Published in final edited form as:

Nat Protoc. 2017 July ; 12(7): 1437–1450. doi:10.1038/nprot.2017.042.

Interfacing 3D magnetic twisting cytometry with confocal fluorescence microscopy to image force responses in living cells

Yuejin Zhang^{1,2,#}, Fuxiang Wei^{1,#}, Yeh-Chuin Poh^{3,&}, Qiong Jia¹, Junjian Chen¹, Junwei Chen¹, Junyu Luo¹, Wenting Yao¹, Wenwen Zhou¹, Wei Huang¹, Fang Yang¹, Yao Zhang¹, and Ning Wang^{1,3,*}

¹Laboratory for Cellular Biomechanics and Regenerative Medicine, School of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, Hubei 430074 China

²School of Information Engineering, East China Jiaotong University, Nanchang, Jiangxi 330013, China

³Department of Mechanical Science and Engineering, College of Engineering, University of Illinois at Urbana-Champaign, Urbana, IL 61801 USA

Abstract

Cells and tissues can undergo a variety of biological and structural changes in response to mechanical forces. Only few existing techniques are available for quantification of structural changes at high resolution in response to forces applied along different directions. Three dimensional-Magnetic Twisting Cytometry (3D-MTC) is a technique for applying local mechanical stresses on living cells. Here we describe a protocol for interfacing 3D-MTC with confocal fluorescence microscopy. In 3D-MTC, ferromagnetic beads are bound to the cell surface via surface receptors followed by their magnetization in any desired direction. A magnetic twisting field in a different direction is then applied to generate rotational shear stresses in any desired direction. This protocol describes how to combine magnetic field-induced mechanical stimulation with confocal fluorescence microscopy and provides an optional extension for super resolution imaging using stimulated emission depletion (STED) nanoscopy. This technology allows for rapid real time acquisition of a living cell's mechanical responses to forces via specific receptors and for quantifying structural and biochemical changes in the same cell using confocal fluorescence microscopy or STED. The integrated 3D-MTC – microscopy platform takes around 20 days to construct and the experimental procedures require ~4 days when carried out by a life sciences graduate student.

*Send correspondence to: Dr. Ning Wang at nwangrw@illinois.edu.

&Current affiliation address: Semma Therapeutics, Inc., 450 Kendall Street, Cambridge, MA 02142 USA

#These authors contributed equally to this work.

Author contributions

NW conceived the project. NW, YZ, and FW designed the experiments and analyzed the data. YCP developed some analytical tools. YZ, FW, QJ, JJC, JWC, JL, WY, WZ, WH, FY, and Yao Z carried out the experiments. NW, YZ, and FW wrote the manuscript. All authors discussed the results and implications and commented on the manuscript at all stages.

Competing financial interests

The authors declare that they have no competing financial interests.

Keywords

cell stiffness; nuclear strains; force response; mechanotransduction; cell mechanobiology; 3D magnetic twisting cytometry; three-dimensional magnetic twist cytometry; 3D-MTC; 3D magnetic tweezers; three-dimensional magnetic tweezers; high resolution deformation imaging

INTRODUCTION

Development of the protocol

Cells and tissues constantly experience mechanical forces generated by both endogenous and exogenous sources. It is by now well-known that these mechanical forces can trigger signaling events that are critical for the proper functioning of living cells and tissues^{1–4}. Several methods have been established to apply mechanical forces to a living cell, including micropipette aspiration⁵, atomic force microscopy (AFM)^{6–8}, optical tweezers^{9–11}, optical stretchers¹², magnetic gradient tweezers¹³, and magnetic twisting cytometry (MTC)^{14–16}. Each of these techniques has its own strength and is most appropriate in applying and quantifying mechanical responses in certain conditions. MTC was originally developed to apply local shear forces to living cells via magnetic beads and to probe mechanical responses by quantifying the remnant magnetic fields (magnetic fields generated by the magnetized magnetic beads themselves after the external magnetic field is turned off) of the magnetic beads¹⁴. Later, MTC was combined with light microscope in order to apply shear forces in one dimension while probing the mechanical responses by optical recording of magnetic bead displacements¹⁷. In comparison to other existing methods, MTC can be used to apply forces with a wide range of force frequencies (the rate of force application) (0 to 1000 Hz) and to simultaneously probe many (> 100) cells, making it unique as a mechanical technique.

