METADATA

Editorial Summary: Many cellular processes rely on cells generating or responding to nanoscale mechanical forces. This protocol describes STED-Traction Force Microscopy (STFM), which allows these forces to be measured with higher resolution and accuracy than standard TFM.

Proposed Tweet: #NewNProt: Measuring nanoscale forces in living cells with combined #STED and #TractionForceMicroscopy [LINK] #biophysics @MRC_WIMM

Proposed Ontology terms:

Biological sciences/Biophysics/Nanoscale biophysics

Biological sciences / Biological techniques / Biophysical methods

Biological sciences / Cell biology / Cellular imaging / Super-resolution microscopy

Biological sciences / Cell biology / Cell adhesion / Mechanotransduction

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Supporting Primary Paper

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Dissecting mechanical force in living cells by super-resolved traction force microscopy

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ABSTRACT

Cells continuously exert or respond to mechanical force. Measuring these nanoscale forces is a major challenge in cell biology yet they are essential for the understanding of cell regulation and function. Current methods for examining mechanical force generation either necessitate dedicated equipment or limit themselves to coarse-grained force measurements on the micron-scale. In this protocol, we describe stimulated emission depletion traction force microscopy STED-TFM (STFM), which allows higher sampling of the forces generated by the cell than conventional TFM, leading to a 2-fold increase in spatial resolution of up to 500 nm. The procedure involves the preparation of functionalized polyacrylamide gels loaded with fluorescent beads, as well as the acquisition of STED images and their analysis. We illustrate the approach using the example of HeLa cells expressing paxillin-EGFP to visualize focal adhesions. Our protocol uses widely available laser-scanning confocal microscopes equipped with a conventional STED laser, open-source software, and common molecular biology techniques. The entire STFM experiment preparation, data acquisition, and analysis require 2–3 days and could be completed by someone with minimal experience in molecular biology or biophysics.

INTRODUCTION

Mechanical force is a fundamental regulator of cellular function. Living cells continuously sense mechanical force and convert them into biological responses[1–3]. Similarly, external stimuli can influence cell homoeostasis and their ability to respond[4,5]. Gaining a quantitative understanding of how force is regulated in cells and how it persists and dissipates, ultimately necessitates the systematic determination of each molecular component participating in the force generating processes. To add further complexity to this picture, force and mechanical stress act on biological systems at very different length- and time-scales, from cells, down to single molecules, and up to entire organisms lasting over a few seconds to hours and even over their whole lifetime. Cell mechanical properties in turn, such as cell stiffness or elasticity (the ability to resist mechanical forces), are utterly different depending on the speed of the applied forces, including poroelastic and viscoelastic stress responses, as well as protein turnover-dependent responses[6–9]. Current methods for examining force generation either necessitate highly specialized equipment or limit themselves to coarse-grained force measurements on the micron-scale, which inevitably precludes identification and characterization of the underlying mechanisms. Consequently, new methods for characterizing molecular force within living cells are necessary to help us understand macroscopic mechanics from the bottom up.

Mechanical probing of living cells depends on the compliance and the length-scale of the biological material, the frequency (time-scale) at which mechanics is characterized, as well as on the environmental and experimental conditions. Hence, a myriad of experimental tools and theoretical models adapted from soft matter physics and engineering are now available to quantify cell mechanics, including mechanical force generation and cell mechanical properties. Modern instruments are capable of probing and manipulating single cells and biomolecules at forces and displacements smaller than a piconewton (1 pN = 10^{-12} N) and a nanometre (1 nm = 10^{-9} m), respectively[2]. TFM is perhaps the most commonly used technique for examining mechanical force generation in cells, owing to the simplicity of its implementation on most laser-scanning confocal microscopes. In TFM experiments, a thin (20−30 μm) elastic gel is formed on a glass coverslip onto which proteins facilitating cell adherence can be attached (**Fig. 1a**)[20]. Within the gel, which are typically made from polyacrylamide (see **Materials** and **Procedure** section), fluorescent beads serve as fiducial markers and imaging of the bead positions over time during the application of cellular tractions allows the displacement of the gel to be quantified. By combining the measured displacement field with knowledge of the mechanical properties of the gel, the tractions responsible for the displacements can be calculated. Notably, TFM is the ideal technique to investigate physiological interactions of cells within a two-dimensional (2D) geometry and is extremely versatile because the polyacrylamide gel can be functionalized with any protein of interest. Fluorescently-labeled proteins on the gel or integrated within intracellular structures of cells can then be simultaneously monitored due to the multi-color ability of confocal microscopes[10].

Despite its widespread usage,

TFM is limited in sensitivity due to the finite density at which the displacement field can be sampled within the gel[11]. The density of fiducial markers 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 is lost. Conversely, if the bead density is too high information is also 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. In general, this can be thought of as a sampling problem, where to meet the Nyquist criteria the spatial sampling frequency of the displacement field must be twice that of any features that may be resolved in the displacement field[21]. Consequently, the resolution limit of conventional fluorescent microscopes imposes a fundamental limit on the length-scale at which forces can be measured using TFM, which is typically greater than 1 μm [11]. Given the molecular origin of cellular forces, the length-scales at which they are active is likely to be sub-micrometre, thus there is a need to advance TFM to address questions on the nanoscale.

A first attempt to overcome this limitation involved the use of two different colors of marker beads which proved that the recovery of micron sized tractions is feasible[11]. However, due to its reliance on the spectral separation of the beads, this technique is ultimately limited by the spectral range of the microscope.

Development and overview of STED-TFM

To overcome these challenges, we recently improved the spatial resolution and accuracy of TFM using super-resolution optical STED (Stimulated emission depletion) microscopy (**Fig. 1b**)[10]. The increased spatial resolution of STED-TFM (STFM) allows a greater than 2-fold

higher sampling of the forces generated by the cell than conventional TFM, leading to more accurate quantification of cellular tractions (**Fig.1c**). Experiments yield quantitative values of mechanical force, commonly presented in pascals (1 Pa = 1 N/m^2), computed from the displacements of the fluorescent marker beads within the polyacrylamide gel using a theoretical framework derived from linear elastic theory[12,13]. Optimal experimental conditions refer to the appropriate gel stiffness for optimal sensitivity, the imaging parameters for optimal signal to noise ratio, and the appropriate implementation of STFM analysis.

In a typical STFM experiment, a polyacrylamide gel is first fabricated by a sandwich method and then validated to ensure that it has the correct mechanical (elasticity, thickness, flatness) and optical qualities (homogeneous, refractive index). Fluorescent beads must be added to the gel at this stage and here we describe a method of ensuring that the fluorescent beads are present only at the top surface of the gel which leads to reduced background due to out of focus light, and improved STED imaging (see **Procedure** section). The gel can then be functionalized with proteins relevant to the biological system in question. Functionalization can be achieved by various methods including biotin-streptavidin interactions, hydrazine hydrate or bifunctional crosslinkers[14–16]. We here outline one of the most common methods, using the bifunctional crosslinked, sulfo-SANPAH (see **Procedure** section). Once the gel has been validated and functionalized, cells can be allowed to interact with the gel and force measurements recorded. Cells can either be plated on the gel prior to imaging, for example in the case of adherent cells, or can be pipetted down at the microscope, where forces involved in the spreading or early interactions of the cells and the substrate can thus be studied. In this protocol, we focus on the study of HeLa cell focal adhesions and hence the cells are allowed to adhere to the functionalized gel prior to imaging. To measure the forces generated by the adherent cells, we first record a STED image of the gel in a tensioned state, followed by treatment with trypsin-EDTA, allowing the cell to detach, and then record a STED image of the relaxed state of the gel. Combining knowledge of the mechanical nature of the gel and displacement between the tensioned and relaxed state allows the computation of the forces to be recovered by applying a mathematical model[12,13].

