allowing the quantification of forces generated by small focal adhesions in living cells [26].

Future of TFM

Combining TFM and super-resolution microscopy such as STED might allow this force probing methodology to not only remain a leader in the field but also extend its applicability to more complex biological questions in the near future. Hence, the impact and future of TFM is very promising. Especially, the extended sensitivity offered by STFM will allow the technique to tackle questions at length-scales currently prohibited by conventional TFM, providing unprecedented measurements of forces exerted by small cell organelles or individual receptors. This will be vital in the understanding of the role of mechanics across many disciplines at the interface of immunology, neuroscience, and cancer biology.

To further improve the impact of TFM, future approaches may aim to increase the statistical output of the technique. This involves experiments and analysis, because currently only individual cells can be measured at a given time, and the analysis pipe-lines lasts multiple hours. Overcoming these shortcomings would allow to systematically extend TFM experiments to a standardised readout of mechanical force production and investigate aspects of mechanobiology such as mechanosensitivity, feedback mechanisms, or more generic the ability of cells to adjust their force

production to the hydrogel stiffness. For example, this could allow to quantify how T cells dynamically adjust their elastic properties such as surface stiffness by alternating the turnover time and filament length of their cortical actin in response to the strength of activation with antigen presenting cells [5,27]. In the same way, dysregulation of the mechanosensitivity of myofibroblasts could be measured that are thought to contribute to the progression of human fibrosis.

Conclusions

In light of the increasing discovery of the importance of mechanobiology in cell physiology, we envisage traction force microscopy to remain a major player for quantifying mechanical forces in living cells. TFM imaging is established as a valuable tool for biological research and its application must be extended to a wider range of scientific questions. Development of (S)TFM will involve the implementation of improved fluorescent dyes with increased photo-stability allowing a reduction in photo-toxicity. In addition, new microscope technologies will be necessary, as extensions to more complex biological specimens will benefit from further improvements in the spatial and temporal resolution, as well as the ability to image thicker specimens. Combining (S)TFM with high-performance objectives for deep-substrate imaging and/or the application of adaptive optics hold significant potential benefit for mechanical probing in immunology, biophysics, and cell-biology. Further, the increasingly improved usability and availability of super-resolution systems will reduce the need for external technical support, thus facilitating the potential of TFM to remain the standard tool for mechanical force probing in the field of biomedical research.

Conflict of interest

The author declare no conflict of interest.

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Figures

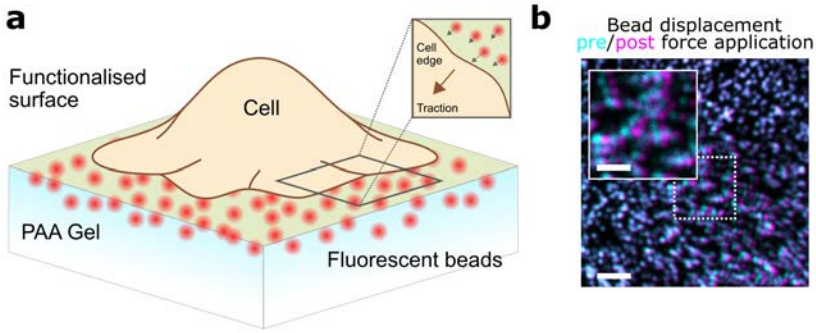

Figure 1: Traction force microscopy. **a)** Schematic outlining a typical traction force experiment. A thin (20-30 µm) PAA gel formed on a glass coverslip is loaded with fluorescent marker beads and its top surface functionalised with proteins that facilitate cell adherence. Traction forces generated by the cell result in displacement of the PAA substrate which can be quantified by imaging the displacement of the fluorescent beads within the gel. **b)** Representative **c**onfocal fluorescent image showing bead positions before (cyan) and after (magenta) the application on cellular traction. Scale bar is 2 µm. Inset shows a zoom-in of the dotted region. Scale bar is 1 µm.

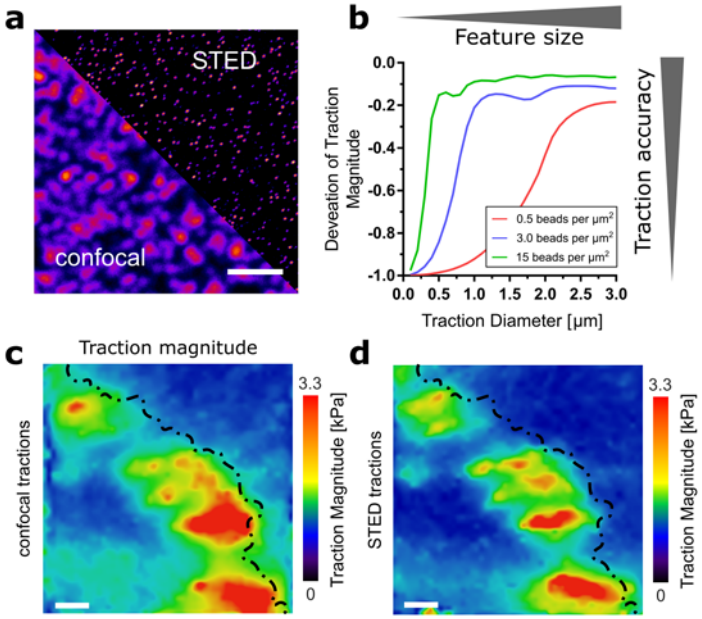

Figure 2: Super-resolved Traction Force Microscopy. a) Representative confocal and STED imaging of the distribution of beads at the top surface of the PAA gel. High resolution STED microscopy allows for the use of higher bead densities in TFM. b) Simulations highlighting the relationship between the sampling density and the level of traction recovery. Higher sampling densities made possible using STED microscopy (green and blue lines) lead to the recovery of spatially smaller traction compared to confocal (red). c) and d) Traction magnitude resulting from HeLa cell focal adhesions recorded by confocal (left) and STED (right) imaging. The spatial resolution of the traction map is enhanced using STFM, allowing improved localising of tractions and cellular architecture.