More than a decade ago, a three-dimensional MTC (3D-MTC) was developed in order to be able to apply forces and quantify mechanical responses in all three dimensions¹⁸. This technique was for instance used to reveal that actin bundles are critical for establishing the mechanical anisotropic behavior (i.e., the mechanical properties are dependent on the direction of the applied force) of an elongated cell¹⁸. 3D-MTC was originally designed to be used in combination with conventional widefield fluorescence microscopy, which limited its imaging spatial resolution due to out-of-focus fluorescence. To overcome this limitation and to significantly advance the applications of 3D-MTC using fluorescence microscopy, we developed an approach to interface and synchronize 3D-MTC with confocal fluorescence microscopy¹⁹. This allows for the quantification of dynamic structural changes in single cells in response to the applied forces. Here we describe a protocol on how to combine confocal fluorescence microscopy with a mechanical stimulation probe.

The development of super-resolved fluorescence microscopy such as stimulated emission depletion (STED)^{20, 21} and single-fluorophore microscopy (photo-activated localization microscopy (PALM))²² or STORM (Stochastic Optical Reconstruction Microscopy²³) has revolutionized light microscopy for observing dynamic processes occurring in a living cell. The diffraction-limited focal region, i.e., the spatial resolution of

ensemble-fluorophores in STED or of single-fluorophore in PALM, in theory has no lower limit. Although in practice the current optimal spatial resolution of either STED or PALM is ~20–70 nanometers^{24, 25}, it is still 3–10 fold better than the resolution (~200 nm) of the conventional light microscopy set by Abbe's limit. Spatial resolution enhancement may help identify molecule-molecule (such as protein-protein) structural interactions that would be impossible with conventional fluorescence microscopy. For example, STED is used to discover that *Drosophila* coiled-coil domain protein Bruchpilot (BRP) in donut-shaped structures is centered at active zones of neuromuscular synapses²⁶, not possible using the conventional confocal fluorescence microscopy. A recent paper²⁷ by Hell and colleagues reports a new method of MINFLUX that combines STED with PALM/STORM, minimizes the number of fluorescence molecules needed for high resolution imaging, and thus can localize single molecules to ~1 nm precision.

Hundreds of labs have access to widefield or confocal fluorescence microscopy and dozens of labs have built their own 1D-MTC or have purchased commercial 1D-MTC devices. This protocol can be used by these labs to interface 1D-MTC with the widefield and/or confocal fluorescence microscopy. Some labs are building or obtaining 3D-MTC. Since the ability to apply forces in any direction is important in studies of mechanotransduction and mechanobiology, we anticipate that our protocol will find broad adoption in the near future. In addition, we introduce an extension of the protocol to combine 3D-MTC with STED nanoscopy.

Overview of the Procedure

Here we outline the use of 3D-MTC to study the spatial and temporal mechano-chemical response within the cell nucleus. Magnetic beads coated with integrin-binding peptides are added to a tissue culture dish to allow the beads to firmly attach to the cell surface. After placing the dish on the microscope, a brief and strong magnetic impulse (2500 G, < 0.5 ms) is applied to magnetize the beads. Next, a twisting torque is applied to the magnetic beads by applying a homogeneous sinusoidal magnetic field perpendicular to the beads magnetic moment (Fig. 2). This magnetic torque causes the bead to rotate, thus deforming the cells. The cell shear modulus (stiffness) can be computed by quantifying the embedded area of the bead and the displacement of the bead (Fig. 3).

We developed a new user-friendly software source code that is responsible for controlling all the required operations of the system. The software was developed in C++ programming language and can be downloaded free of charge from <http://pan.baidu.com/s/1hrJlgss> and is named "Viewer_BeadsTracking_Sine_Square.zip". The confocal microscope manages the communication between the frame grabber and the camera, allows setting the parameters of image acquisition and storage. The system is easily customized according to the required experimental conditions. Images were acquired with a Hybrid Detector(<http://www.leica-microsystems.com/products/confocal-microscopes/details/product/leica-hyd/>) which was also responsible for transferring the image data. Since all our codes are open to the scientific community, for others to create a similar interface, we anticipate that it would only take ~20 days to integrate the hardware.

After data filtering, the maximum displacement of a magnetic bead without twisting fields is approximately 5 nm and the maximum displacement amplitude in the Fourier domain is only ~1 nm. The noise is extremely small relative to the stress-induced bead displacement on the cell that ranges ~30–600 nm, depending on cell types, various plating conditions, and substrate stiffness. Therefore, although different twisting frequencies might generate noise in the bead position, this noise is very low (the signal-to-noise ratio is between 6:1 and 120:1). This means that the noise from the microscope stage or other sources can be effectively ignored during the course of the experiment.