This protocol will restrict itself to 2D force generation by STFM, and it is broadly applicable to any cell that undergoes physical interactions with any other large cellular organelle or the extra cellular matrix. For reasons of simplicity and generality, we have used focal adhesions as an illustration of this protocol, but it is of course not limited to the study of adhesion-associating proteins.

Applications of the protocol

Applying STFM allows the investigation of mechanical force generation within living cells. It is well suited to biological questions concerning 2D molecular force generation on the nanoscale for example cell adherence, but does not provide further insights on forces acting on the micro-meter scale compared to conventional TFM. In a previous study[10], we applied STFM in the single cell environment to measure traction forces generated during immune cell activation[17]. Quantification of the STFM experiments yielded more detailed force maps with a substantially higher degree of information due to 5-fold increased force sampling compared to classic TFM. Hence, STFM allows an increase in accuracy of STFM, which is important when considering cellular forces on small length-scales, as is the case for receptor-antagonist interactions in immune cells. Using STFM, we are now better able to make links between the forces generated by the cell, and those molecules which are responsible for force generation. This is particularly valuable when force measurements are coupled to fluorescent data.

Most biophysical interactions involve mechanical force generation and depend on the mechanical properties of cells. Overall, mechanical force depends on the fraction of bound/unbound protein-agonists, the total number of possible molecular interactions, the proportion of protein undergoing each interaction and the associated dissociation rate constant (time of duration of the reaction) of each interaction. Each of these variables can be modified by signaling, such as an increase in the proportion of phosphorylated protein in response to progression through the cell cycle. Post-translational modifications can result in changes in protein structure and consequently lead to changes in the binding affinity of some or all of a protein's subdomains or the exposure of cryptic binding domains[18]. If a sufficient portion of the total protein is affected by these post-translational modifications, this will lead to changes in association/dissociation and thus in force generation. However, coarse-grained measurements of mechanical force at low spatio-temporal accuracy cannot report on the origin of changes in force. By allowing the determination of this information by STFM, we anticipate that our protocol will enable a more precise understanding of the molecular origins of changes in mechanical force generation.

Computational models of cellular interactions with their mechanical environment have become an integral part of research into the organization and mechanics of the cytoskeleton during

cellular processes such as adhesion, locomotion[19–21], cytokinesis[22,23] or cell-cell interactions[24,25]. These models attempt to test our understanding of cellular processes from the bottom up by incorporating quantitative force values known to participate in the process to predict macroscopic mechanical properties or mechanical force generation during these processes that can be measured experimentally. Thus, measurement of each component participating to these force-dependent processes is necessary as an input for such simulations. We anticipate that our technique will complement these computational approaches by experimentally characterizing the necessary mechanics and testing model predictions.

Limitations of STFM

By increasing the sampling density of the displacement field, STFM addresses a fundamental limitation of conventional TFM, however STFM naturally suffers from the restrictions inherent to all TFM experiments, and a discussion of these is a given in the following.

Interpreting mechanical force generation in terms of 2D-displacements is naturally limited to the tangential components of forces, which only approximates the three-dimensional force exerted by the cells onto the substrate. TFM has been successfully extended to measure 3D-bead displacement generated by cells within a 3D elastic environment[26]. In the same way that 2D-STED can increase the accuracy of the tangential force reconstruction, using 3D-STED would allow for a greater sampling of the forces perpendicular to the gel surface as it has been demonstrated for TFM[27–29].

The location accuracy of STFM is theoretically not limited since it scales with the applied STED power[30]. This needs to be balanced with other optical factors such as maximal bead density within the gel, the ability to track the beads, and fluorescence light sensitivity of the biological specimen. Moreover, the nature of the (S)TFM setup requires all imaging to be done at the top surface of the gel, meaning imaging is subject to aberrations induced by the mismatch in refractive index of the gel and the immersion media. Improvements in aberration correction, for example using adaptive optics would reduce the effect of these aberrations and would result in an improved STED resolution, possibly along all three spatial dimensions[31], allowing even higher bead densities to be used and the accuracy of experiments to approach those shown possible by simulations[10]. Critical, case by case, investigation will decide if such an extension provides in practice additional information and increased quality of the force data. Specifically, in scenarios of adhering HeLa cells, whereas focal adhesions generate predominantly force tangential to the substrate, 3D-STFM may not be required. Furthermore, TFM experiments cannot provide information about cooperative force generation dynamics between different domains of the molecules of interest but should be able to provide insights into avidity effects of the force generating molecules. If such phenomena are at play, FRET measurements will be necessary[32].

Another limitation of our super-resolved approach is that we can only characterise molecular processes that occur on sufficiently different time-scales. If several molecular processes occur at similar frequencies, they cannot be separated, and the apparent force measured reflects a convolution over all of the molecular processes acting at that frequency. In that case, the force results from multiple molecular processes and does not represent a real molecular force measurement. In these situations, interpretation of the results will necessitate in-depth consideration of all of the force producing reactions that can lead to observed displacements, which is something best achieved by using computational modelling approaches.

Alternative methods to STFM

In the following, we briefly introduce a number of alternative approached to measuring cell generated forces and outline their advantages and disadvantages compared to STFM.

Whilst TFM relies on a continuous elastic surface, micro fabricated arrays of pillars made from an elastic material such as silicon or Poly(dimethylsiloxane) (PDMS) can also be used to measure cell tractions[33]. The tops of the pillars serve as contact points for the cell and can be functionalized using similar methods to those used in conventional TFM. By measuring the displacement of the top of each pillar, the force acting on it can be calculated, with each pillar behaving independently. The technique can offer resolution comparable to TFM, however, the complex topology of such an assay is not applicable to all cell types, and the influence of such a topology on cell behaviour is unclear[34].

Quantification of intracellular force generation within cells can also be achieved using fluorescence resonance energy transfer (FRET) based force sensors. FRET is a single molecule technique and relies on a donor fluorophore in an excited electronic state, which transfers its excitation energy to an acceptor chromophore in a non-radiative fashion through long-range dipole-dipole interactions. FRET force sensors typically contain two fluorescent molecules coupled by a flexible linker which extends under mechanical load. The extension of the linker results in a measurable decrease in FRET signal between the two fluorophores[32,35]. These tools allow forces to be quantified on the single molecule level within living cells and have been successfully used to measure forces in focal adhesions as well as receptor-ligand interactions[36–38]. Recently, a FRET force sensor was combined with optical super-resolution techniques, giving a spatial resolution of 50 nm[39]. Whilst this is an exciting development in the field, currently production of force sensitive FRET probes is challenging and requires in-depth biochemical knowledge of the specific biological system. Molecular probes of this type are also limited to measuring only the magnitude of forces, whereas TFM can measure both the magnitude and directionality[27].

Others techniques focus on quantifying the mechanical properties of cells. Atomic force microscopy (AFM) is a high-resolution surface characterization technique that is predominantly applied for imaging and measurements of mechanical properties but also allow the mechanical probing of biological samples[40]. AFM measurements employ a micro-fabricated cantilever beam equipped with a micron-sized tip to deform and interact with the sample of interest. Specifically, force spectroscopy can be employed as a single molecule tool to characterize mechanical properties and active force generation by cells with sub-nanometer and pico-newton resolution[1]. Cellular elasticity and rheology are measured by pressing the cantilever tip against the cell surface while the imposed cellular deformation (and thus force) are monitored. Considering an appropriate contact model and the geometry of the cantilever tip, this yields information about measured force versus indentation from which the elasticity of the cell is computed[41]. Force generation can be quantified by functionalizing the cantilever tip with integrins or protein binding agonists expressed on the cell surface[42,43]. Moreover, AFM allows time-dependent mechanical characterization and oscillatory tests[44] in stress-relaxation and creep experiments[45,46]. Instrumentation of AFM cannot easily be custom-built, are less available than TFM, and require specialized microscopy equipment and training, but is commercially affordable.

Optical tweezers (OTs) employ the concept of light trapping to mechanically manipulate, stretch, and move cells[47]. OTs focus collimated light that enters a medium of different refractive index resulting in a restoring force towards the center of the beam created by the light passing through the material that resists higher levels of refraction. Typically, the applied forces are in the pico-newton range and allow spatio-temporal measurements at high accuracy of displacements in the nanometer range of objects ranging in size from 10 nm to over 100 mm[48–50]. Similarly to AFM, OTs have been used to quantify force generating processes and mechanical properties of cells[51]. For instance, the adhesion energy was measured between the plasma membrane and the actin cortex by pulling particles, attached to the cell membrane, away from the cell surface with the optical laser beam[49,50]. Instrumentation of OTs with high spatio-temporal precision and accuracy can no longer be built by modifying a standard optical microscope but rather require sophisticated devices under computer control. Additionally, although optical tweezers provide a valuable tool for high precision measurements of small forces, they have an inherent limit on the amount of force that can be applied to cells without inducing local heating, affecting cell viability and the quality of force probing.

Particle tracking micro-rheology (PTM) relies on three-dimensional (3D) tracking of fluorescent particles embedded into intracellular structures of cytoskeleton components in the cytoplasm of cells. The particles can be cell membrane permeable or injected into the cells via micro-pipette aspiration[52]. Micro-rheological analysis of movement of the particles within cells report on the existence of Brownian fluctuations of free moving beads or directed motion of trapped beads. Indeed the movement of particles within the cell is perturbed by both elastic and viscous mechanical resistances[53]. Other possible contributions could include the influence of energy-consuming active processes and macro-molecular crowding on tracer particles inside the cytoplasm. Generally PTM experiments on cells reveal a dominant elastic response at high frequencies (short time-scales) and a more viscous behavior on small frequencies (over longer time periods)[1]. Instrumentation of PTM can be achieved on a standard light-field microscope but is limited by the ability to transport the beads to the location of interest.

One promising new method that overcomes such limitations is Brillouin optical cell microscopy (BM). BM measures intracellular and extracellular hydro mechanical properties of cells[54]. It is a label-free technique that does not require physical contact with cells and quantifies changes in light scattering due to cytoskeletal modulation and cell-volume regulation. Yet, this method is sensitive to the biomechanical changes in a cell caused by factors such as polymerization, branching and liquid-solid volume fraction, and measures only relative changes in the elastic behavior of cells. BM is a very promising new approach whose success still needs to be proven in the field of cell biology.

Table 1: Summary of force probing techniques in living cells.

Technique	**Resolution – force – space**	**Geometry**	**Magnitude/Direction of force**	**Reference**
FRET force sensors	Single molecule – 10s pN	2D	Magnitude only	[32,35–37]
Micro pillar	1 – 2µm - 50 pN	2D	Magnitude and direction	[33]
TFM	1 – 2µm - 1 nN	2D and 3D	Magnitude and direction	[11]

STFM	0.4–1μm - <1 nN	2D	Magnitude and direction	[10]

Experimental design

There are a number of key considerations when designing a TFM experiment, including the gel stiffness, the functionalization of the gel surface, and the computational methods required to extract the gel displacements and to compute the traction forces. STFM is a straight forward extension of the TFM technique, but as previously mentioned it requires extra consideration of a number of experimental parameters, primarily the bead density, and distribution in the gel including its mesh-size, as well as the imaging setup and equipment. In the following, we discuss the importance of each of these parameters.

Gel stiffness. As with all TFM experiments, the gel stiffness is of critical importance and must be tuned to the biological system under investigation. Cells can exert forces over a wide range and as a result the gel stiffness must be chosen such that any exerted forces result in appropriate displacements of the gel-embedded beads[11]. If the gel is too stiff, the cell will be unable to sufficiently displace the beads, preventing the accurate calculation of forces. Alternatively, if the gel is too soft, the displacements may be too large, contravening the assumption of linear elasticity meaning in practice the bead displacements should be 0.1-0.5 μm[11]. In some cases, the cells may have mechanosensitive properties and hence respond directly to the stiffness of the gel and only adhere to gels that are of the appropriate stiffness[55]. To this end, we fabricate gels using polyacrylamide, which can be produced over a wide range of stiffness' by simply altering the ratio of acrylamide monomers to the cross linker bis-acrylamide. Whilst the ratios of acrylamide and bis-acrymaide that yield a certain stiffness have been well characterised, if required gel stiffness can be verified using AFM indentation[56]. It is important to note that the mesh size of the polyacrylamide gel is proportional to the stiffness, with stiffer gels having smaller mesh sizes. Care must be taken to ensure that the chosen stiffness has a mesh size sufficiently small to trap the chosen fluorescent beads within the gel structure. Mesh size may also determine the surface density of functionalized proteins[57].

Fluorescent bead distribution.

Fluorescent beads required to track the displacement of the gel are usually added to the un-polymerized acrylamide/bis-acrylamide mixture at a given ratio. As the gel polymerizes the beads are trapped within the gel matrix and remain in place. Whilst this is a very effective way of adding the beads to the gel, it results in beads being evenly distributed throughout the thickness of the gel. This is undesirable as only beads at the top surface are useful for measuring forces. In addition, out of focus light from beads that are not at the top surface contribute to a high level of signal background, making high contrast imaging of the beads at the top surface challenging. This becomes especially problematic when high bead densities are used, as is required for STFM. To alleviate this, we modify this typical strategy to ensure beads are only present at the top surface of the gel[58]. To achieve this, the coverslip used to form the top of the gel sandwich is first functionalized with poly-L-lysine followed by a coating of fluorescent beads, creating a dense and uniform layer of beads on the coverslip (**Fig. 2a,b**). When the sandwich is formed, the beads are captured within the gel solution as it polymerizes, resulting in a dense layer of beads that is restricted to the top layer of the polyacrylamide gel.