After the magnetic beads are magnetized and twisted, one can obtain synchronized bead displacements that are also sinusoidal (Fig. 3c). Using the cell stiffness calculation based on bead displacements, one could obtain cell stiffness values (Fig. 3d). As discussed earlier, care should be taken to avoid recording potential loosely-bound beads or non-magnetized beads. One could also quantify GFP-labeled histone 2B (H2B) displacement, which synchronizes with the stress applied via integrins (Fig. 4). H2B is one of the core histones where a segment of DNA wraps around to form nucleosomes. Therefore GFP-H2B can be used as a marker for chromatin. Alternatively, GFP-labeled H2A, H3, H4, or H1 can also be used as markers of chromatin. From the displacement maps, one strain maps can be computed (shear strains and 2D bulk strains) in response to force directionality (Fig. 5).

Comparison with alternative methods

Another way to probe single-cell mechanics is Atomic force microscopy (AFM). AFM uses a flexible cantilever with a sharp tip to approach the cell surface to apply forces to the cell. AFM can apply higher forces than MTC, but it can only probe one cell at a time. Moreover, because the cantilever is immersed in the cell-culture medium, it is not suitable for high frequency (>10 Hz) force application due to the dampening effects of the medium on the cantilever movement. Alternatively, aspiration techniques work by sucking the cell into a micropipette and are most useful for cells in suspension. Finally, an optical trap can be used to apply forces to single cells. The strength of the optical trap depends on the power of the laser. Most labs use optical traps to generate forces between 100 to 300 pNs. Since the application of higher forces relies on increased laser intensity, this can easily result in local heating problems and thereby cause potential damage to the sample. Each of the above mechanical probes (including 3D-MTC) has its own strengths and limitations depending on what kind of biological questions one wants to address.

3D-MTC can probe and quantify hundreds of magnetic beads in a culture dish in one run of the twisting field, making it much more efficient and time-saving than AFM or optical tweezers, which can only measure one cell at a time. Importantly, cell stiffness within a wide range of frequencies (up to 1000 Hz) can be measured using 3D-MTC^{28, 29}. This is a unique feature that is not easy to achieve with either AFM or optical tweezers.

When interfaced with confocal fluorescence microscopy (and STED nanoscopy, optional) (Supplementary Fig. 10), 3D-MTC can be used for quantifying cellular responses to forces applied at the cell surface via specific receptors. Our system is complementary to another recently published method wherein optical tweezers were integrated with STED nanoscopy³⁰ but can cover wide loading frequency ranges and complex forcing patterns in

all 3 dimensions for living cell applications. In addition, an optical tweezer can only probe one cell at a time, whereas 3D-MTC can probe hundreds of cells at a time. Acousto-optical or electro-optical deflectors have been used for 2D force application at high forcing rates³¹. While these alternative techniques have the advantage of being able to precisely place the probe at a pre-specified location on the cell surface over the 3D-MTC technique, these optical probes can only test one cell at a time and it is challenging to precisely control magnitudes of applied forces in the z direction. An optical device for trapping and rotating single molecules in 3D has also been developed³². Such an optical device could in principle be used to manipulate the Z direction in living cells at high forcing frequency, but this has until now not been reported.

Advantages and limitations

Combining 3D-MTC with confocal fluorescence microscopy has several advantages over conventional widefield microscopy approaches. Our approach supports a wide range of stress magnitudes (0–25 Pa), waveform (step function, sinusoidal, etc), and frequencies (0–1000 Hz), and 3D force directionality in living cells with improved displacement/deformation quantification over conventional widefield fluorescence microscopy¹⁸. The average shear stress on endothelial cells in the blood vessel is ~ 1 Pa, which can increase by several folds during exercise, even higher at some sites of the blood vessel in cardiovascular diseases. A step-function waveform can simulate a sudden force impact on living cells in vivo (e.g., head injury and concussion in a football match) and a sinusoidal waveform can simulate force patterns of the heart and of the respiratory system. A human heart beats at ~1 Hz at rest and ~3 Hz during exercise. Some maneuvers such as jumping or running can generate high impact forces on the human body up to many dozens of Hz. 3D-MTC is useful in that it can cover ranges of these parameters in physiology and in diseases. We have currently only interfaced 3D-MTC with a Leica SP8 confocal microscope. We therefore recommend users of different systems to review the software development kit (SDK) from their microscope manufacturer. It is important to note that the coils are heavy and thus limit the speed of the Z-height scanning when they are placed on the stage of the confocal microscope. In addition, confocal microscopy using an oil-immersion objective that limits the accessible Z-range to ~100 μm .