Optimal bead density. The density of beads on the top surface of the polyacrylamide gel directly determines the accuracy of force recovery. When deciding on the bead density to use, consideration must be given to the spatial scale and nature of the forces which are to be measured and the image analysis method to be used to extract the bead displacements. Specifically, the accuracy of the traction measurement depends on the sampling of the displacement field which in turn is determined by the bead density. The relationship between bead density and the spatial size of traction that can be accurately recovered can be demonstrated by simulations. We computer-simulated a circular traction field at varying diameters and subsequently recovered the tractions by using displacements extracted from different bead densities (**Fig. 3**.**a,b**). The results illustrate how increasing spatial sampling allows smaller tractions to be effectively recovered. The quality of traction recovery is measured by the so called deviation of traction magnitude (DTM), which is the normalized difference in the norms of the simulated and recovered traction fields. A DTM of 0 represents a perfect recovery whereas -1 indicates that all information of the traction has been lost. This plot should serve as a guide to those wishing to resolve details on a certain spatial scale, for example, submicron resolutions require a sampling density of 3 beads per μm^2 or better. Crucially, in order to gain accurate displacement measurements at these high bead densities it is desirable to increase the optical resolution beyond that of a conventional confocal microscope using STED.

STED microscopy. STED microscopy is becoming increasingly available and has been implemented in a series of different commercial systems. This offers an opportunity to improve the accuracy of established methods such as TFM, which rely on confocal microscopy. To maximize the resolution improvements of STED, whilst maintaining the biological integrity of the samples, a number of considerations must be taken into account, including the choice of laser wavelengths, the optical properties of the substrate, the fluorescent beads, and the image acquisition settings.

STED has been shown to work across the fluorescent spectrum and indeed fluorescent dyes can give resolution enhancement from the blue to the red range using for example STED wavelengths of 592 nm, 660 nm and 775 nm, respectively[59]. Because biological samples will absorb more strongly towards the blue end of the spectrum, it is desirable to use a STED wavelength that is red shifted. In this study, we use a combination of 40 nm red-fluorescent beads excited at 594 nm and a STED wavelength of 660 nm. We demonstrated that this combination offers a resolution enhancement of >5x when optical conditions are optimal[10].

Our protocol takes advantage of continuous wave (CW) laser based STED. This greatly reduces the cost and complexity of the optical system, removing the need for pulsed lasers. The disadvantage of CW-STED is the decrease in depletion efficiency, and hence resolution, due to the lack of temporal coherence between the excitation and depletion lasers. However, this can be overcome by gating the detection of the signal using so called gated-CW-STED or gCW-STED[60]. This system has been routinely implemented and is for instance commercially available in the form of the Leica SP8 microscope, as was used in the protocol. Yet, there is no principle limitation of using pulsed STED laser systems instead.

Because STED microscopy is usually applied as a confocal-based technique, it can be performed at any focal plane within the sample. This is a key requirement for TFM, where imaging is performed at a focal plane at the top of the polyacrylamide gel and away from the

coverslip. Because the polyacrylamide gel has a refractive index that differs significantly from glass (around n=1.33), using an oil objective optimized for glass will lead to optical aberrations and result in reduced signal and STED resolution when imaging at top of the gel (**Fig. 4 a-d**). It is hence desirable to minimize these aberrations in two ways, 1) limit the thickness of the gel to around 30 μm to reduce scattering losses while maintaining sufficient thickness to avoid problems with the assumption of gel linear elasticity and effects of the glass surface; 2) to use an objective immersion medium which is well matched in refractive index to that of the gel. We have found that using a water immersion objective works well in this case, exhibiting minimal spherical aberrations and allowing a STED enhancement of 4x at a depth of 25-30 μm (**Fig. 4 a-d**).

When imaging biological samples using high intensity laser light, it is important to conduct controls to ensure laser light is not interfering with the biological function of the sample. This is particularly important in STFM, where imaging should be conducted in both confocal and STED modalities to ensure that the higher laser intensities used in STED do not perturb the sample. As with all scanning based florescence techniques, to further minimize the photo-toxicity effects on the sample, the scan speed should be maximized combined with line averaging i.e. the pixel dwell time should be as low as possible to minimize the effects of local exposure. To increase photon count and enhance image contrast, multiple line averages should be taken rather than increasing the dwell time.

STFM analysis.

Image analysis forms a crucial part of the STFM technique and there are a number of methods by which bead displacement can be quantified. These methods can broadly be divided into those that depend on tracking each individual bead, so called Single Particle Tracking (SPT) techniques, and those that depend on correlating displacements within regions of an image such as Particle Image Velocimetry (PIV). The choice of methods depends on the expected nature of the forces and on the density of beads within the image, thus it is important to consider which analysis is more suited to the particular experiment at the outset.

In the case of focal adhesions, where the forces are likely to result in the collective motion of a number of beads and hence smooth displacements, it is appropriate to use an image analysis method that depends on correlating regions of the image rather than individual bead movements. PIV has been widely implemented in this scenario and is a good choice for such measurements. Because PIV does not depend on resolving each individual bead, higher bead densities, in the range of 3-15 beads per $\mu m^{2,}$ can be used (**Fig. 3c**). However, because PIV requires the image to be subdivided into regions of finite size, high frequency spatial information within the sub regions may be lost. To minimize this loss, the selection of the PIV interrogation window is critical. If the windows is too large, fine detail within the displacement of the beads will be lost and the overall resolution of the traction will be reduced. Alternatively, if the window is too small such that it contains no discernible feature, the correlation between frames will be unreliable and prone to error. As a guide, the PIV windows should be chosen such that they contain on average three beads, although optimization may be required for each particular experiment[61]. Therefore, if the bead density is 5 beads per μm^2, recorded at 20 nm pixel size, the interrogation window should be 30 pixels x 30 pixels. Alternatively, if the forces are more spatially complex or are likely to contain localised high frequency displacements, it

is desirable to use lower bead densities that allow each individual particle to be tracked using a SPT approach. In this case a bead density of 0.5-3 beads per μm^2 is appropriate (**Fig. 3c**).

In this protocol, the data acquired is more suited to a PIV approach to extract the displacement however it has been shown that whether using PIV or SPT, the higher resolution offered by STED enhances the accuracy of force reconstruction[10]. To extract the displacement we use in this protocol an open source ImageJ implementation of a PIV routine[73,] (PIV (Particle Image Velocimetry) ImageJ plugin. Available at: https://sites.google.com/site/qingzongtseng/piv) (see **Procedure** section).

Once displacements have been extracted from the bead images, this can be combined with the mechanical properties of the gel to calculate the forces exerted on the gel. The traction field is a convolution of the displacement field and the so called Green's function which contains information about the mechanical properties of the gel, and therefore the traction solution requires solving a so called ill-posed inverse problem. Inverse problems are subject to errors due to noise, in this case in the displacement field, and hence there are a number methods proposed to optimize the solution to this inverse problem[12,13,62–64]. The most common and most general is by so called Fourier Transform Traction Cytometry (FTTC) in combination with regularization[11,13]. Regularization is a mathematical technique by which the solution of a linear problem can be limited by some pre-defined constraint. The degree of regularization is chosen such that a balance is found between how well the solution fits the noise corrupted experimental displacement data and the overall magnitude of the traction solution. If the solution is over regularized, high resolution information will be lost as we are in effect over smoothing the data. In contrast, under-regularization leads to a solution that over fits the noise in the data and will not be representative of the data. Once an adequate regularization parameter has been found this value should be used for all subsequent traction measurements to make analysis across cells consistent. As with the PIV algorithm, the regularized FTTC has been implemented as an ImageJ plugin[61,] (TFM (Traction Force Microscopy) --- ImageJ plugin. Available at: https://sites.google.com/site/qingzongtseng/tfm), making processing of the STFM data straight forward (see **Procedure** section).

MATERIALS

Reagents

Gel Fabrication

- (3-Aminopropyl)trimethoxysilane (APTMS) (cat. no. 281778, Sigma Aldrich, United Kingdom)
- Ammonium persulfate (APS) (cat. no. 215589-100G-D, Sigma Aldrich)
- Acrylamide solution, 40 % in ddH_2O (cat. no. A4058-100ML Sigma Aldrich)
- N,N-Methylenebisacrylamide solution, 2 % in ddH_2O (cat. no. M1533-25ML Sigma Aldrich)
- N,N,N,N-Tetramethylethylenediamine (TEMED) (cat. no. 411019-100ML, Sigma Aldrich)
- FluoSpheres Carboxylate-Modified Microspheres, 0.04 µm, red fluorescent beads (580/605), 5% solids, azide free (cat. no. F-8793, ThermoFisher, United Kingdom)

- Poly-L-lysine solution mol. wt. 70,000-150,000, 0.01% (wt/vol) (cat. no. P4707-50ML, Sigma Aldrich)
- Sulfo-SANPAH, 50 mg (cat. no. 22589, ThermoFisher)
- Fibronectin from bovine plasma (cat. no. F1141-1MG, Sigma Aldrich)
- Sulfuric Acid, 99.99 % (cat. no. 339741-500ML, Sigma Aldrich)
- Hydrogen Peroxide, 30 % (wt/wt) in H_2O (cat. no. H1009-500ML, Sigma Aldrich)
- Glutaraldehyde, 25 % in ddH_2O (cat. no. G5882-50ML, Sigma Aldrich)
- Phosphate-buffered saline tablets (cat. no. P4417-50TAB, Sigma Aldrich)
- Double distilled water ddH_2O

Cell Culture and transfection

- Cells of choice. Here, we use HeLa Human cervical carcinoma cell (e.g. ATCC database; cat. no. PTA-5659)
  CAUTION: The cell lines used should be regularly checked to ensure they are authentic and are not infected with mycoplasma.
- Plasmid encoding the fluorescently-tagged protein of interest. Here, we use paxillin-EGFP (addgene cat. no. 15233)
- Cell culture medium, DMEM (cat. no. D6046-500ML, Sigma Aldrich)
- Fetal Bovine Serum (FBS) (cat. no.16140071, ThermoFisher)
- L-Glutamine (200 mM) (cat. no. 25030081, ThermoFisher)
- Penicillin-Streptomycin (10,000 U/mL) (cat. no. 15140122, ThermoFisher)
- Leibovitz L15 Medium, without phenol red (cat. no. 21083027, ThermoFisher)
- Lipofectamine 3000 Transfection Reagent (cat. no. L3000015, ThermoFisher)
- Opti-MEM I Reduced Serum Medium (cat. no. 11058021, ThermoFisher)
- Trypsin-EDTA 0.05 % (wt/vol) (cat. no. 25300054, ThermoFisher)

Software

- PIV (Particle Image Velocimetry) ImageJ plugin. Available at: https://sites.google.com/site/qingzongtseng/piv.
- TFM (Traction Force Microscopy) --- ImageJ plugin. Available at: https://sites.google.com/site/qingzongtseng/tfm.

Equipment

- Coverslips No 1.5 18mm and 12mm (cat. no MIC3342 and MIC3334, SLS)
- UV crosslinker with 365 nm emission (Stratalinker 1800/2400, Stratagene or equivalent)
- Confocal microscope equipped with a conventional continuous-wave 660 nm STED laser and an excitation Wight Light Laser (WLL) as well as an environmental control

for live cell imaging (Leica SP8, Leica Microsystems UK, or equivalent)

- 63x or 100x Water immersion objective (Leica HC PL APO 63X/1.20 W motCORR CS2, Leica Microsystems UK or equivalent)
- Sonication bath (Grant XUB5UK Grant Instruments, United Kingdom)
- Pipettes 1000 µl, 200 µl, 10 µl and 2 µl (cat. no. 17014382, 17014391, 17014388, 17014393, Rainin)
- 18 mm coverslip holder (cat. no. CSC-18L, Biosciencetools)
- Centrifuge tubes, 15 ml and 50 ml (cat. no. 430053, 430304, Corning, United Kingdom)
- Micro-centrifuge tubes, 1.5 ml (cat. no. 3621, Corning)
- 6-well tissue culture plates (cat. no. 353846, Corning)
- T25 culture flasks (cat. no. 430639, Corning)
- Petri dishes (100 mm x 15 mm) (cat. no. BP94A-01, Corning)
- CO_2 incubator (cat. no. MCO-170AIC-PE, Panasonic, United Kingdom)
- Centrifuge (cat. no. 75004503 ThermoFisher)
- Biosafety cabinet (Biomat-2, CAS, United Kingdom)
- Parafilm (cat. no. P7793-1EA Sigma Aldrich)

Reagent Setup

- **APS solution** Prepare a 10 % (wt/vol) APS solution by dissolving 1 g of APS in 10 ml of ddH_20. Freeze in 50 µl single use aliquots and store at -20 °C for up to 1 month.
- **Fluorescent bead solution** Dilute bead solutions can be prepared by diluting the original manufacturers solution in ddH_20. For the case of above listed 40 nm red fluorescent beads a vol/vol dilution of 1 in 10,000 is adequate. To achieve this dilution it is advisable to first make a 1 in 100 dilution by adding 10 µl of the concentrated fluorescent bead solution to 990 ml of ddH_20. A further 1 in 100 dilution can be performed in the same way, adding 10 µl of the 1:100 solution to a further 990 ml of ddH_20. When performing this serial dilution it is critical that the solutions are well mixed at every stage and that the beads are not aggregated. To minimize aggregation, the solutions should be sonicated using an ultrasonic bath for 10 mins prior to further dilution. Bead solutions can be stored at 4 °C for up to 1 month.
- **Polyacrylamide solution** The relative concentrations of acrylamide and bis-acryalmide determine the gel stiffness and this must be optimized for each specific experimental condition. In the case of HeLa cells a solution containing final concentrations of 8 % and 0.48 % acrylamide and bis-acrylamide, respectively, will produce a 40 kPa polyacrylamide gel. To produce 500 µl of this solution, add 100 µl of 40 % acrylamide and 120 µl of 2 % bis-acrylamide to 280 µl of PBS. Formulations can be stored at 4°C for up to 6 months. For a comprehensive list of formulations and their corresponding stiffness see[68]. CAUTION! Acrylamide is a potent neurotoxin and care should be to taken to minimize contact.
- **Sulfo-SANPAH crosslinker solution** Dissolve the sulfo-SANPAH in 2000 µl of DMSO, creating a 25 mg/ml solution. Freeze 40 µl single use aliquots and store at -80 °C.