Using high NA oil/water immersion objectives could also pose a large heat-sink that dominates the local temperature in the sample. It should be noted that heat production by the coils can result in overheating of the setup when the coils operate at high frequencies (>100 Hz) with strong magnetic fields (>50 Gauss). This might limit the continuous magnetic field application to ~10 minutes and the magnetic field needs to be turned off for a few minutes to allow for coil cooling before another round of magnetic field application can be started. 3D-MTC has an auto temperature controller that applies a flow of 37° C air to mitigate potential local heating problems.

A limit of our system is the frame transfer time between the HyD and the confocal computer. When the frame transfer rate is below the sampling rate (for acquisition of a 512 pixel $\times$ 512 pixel image, the maximum sampling rate is 1 image per sec), it will not be possible to track the movement of the magnetic bead. To reduce the acquisition time we have

used a strategy for image acquisition. For reconstructing a period, a software is written that calculates the frame rate allowed by the frame transfer time and the minimum number of periods, n , in order to satisfy the requested frames, m , per period, and the proper trigger, together with the time shift of each period. Using this strategy, we find that the acquisition time is reduced by a factor equal to $(m-n)/m$, as multiple points for the period are acquired, with no missing data.

The magnetic bead generates rotation, resulting in x -, y - and z -displacements. To measure the z -displacement, one must take multiple z -scans, and then calculate the obtained Z -deformation. This is doable, but it means that one must apply forces for many more cycles compared to when just one z -height scan is used. Currently, we only take images at one focal plane or one Z -height, in order to get rapid real time images of deformation induced by the magnetic bead. Therefore, the deformation analysis that we performed to obtain the strain maps is an underestimate of the actual deformation.

Because the cultured CHO DG44 or B16-F1 cells are generally very spread, the observed Z -displacement is much less than X - or Y -displacements. However, if rounder cells are used the z -displacement can be very significant and must be taken into account for calculating the total (x - y - z) displacement and the resulting strains. This is not incorporated in the current protocol and could be included in the future.

With 3D-MTC, the cell stiffness that is measured via an individual magnetic bead on a particular cell varies from cell to cell. Since the position of the bead on a specific part of the cell cannot be precisely controlled, different stiffness values will be reported due to for instance the heterogeneity of a living cell. AFM on different positions on the cell surface has revealed such regional differences in cell stiffness^{33–35}. Other contributions to cell-to-cell variation in measured cell stiffness include variation in cell-matrix adhesion, differences in cell spreading and/or tractional forces, and differences in the cell cycle stage. Some of these differences can be mitigated or minimized by carefully controlling the chemical environments of the cell, cell adhesion, plating time, and spreading areas (e.g., use micropatterned surfaces).

In this study, we have only extended the protocol by integration of 3D-MTC with STED, as we do not have access to the PALM or STORM techniques. It will be interesting to see if 3D-MTC can be integrated with PALM/STORM or with the new MINFLUX method in the future. However, to obtain super-resolution using PALM/STORM, only a sparse set of fluorophores is excited at a given time in order to determine their accurate position. In order to gather sufficient data, the long calculation times associated with this approach may complicate the rapid real time capturing of the displacements in response to the mechanical signal.

Applications of the method

Experimental findings from several laboratories over the last two decades have shown that 3D-MTC is robust, reliable, fast, and convenient to quantify cell stiffness changes. Cell stiffness can be quantified in individual cells both before and after adding a soluble chemical factor (e.g., a growth factor, a cytokine, or a drug) to the culture dish^{36–41}. 3D-MTC can be

interfaced with confocal fluorescence microscopy (or STED), as discussed in this protocol. This allows for the quantification of cell stiffness and strain mapping in response to force directionality and in improving resolution of deformation mapping in a living cell. The 3D-MTC technology can be combined with other single cell assays, such as fluorescence resonance energy transfer (FRET) or FISH (fluorescence in situ hybridization). The combined platform can quantify intracellular enzymatic activities (such as Src and Rac^{42–44}), measure the dissociation of intranuclear protein-protein complexes⁴⁵, and assess changes in gene expression¹⁹ in response to forces applied via integrins.