- **HeLa cell culture medium** To a 500 ml bottle of DMEM, add 50 ml of FBS, 5 ml of L-Glutamine and 5 ml of Penicillin-Streptomycin. Cell culture medium can be stored at 4 °C for up to 1 month.

PROCEDURE

Acid cleaning of 12 mm and 18 mm glass coverslips
Timing – 1.5 hours

1. In 500 ml pyrex beaker, add 20 ml of 99.99 % sulfuric acid. To the same beaker, slowly add 10 ml of 30 % hydrogen peroxide solution. CAUTION! Hydrogen peroxide must be added second to avoid causing dangerous local heating within the mixture. Acid cleaning must be done in a fume hood and suitable protection must be worn.
2. To this solution, add one by one the 12 and 18 mm coverslips ensuring all are completely submerged. Gently agitate. Incubate at room temperature for 1 hour agitating periodically. Bubbles on the glass surface indicate the degradation of organic material on the glass. When bubbles have significantly reduced, the glass has been cleaned.

3. Prepare 2 l of ddH_20 in a suitable container. To the beaker containing the coverslips, add 500 ml of the ddH_20 to dilute the acid solution as much as possible. Pour away the diluted acid solution taking care that the solution being rinsed away is very dilute to avoid any damage to the sink and surroundings. Repeat this process 3 times. On the final wash do not discard the contents of the beaker but keep the coverslips submerged in ddH_20. Cover the beaker with parafilm and store at room temperature for up to 2 weeks.
4. Repeat step 4 for the 18 mm coverslips

Activation of 18 mm coverslips Timing – 1.5 hours

CRITICAL. Steps 5 – 11 and 12 – 16 can be done in parallel to save time.

5. Place 10-15 acid cleaned 18 mm coverslips in a petri dish ensuring no coverslips are overlapping.
6. In a 50 ml centrifuge tube, add 40 ml of ddH_20 followed by 200 µl of APTMS, forming a 0.5 % (vol/vol) solution. CRITICAL STEP APTMS can break down plastics so pipetting should be done as quickly as possible.
7. Submerge the coverslips in the 0.5 % APTMS solution ensuring all coverslips are coated. Incubate at room temperature for 30 mins on an orbital shaker.
8. Remove the coverslips from the shaker and aspirate the APTMS solution. Re-submerge in ddH_20 and then remove washing solution. Repeat three times.

9. In a 50 ml centrifuge tube, mix together 40 ml of ddH20 and 200 µl of glutaraldehyde, forming a 0.5 % (vol/vol) solution. CAUTION! Glutaraldehyde is toxic and should be handled in a fume hood and disposed of correctly.
10. Submerge coverslips in the glutaraldehyde 0.5 % solution, again ensuring all coverslips are coated and return to the orbital shaker for a further 30 mins at room temperature.
11. Aspirate the glutaraldehyde solution and replace with ddH_20. Repeat three times. After the final wash, leave the coverslips submerged in the ddH_20. Seal the petri dish with parafilm.
    PAUSE POINT. Coverslips can be stored at room temperature for up to 2 weeks. CRITICAL STEP. if the washing is not done correctly, or incorrect concentrations have been used, a brown precipitate with form on addition of the glutaraldehyde (step 10). If this occurs, the coverslips are unusable, and steps 5-11 must be repeated with fresh 18 mm coverslips.

Coating 12 mm coverslips with fluorescent beads Timing – 1.5 hours

12. Dry 6 12 mm acid cleaned coverslips using an air gun or equivalent device
13. Place 25 µl of 0.01 % (wt/vol) Poly-L-Lysine solution on each coverslip and incubate at 4 °C for 30 mins.
14. Remove the Poly-L-Lysine by air gun. Alternatively, the Poly-L-Lysine solution can be removed by pipette and the coverslip submerged in ddH_20 then allowed to air dry.

15. Place a 25 µl drop of 1:10,000 diluted fluorescent bead solution (see Reagent setup) on each coverslip and incubate at 4 °C for 30 mins. CRITICAL STEP – the precise dilution ratio may require optimisation for a given bead size and gel stiffness.
16. Remove the bead solution by air gun. Alternatively, the bead solution can be removed by pipette and the coverslip submerged in ddH_20 then allowed to air dry. CRITICAL STEP Bead coated coverslip should be prepared fresh prior to gel fabrication.

TROUBLESHOOTING

Gel sandwich formation
Timing – 1 hour

17. In a 1.5 ml micro-centrifuge tube prepare 50 µl of the previously prepared acrylamide/bis-acrylamide solution (see **Reagent Setup**). The exact ratio of acrylamide and bis-acrylamide with determine the gel stiffness and must be tuned to the specific biological question. However, the polymerization procedure is independent of the gel formulation. Note, that different acrylamide and bis-acrylamide concentration yield the same elastic stiffness of gels at different gel mesh-sizes which can affect the mobility of the fluorescent marker beads within the gels.
18. Degas the gel solution (e.g. using a sonication bath) for 30 mins to remove oxygen.

19. For each gel to be fabricated, place one bead coated 12 mm coverslip (from step 16) and one air dried 18 mm APTMS/Glutaraldehyde treated coverslip on a convenient surface (e.g. tissue paper) ensuring the bead coated surface is facing upwards. Typically, 6 gels can be made at any one time and is limited by the speed at which the solution polymerizes.

20. Thaw one aliquot of the 10 % (wt/vol) APS solution ready for gel polymerization.

21. After degassing, add first TEMED at a 1:250 dilution (0.4 µl in 100 µl) to the un-polymerized gel solution. Mix gently by pipette to avoid introducing any air bubbles.

22. Working quickly, add APS to the solution at the volume ration of 1:100 (1 µl in 100 µl), again mixing gently by pipette. Pipette 3 µl of the solution onto the 12 mm coverslip and quickly place an 18 mm coverslip (from step 11) on /top to form a sandwich. Allow gel to polymerize fully for 30 mins; when the polyacrylamide gel solution in the micro-centrifuge tube has polymerized it can be assumed that the sandwich has also polymerized.Note, the volume of gel solution directly determines the thickness of the gel. 3 µl on a 12 mm coverslip will form a gel 26 µm thick..

TROUBLESHOOTING

23. Using a scalpel carefully remove and discard the 12 mm coverslip from the top of the gel and submerge the 18 mm coverslip with gel attached in a 6 well plate in 2 ml of PBS. The beads have now been transferred to the gel attached to the 18 mm coverslip.

24. Wash each gel by exchanging the 2ml of PBS in each well 6 times to remove all unpolymerized acrylamide.
PAUSE POINT. Gels can be stored in PBS at 4 °C for up to 2 weeks.
At the microscope validate a fabricated gel to assess its fluorescent bead coverage, STED resolution enhancement and thickness. This is a good opportunity to optimize the imaging conditions to maximize resolution and signal to noise. For examples of recommended bead densities see **Fig 2c**.

Gel functionalization Timing – 12 hours (or overnight)

25. Thaw one 40 µl aliquot of sulfo-SANPAH bifunctional crosslinker and dilute in 960 µl of ddH20 (see **Reagent Setup**). Store on ice.