Experimental Design

We present a detailed procedure to accurately validate the system, in order to standardize its calibration procedure and to obtain quantitative and reproducible data. Interfacing and synchronizing the confocal fluorescence microscope with the state-of-art biophysical technologies that can apply precisely controlled mechanical stimuli will greatly enhance the capacity of the integrated system in imaging, quantifying, and understanding the complex physiological responses and behaviors in a living cell.

Establishing 3D-MTC and confocal fluorescence microscopy hardware—3D-MTC and the confocal fluorescence microscope hardware (Fig. 1) includes an inverted confocal microscope with a HyD photon counter, an anti-vibration table, a magnetic field generator, a 3D-MTC, a 3D-MTC personal computer (PC) and a confocal PC (Supplementary Fig. 1). The Leica HyD combines the advantages of photomultiplier tube (PMT) and avalanche photodiodes (APD) as described in <http://www.leica-microsystems.com/products/confocal-microscopes/details/product/leica-hyd/>. The software (file name, “Viewer_BeadsTracking_Sine_Square.zip”, can be downloaded at <http://pan.baidu.com/s/1hrJlgss>) that is installed in 3D-MTC PC controls the operations of the entire system, using high-level functions to communicate with the confocal PC to manage image acquisition. It also communicates with the DAQCard-6713 (NI PCI-6713 analog output board with NI-DAQ 8 analog outputs) signal generation board. This specifies the frequency and amplitude of the sinusoidal wave signal generated by SCB-68-A used to drive the twisting coils through the voltage controlled current source (Fig. 1b). The signal generation board is also responsible for generating the requested signals from SCB-68-A into DAQCard-6052E (NI PCI-6052E multifunction I/O with NI-DAQ)-A, in order to synchronize image acquisition by means of an external trigger.

The signal detection and data processing systems are the core of the whole system that includes main functions like data acquisition, receiving and sending control instruction, data storage, data transmission, data processing, and implementing the user-machine interaction. The software of the synchronous system controls the execution of the synchronization module. The HyD is connected to the objective and is controlled by the confocal PC. The DAQCard-6713 is employed to satisfy the timing constraints of the system, since a computer operating system cannot guarantee the real time execution of the firmware. Additional advantages are that this setup is low cost and easy to implement. The DAQCard-6713 board generates the sinusoidal analog voltage signal to induce the oscillatory magnetic field (0.001 Hz–1000 Hz, 1–75 Gauss). HyD utilizes the digital trigger signal to acquire images at a

specified time. A DAQCard-6052E-A board captures the sinusoidal wave and the corresponding trigger signal. From the values set in the DAQCard-6713 timer registration, a software calculates the exact wave frequencies and trigger periods. Then a set of signals from SCB-68-A is generated by the DAQCard-6713 through the 3D-MTC after the coils of 3D-MTC were positioned on the microscope stage. The coils are fed with voltages to generate magnetic fields at different intensities. The current flowing through the coils is simultaneously acquired by the DAQCard-6052E-B from SCB-68-B. All the above procedures are performed automatically without user intervention.

The experimental setup shows beam paths and crucial components. For fluorescence imaging, the laser system supplies excitation beams (514 nm, 496 nm, 488 nm, 476 nm, and 458 nm, respectively, filtered from an argon laser using an acousto-optical tunable filter (AOTF)) (Fig. 1b). A STED depletion laser (592 nm) can be added and used as optional to improve lateral spatial resolution. The module diagram of the system is depicted in Supplementary Fig. 2. 3D-MTC has an auto temperature controller that applies a low flow rate of 37° C air to mitigate the potential local heating problem.

Development of image acquisition software in the 3D-MTC PC with the Leica software development kit

The Leica software development kit (SDK) provides the user interface and contains functional modules for software development. In the SDK, the interface, the binary code, the related device drivers, and the configuration resources all ensure that experimentalists can control HyD image acquisition by using other programming languages and software platforms. The SDK includes drivers, a dynamic link library, a static input database, interface declarations (these are names of various components of the SDK), and debugging software. The installed HyD image SDK includes Bin, C++, Include, Lib, Samples, Docs, and the Proxy bin file folder. In order to install the SDK of the Leica system into the PC, one needs to include the header files in the development system for providing the interface declaration, importing the static input database and copying the dynamic link library to the executing program directory.

Interfacing 3D-MTC with confocal microscopy—A confocal personal computer (PC) is used to link the 3D-MTC-PC to the HyD photon counter. The static input database helps to link the dynamic link library during compiling. The client application program uses the SDK to control the process of the HyD image acquisition by calling the relevant functions through the interface declaration, compiling through the static input database, and importing the real functions through the dynamic link library, and then to complete all the operations.