26. On a petri dish, stretch out parafilm, so that it forms a suspended layer across the top of the petri dish.

27. Remove the gel (from step 24) from solution and remove as much excess buffer as possible. Place the coverslip on the parafilm coated dish, making sure the gel is facing upwards. Pipette 170 µl of the sulfo-SANPAH solution on the gel and immediately place under the UV lamp. Expose with 365 nm UV radiation for 10 mins. This process can be done in parallel for 6 gels at a time.

28. Remove each gel from under the UV lamp and submerge in 2 ml of PBS, exchanging the PBS 3 times to ensure all unbound sulfo-SANPAH has been removed.. Note, after exposure to UV the sulfo-SANPAH solution will darken in colour from red to brown.

29. On a second petri dish, stretch out parafilm so that it forms a suspended layer across the top of the petri dish and place a 20 µl drop of 500 µg/ml of fibronectin for each gel. Place each coverslip, gel side down on the drop of fibronectin and incubate at 4 °C for 12 hours or overnight.
30. Remove each gel from the fibronectin solution and place in a 6-well dish containing 2 ml PBS. Once again wash by exchanging the PBS 3 times.. Coverslips are now ready to use.
    PAUSE POINT. Coverslips may be stored in PBS at 4 °C for up to 2 weeks.

Cell preparation Timing – 12-24 hours

31. Remove the PBS from each well of the 6-well plate containing the coverslips (from step30). Add 2 ml of cell growth medium to submerge the gels and sterilise by placing under a UV lamp for 1 hour.
32. Detach HeLa cells, grown to 80 % confluency in a T25 flask, by removing the growth medium and then wash by adding 5 ml of PBS. Next, remove the PBSand add 2 ml of trypsin-EDTA 0.05 % (wt/vol) warmed to 37 °C. Return to the incubator at 37 °C for 5 mins,
33. After validating the cell detachment using a benchtop light microscope, add a further 5 ml of medium to the cells and aspirate from the flask. Transfer to a 15 ml centrifuge tube and centrifuge at 200 xg for 3 mins. Remove and discard supernatant and re-suspend cells in 5ml of fresh medium.
34. Count the cells using a haemocytometer. Add 0.5 million cells to the centre of each coverslip (from step 31). Gently agitate the 6-well plate to ensure that cells are well distributed over the gel surface.
35. Return the 6-well plate to the 37 ºC incubator and give the cells sufficient time to attach (6-12 hours). PAUSE POINT The degree of cell attachment can be assessed using a benchtop light microscope.

TROUBLESHOOTING

36. Once the cells have attached the plasmid of choice (in this case paxillin-EGFP plasmid DNA) can be transfected into the cell, as described in steps 36-39
37. For each coverslip prepare 2 micro-centrifuge tubes, each containing 125 µl of Opti-MEM. To the first tube add 1 µg of plasmid DNA followed by 5 µl of the P3000 transfection reagent. To the second tube add 4 µl of the L3000 reagent. Allow to diffuse throughout the solution at room temperature for 5 mins. Combine the two solutions and allow to stand for a further 20 mins to allow the DNA and transfection reagent to form complexes.
38. Add the combined mixture to each coverslip, pipetting slowly to distribute the solution evenly over the surface of the coverslip. Do not mix by pipetting. Return to the 37 ºC incubator for 4 hours.
39. Remove the medium containing the DNA and transfection reagent and replace with fresh medium. Return to the incubator for a further 12 hours or overnight to allow the cells to express the fluorescent protein.
40. Validate the expression by checking the signal from the fluorescent protein using a simple inverted microscope equipped with a fluorescent lamp.

TROUBLESHOOTING

Data acquisition Timing – 1 hour

CRITICAL. This procedure details how to set up and perform a STFM experiment on a Leica SP8 STED microscope. Similar functions should be available in most laser-scanning STED-based microscopes. For a practical guide to building a custom-built STED setup see [71].We present optimal settings for acquiring STFM data on a microscope equipped with a 660 nm STED line and a white light laser for fluorescence excitation.

41. Remove the gel coated coverslips with cells attached form the 37 ºC incubator (from step 39) and load one of the coverslips into a suitable sample chamber. Add 500 µl of L15 serum free medium and return the sample to the incubator while the microscope reaches 37 °C.

42. Switch on all required laser lines and allow the laser powers to stabilize (Configuration > Laser Configuration > WLL -> on, set to 100 % power, 660 nm -> on, set to 100 % power) Specific laser line powers measured to be 488 nm – 36 µW, 594 nm – 55 µW, 660 nm – 80 mW.

CRITICAL STEP Prior to experimentation, ensure that the microscope is well aligned using the automatic alignment tool (Configuration > STED > Align Beams) and that the environmental control is set to 37 °C.

43. When the microscope has reached the correct temperature load the sample and locate the cells using a water immersion objective of suitable NA and magnification e.g. Leica HC PL APO 63x/1.2 Water motCORR CS2 Objective. Locate a cell and select a region of interest (ROI). For dynamic measurements choose a region that is not too large as to allow for sufficiently fast scan time to capture the force dynamics in a representative region such as 20 x 20 µm.

44. For comparison acquire a confocal image of both the cell and beads beneath the cell. This image should be acquired using the same parameters as for the STED, only differing in laser powers (as tabulated below).

	Confocal	STED
Pixel size	25 nm	25 nm
Repetition rate	1000 Hz	1000 Hz
488 nm excitation power	5 %	5 %
594 nm excitation power	5 %	5 %
660 nm STED power	0 %	80 %
Gating time	No gating	1 ns – 6 ns
Gating time	48 x	48 x
ROI size	20 x 20 µm	20 x 20 µm

45. Acquire a STED image of the beads, as tabulated above. Imaging parameters should be tuned to maximize the resolution.

CRICIAL STEP. Note, this is an important point at which to take note of any adverse effects of the laser light e.g. cell retraction. If these effects are observed then lower laser intensities should be used in combination with faster scan speeds

46. Add 50 µl 0.05 % trypsin-EDTA to the cells and wait 5 mins or until the cell has fully detached.

47. Acquire a further confocal and STED image of the relaxed gel. Save the data and associated meta-data.

Data analysis Timing – 1 hour

48. Open ImageJ and load both the pre and post trypsin-EDTA treatment images. Using the stack tool (Image>Stacks>Images to Stack), combine both images.

49. If required, correct for any drift between the two images using an appropriate tool such as StackReg (Plugins> Registration> StackReg> Translation). This is critical as any drift between the two frames will affect the measured displacements.

50. To assess the displacement of the beads between the two frames use the PIV ImageJ plugin (Plugins > PIV > Iterative PIV (cross-correlation)). Save the result. Note, the minimum interrogation window of the iterative PIV routine must be sufficiently large to contain one bead.

51. Use FTTC Traction Force Microscopy Plugin (Plugins > FTTC > FTTC) to calculate the corresponding traction map. Insert the image pixel size, Poisson ratio (assume to be 0.5), gel stiffness and regularisation factor (1e-10 is suitable value in most cases) and load in the calculated displacements. Save the result.

TROUBLESHOOTING

Troubleshooting advice can be found in Table 2.