The confocal microscope embedded part of the 3D-MTC is composed of 3 pairs of coils, X, Y, and Z directions (Supplementary Fig. 2b). One of Z direction coils is positioned below the microscope's stage and the objective lens is located inside the Z coils. Each of the coil pairs can generate magnetizing and twisting fields independently. Each of the coils is wound by a Helmholtz coil with 180 turns and generates an inductive strength of 1.43 mH. The magnetic field strength of the coil at the center plane is about 1.82 mT/A. The coils have a 16 F capacitor that is used to store energy for magnetization, and the highest voltage of the

capacitor can reach about 2.4 kV. When the coils are charged with magnetization, the coils generate a 0.5 ms pulse magnetic field to magnetize the magnetic beads, the strength of the magnetic field is 2500 Gauss, resulting in the permanent magnetization of the beads. The magnetic coils can guarantee a bandwidth of about 1 kHz and the maximum twisting field is 75 Gauss, which is large enough to exert physiologically-relevant local stresses to the cell surface through the magnetic bead but still small enough to prevent re-magnetization of the bead.

Beads Tracking program—The following task is executed automatically without user intervention. The first approximation value of x coordinates of the center of the magnetic beads can be defined as:

$$\bar{x} = \frac{\sum_{k=1}^n x_k I_k}{\sum_{k=1}^n I_k}$$

Where x_k and I_k are x coordinate values and the inversion saturation with n pixels window of the k th term pixel value; the results of x are of sub-pixels. The bright-field image is inverted into a gray image. The magnetic bead has the biggest gray scale in the image. The threshold is adjusted to determine the position of the center of mass of the magnetic bead. The Matlab program is used to track the spontaneous bead displacement and determines it to be ~5 nm. The inversion saturation pixel makes dark pixels be more than light colored pixel values. The specific method for calculating the inversion saturation is shown as: the largest pixel saturation subtracts the original pixel saturation value; the maximum pixel saturation can be calculated as: $I_{max} = 2^N - I$, where N is the bits of image, the typical value is 8 or 16 bits. Once the first approximation of the x coordinates in the center is found, the moving window center will move to this point, the new window position calculation is continued by the same algorithm, x coordinate center of magnetic beads is calculated. The iterative process is repeated, till no longer moved or have reached the maximum number of iterations, which is allowed in the iterative process (usually 3 times). The magnetic bead tracking software interfaces for magnetic bead image on screen. The magnetic bead with a “+” symbol is the recognized magnetic bead (Fig. 3b) through screening identification algorithm. This recognized bead can be differentiated from other magnetic beads that do not have the “+” symbol, which are either due to the diameter is smaller than the set value, or because the distance between each other is less than single magnetic bead diameter; thus they cannot be selected.

The Beads Tracking program is used to control the HyD and to collect the original image. The software program can automatically locate and record the positions coordinates (x , y) of the magnetic beads in text format, the contents of output text file include experiment running time, current amplitudes and frequencies of the twisting field.

The user sets the parameters using the magnetic bead tracking software. Parameters such as the magnetic bead center and the background pixel threshold, and the distance between the beads are calculated. Under normal circumstances, the minimum distance between the magnetic beads should not be less than the diameter of a single magnetic bead. In other

words, two or more beads that aggregate together will not be tracked by the Bead Tracking software. This way, the potential errors which occur as a result of two or more too-closely-associated beads (due to their remnant fields and the magnetic forces between the two beads) that interfere with effects of the external twisting field can be eliminated or significantly reduced.

In a typical magnetic twisting experiment, using a 10× objective, the Bead Tracker software can keep track of 100–300 magnetic beads simultaneously (depending on cell plating density and how many beads one would like to have on a cell). When a 10× objective is used, each pixel is ~636 nm. The diameter of a magnetic bead is ~4.5 μm and it can span ~7 pixels. With a 10× objective, the field of view of the microscope for bright-field imaging is 1520 μm by 1520 μm. We used a low magnification 10× objective to find the cell of interest that has a magnetic bead on its surface. The 10× lens can be also used to obtain mechanical stiffness values of a larger number of cells (~100–150 cells per field of view).

During the process of rotation, the center of the magnetic bead undergoes lateral translation in the x-y plane and a typical translation magnitude is between 30–600 nm, which is about 0.05–1 pixels. Hence the sub-pixel algorithm for bead location with a high signal-to-noise ratio (SNR > 3; the background noise is < 5 nm) records the coordinate values of each magnetic bead.