Table 2. Troubleshooting

Step	Problem	Possible cause	Solution
12-16	No fluorescent beads visible at the top surface of the gel	- Coating of Poly-L-lysine was not correct - Fluorescent bead concentration too low - Glass not sufficiently clean	- Repeat Poly-L-lysine coating - Repeat with higher fluorescent bead concentration - Repeat glass cleaning (steps 1-5)
12-16	Fluorescent bead coating is not homogenous	Aggregation of fluorescent bead solution	Increase sonication time or prepare a fresh solution

17-24	Gel does not polymerize	Not the correct ratio of TEMED/APS	Repeat at the correct TEMED/APS ratio
31-35	Cells do not attach to the gel	Sulfo-SANPAH not functional Protein concentration insufficient UV exposure insufficient or wrong wavelength used	Repeat with a fresh aliquot of sulfo-SANPAH Increase the concentration of protein. Increase the UV exposure, ensuring it is the correct wavelength
36-39	Paxillin-EGFP expression is low or not present	Transfection not working	Optimize ratio of DNA and transfection reagents.

TIMING

Steps 1-4, Acid cleaning of 12 mm and 18 mm glass coverslips : 1.5 hours

Steps 5 – 11, Activation of 18 mm coverslips : 1.5 hour

Steps 12 – 16, Coating 12 mm coverslips with fluorescent beads : 1 hour

Steps 17 – 24, Gel sandwich formation: 1 hour

Steps 25 – 30, Gel functionalization: 12 hours

Steps 31 – 39, Cell preparation: 12-24 hours

Steps 41 – 46, Data acquisition: 1 hour

Steps 47 – 50, Data analysis: 1 hour

ANTICIPATED RESULTS

Figure 5 provides an overview of the anticipated results for the application of this protocol to the focal adhesion in living HeLa cells. HeLa cells spread and form focal adhesions on a 40 kPa polyacrylamide gel functionalized with fibronectin. The adhesion points can be visualized as bright regions of paxillin-EGFP signal (**Fig. 5a,b**). Fully spread HeLa cells are in a contractile state, transmitting mechanical forces through the focal adhesions to the gel. To measure these forces, we must release the cell from the gel using the protease, trypsin-EDTA, which severs the links between then cell and gel thus allowing the gel to relax to an equilibrium state. Imaging of the beads should be carried out prior and after trypsin-EDTA treatment to ensure that the tensioned and relaxed state of the gel can be measured by the position of the fluorescent beads within the gel (**Fig. 5c,d**). Following extraction of the displacements via the PIV algorithm and calculation of the traction field via the FTTC algorithm, the force map shows a spatial agreement with the fluorescence intensity distribution of paxillin-EGFP. For comparative purposes, the images of the stressed and unstressed bead positions should be recorded in confocal and STED and the reconstructions compared as in (**Fig. 5e,f**). Using STED microscopy, more detail is apparent in the recovered traction field and the distribution of the magnitude of force is in better agreement with the paxillin-EGFP distribution (**Fig. 5g**).

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Author Contributions H.C-Y conducted the experiments. H.C-Y, M.F and C.E wrote the manuscript.

Competing Financial Interests The authors declare no competing financial interests.

Figure 1. Outline of STFM. (**a**) Schematic representation of a typical STFM setup. An elastic polyacrylamide (PAA) gel filled with fluorescent marker beads is covalently attached to a glass coverslip and functionalized with proteins that facilitate cell adherence. Traction forces applied by the cell to the top surface of the gel results in lateral displacements of the gel which can be quantified by imaging the displacement of the beads within the gel. (**b**) Schematic showing the beam path of a STED microscope. An excitation laser line (green) is combined with a STED depletion laser (red) that has passed through a phase plate, giving a characteristic doughnut shape in the focal plane. The combined STED doughnut (red) and excitation (green) result in a reduction in the size of the observed fluorescence spot size (orange). (**c**) Side by side comparison of the resolution enhancement offered by STED when compared to confocal microscopy using respective images of 40 nm red-fluorescent beads excited at 594 nm and a STED wavelength of 660 nm. Scale bar is 2 μm.

Figure 2. Polyacrylamide gel fabrication and validation. (**a**) Overview of the polyacrylamide gel fabrication procedure (steps 1-30). PAA gels are formed by sandwiching a solution of acrylamide and the crosslinker bis-acrylamide. The coverslips forming the sandwich are activated such that the bottom of the gel is covalently attached to the bottom coverslip and the top surface of the gel is uniformly coated with fluorescent beads (steps 5-16). After the gel solution has been prepared and polymerised (steps 17-22) and a sandwich formed between the two coverslips (steps 23-24), the top surface of the gel can then be functionalized with proteins that facilitate cell attachment via the bifunctional crosslinked sulfo-SANPAH (steps 25-30). (**b**) Confocal images of the x/z cross-section demonstrating the distribution of beads and protein on the gel, both of which are localized to the top surface. Scale bar is 30 µm.

Figure 3. Relationship between bead density and traction resolution. (**a**) Quality of traction recovery for circular tractions of varying diameter shown at three bead densities, 0.5 μm^{-2} (red), 3.0 μm^{-2} (blue) and 15 μm^{-2} (green). **b**) Recovery of a 1 µm circular traction probed at the corresponding three sampling densities. Recovery is seen to improve with increasing density. **c**) Simulated fluorescent bead images at the highlighted densities in both confocal (300 nm PSF) and STED (80 nm PSF). Scale bar is 2 µm.

Figure 4. Microscope objective characterization. (**a,b**) Polyacrylamide gel cross-section (left) showing the distribution of the fluorescent marker beads acquired using the (a) Leica HC PL APO 63x/1.2 Water motCORR CS2 and (b) the Leica HC PL APO 100x/1.4 Oil STED WHITE Objective. Scale bar is 10 µm. The corresponding STED image (right) taken at a depth of 30 µm within the gel. Scale bar is 2 µm. (**c**) Normalized intensity of the fluorescent signal as a function of depth within the gel. Signal decays linearly with depth using the oil objective, whereas the signal is maintained even to a depth of 30 µm using the water objective. (**d**) Comparison of the obtained STED resolution at a depth of 30 µm using both the oil and water objectives. STED resolution is improved by using the water objective, presumably because of the reduced spherical aberrations which better maintain the intensity distribution of the STED depletion doughnut. Error bars are standard deviations from the mean.

Figure 5. STFM applied to HeLa cell focal adhesions. (**a**) Representative fluorescent image of HeLa cell expressing paxillin-EGFP (green) adhering to a 40 kPa PAA gel coated with fibronectin and loaded with 40 nm red-fluorescent beads (red). Scale bar is 10 µm. (**b**) Zoom in of inset marked in (a). Scale bar is 2 µm. (**c,d**) Confocal (c) and (d) STED fluorescent bead image of gel area under the cell, before (cyan) and after (magenta) trypsin-EDTA treatment. Scale bar is 2 µm. Inset – Zoom in of dotted square region. Scale bar is 2 µm. (**e,f**) Traction magnitude calculated from the measured confocal (e) and STED (f) recordings of the bead displacements. Scale bar is 2 µm. Black dotted line represents cell boundary. (**g**) Line profiles showing the traction magnitude and the corresponding fluorescent intensities across the focal adhesions for STED (blue) and confocal (red) imaging. The dashed green line represents the fluorescence intensity distribution of paxillin.

- size - 25 nm
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-
-
-
-
-
-

-
-
- **10 %**
-
- **80 %**
- **1 ns – 6 ns**
-
-

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