Matlab program for Beads Tracking analysis—Matlab software (file name: “Matlab_BeadsTracking_analysis_Program.zip”, can be found at <http://pan.baidu.com/s/1qXCGozy>) is used to read the magnetic bead position coordinates file and to filter out unusually large translation of the bead center (for example from a loosely bound or unattached bead). The lateral translation of the magnetic bead is used to calculate the cell stiffness as described previously⁴⁶. In short, the Matlab code eliminates the drift error caused by the microscope stage and then calculates the cell stiffness. The lateral magnetic bead translation it is converted into the cell complex modulus (a quantitative measure of stiffness of the material taking into account both elastic and viscous contributions; its unit is Pa).

MATERIALS

REAGENTS

- Ferromagnetic beads (Fe₃O₄, ~4.5 μm in diameter; purchased from Spherotech, Inc., Lake Forest, IL; Catalog Number: CFM-40-10)
- Histone 2B-GFP plasmid (Life technology; Catalog Number: C10594)
- Dimethyl sulfoxide, sterile-filtered (DMSO; Sigma; Catalog Number: D2438)
- Fetal bovine serum (FBS; Invitrogen; Catalog Number: 10099141)
- Glass bottom dishes (35 mm; Cell E&G; Houston, TX; Catalog Number: GBD00001-200)
- HEPES buffer (100 mM, Gibco; Catalog Number: 15630080)

- Murine melanoma cell line B16-F1 (American Type Culture Collection)
CAUTION: The cell lines used in one's research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.
- CHO (Chinese hamster ovary) DG44 cell line (provided by Dr. Andrew S. Belmont, University of Illinois at Urbana-Champaign, Urbana, IL)
- Ham's F12 media without thymidine and hypoxanthine (Hyclone; special order)
- Lipofectamine 2000 (Invitrogen; Catalog Number: 11668019)
- Opti-MEM medium (Gibco; Catalog Number: 51985042)
- TrypLE (Invitrogen; Catalog Number: 12604021)
- Penicillin-Streptomycin (Gibco; Catalog Number: 15140122)
- MEM medium (HyClone; Catalog Number: 12571063)
- Phosphate-buffered saline (PBS; HyClone; Catalog Number: 10010023)
- Sodium bicarbonate (NaHCO_3 ; Sigma; Catalog Number: S5761)
- Sodium carbonate (Na_2CO_3 ; Sigma; Catalog Number: S2127)
- Synthetic RGD-containing peptide (Ac-G(dR)GDSPASSKGGGGS(dR) LLLLLL(dR)-NH₂, Peptide-2000 (Telios)⁴⁷; Sigma-Aldrich, St. Louis, MO; Catalog Number: A0472)

REAGENT SETUP

Magnetic Beads—Place dry Beads in 95 % ethanol to a concentration of 5 mg/ml for sterilization and then store them at 4 °C for years. CRITICAL Immerse the magnetic beads in 75% alcohol for 8 hours and then wash and dilute them in PBS or culture medium before use.

Carbonate Buffer—Resuspend 159 mg Na_2CO_3 and 293 mg NaHCO_3 in 80 ml distilled water (0.05 M; pH=9.4).

RGD solution (5 mg/ml)—Dissolve 0.5 mg RGD in 100 μl DMSO. (Can store in 4°C for 6 months.)

Culture Medium for B16-F1 cells—Supplement B16-F1 medium with 10 % (vol/vol) FBS, 0.1 mM Penicillin, 0.1 mM Streptomycin and 2 mM L-Glutamin. (Can store in 4°C for 2 months.)

Transfection Medium for B16-F1 cells—Supplement B16-F1 medium with 2 mM L-Glutamine and 10 % (vol/vol) FBS. (Can store in 4°C for 2 months.)

Culture Medium for CHO DG44 Cells—Supplement Ham's F12 media (without thymidine and hypoxanthine) with 10 % (vol/vol) FBS, 0.1 mM Penicillin, 0.1 mM

Streptomycin and 2 mM L-glutamin (HEPES buffer can be added to create CO₂ independent medium for long duration (a few hours) experiments).

EQUIPMENT

- 10× 0.22 Numerical Aperture (N.A.) air objective (Leica; Catalog Number: 15506285)
- 63× 1.4 N.A. oil-immersion objective (Leica; Catalog Number: 15506350)
- 100× 1.4 N.A. oil-immersion objectives (Leica; Catalog Number: 11506328)
- HyD photoncounter (Leica; Catalog Number: 158001401)
- Leica SP8 Confocal Microscope (STED, optional) (Leica)
- Matlab (Mathworks; Version 8.01)
- 3D-MTC device¹⁸ (Commercially available via special order from EOL Eberhard, Obervil, Switzerland)
- A Dell TM T3500 PC (32-bit operating system, support C++) connects to 3D-MTC and a confocal PC with the confocal microscope (and STED, optional). Note that any workstation better than or similar to Dell TM3500 can be used.

PROCEDURE

Connect 3D-MTC with the confocal microscopy TIMING 20 days

- 1** Install the connecting box NI (National Instrument) PCI 6052E-B and the multifunction input NI PCI 6713 data collection board in the Dell TM T3500 PC, install the drivers of them.
? TROUBLESHOOTING
- 2** Two NI SH68-68-EP shielded cables connect to the cards with two connecting boxes NI SCB-68-A and NI SCB-68-B block connectors separately.
? TROUBLESHOOTING
- 3** Six BNC shielded cables connect with 3D-MTC and the connecting box SCB-68-A connectors separately (three input, three output).
- 4** The multifunction input NI PCI 6052E-A synchronous connects the Leica SP8 confocal microscope with the 3D-MTC PC through the NI SCB-68-B board.
- 5** A microscope insert with three pairs of independently controlled coils to magnetize and twist the magnetic beads, Z axis coils diameters are 51 mm, X and Y axis coil diameter are 41 mm.
? TROUBLESHOOTING
- 6** The Dell TM T3500 PC (32-bit operating system, support C++) controls magnetizing and twisting fields.
? TROUBLESHOOTING

Coating Magnetic Beads with RGD (or other ligands) TIMING 1 day—CRITICAL

Steps 7 – 9 are performed in a sterile laminar flow hood.

- 7 Transfer 200 µl of magnetic beads (See Reagent Setup) into a 1.5 ml microfuge tube.

? TROUBLESHOOTING

- 8 Wash the beads by adding 1.5 ml PBS, re-suspend the beads, and centrifuge them at 4000 rpm (1500g) for 1.5 min in a microfuge. Aspirate the supernatant.

- 9 Re-suspend the beads in 1 ml Carbonate Buffer and add 10 µl of the RGD peptide (5 mg/ml stock concentration) solution..

- 10 Mix the beads with RGD peptides in a shaker at 4 °C under continuous rotation at 0.5 Hz.

PAUSE POINT The beads can be used immediately or stored at 4 °C for up to two weeks.

Cell Culture and Transfections TIMING 3 days—GFP-H2B CRITICAL

Steps 11 – 15 are performed in a sterile laminar flow hood.

- 11 Culture B16-F1 cells (~300,000 cells) on glass-bottom culture dish (35 mm dish) in Transfection Medium.

CRITICAL STEP For CHO DG44 cells, most steps are similar to those for B16-F1 cells but using the cell culture medium for CHO cells as described in the Reagent Setup.

- 12 Dilute 1 µg of the H2B plasmid in 200 µl Opti-MEM I medium in a 1.5 ml microfuge tube and mix thoroughly.

- 13 Add 6 µl Lipofectamine 2000 to 200 µl Opti-MEM I medium in a 1.5 ml microfuge tube, and incubate for 5 min at room temperature.

- 14 Mix the Lipofectamin 2000 solution (from step 17) with the H2B plasmid solution (from step 16), and incubate for 20 min at room temperature.

- 15 Add 400 µl Lipofectamine-DNA complex solution to the B16-F1 cells.

- 16 Incubate the cells at 37 °C in 0.5 % CO₂ for 24 to 48 h (depending on transfection effectiveness of fluorescent probes) before used.

3D-MTC experiment TIMING 1 h

- 17 On the day of the experiment (24–48 hr after transfection of fluorescent probes), aspirate 90% of the culture out of the culture dish but leave some medium to protect the cells from drying out.

- 18 Gently pipette out 20 µl of the bead-containing buffer from step 9 and add the beads (20 µg) to the dish center; make sure that the beads are scattered more or less uniformly in the dish center.

- 19 Return the the cell dish back into the incubator and leave it there for at least 20 minutes to allow for sufficient time for integrin clustering and focal adhesion formation around the beads.
- 20 Take the cells out of the incubator and rinse them once with PBS in order to remove loosely-bound or non-attached beads.

CRITICAL STEP Avoid disturbing cells in the center well. Add and remove PBS gently from the side of the dish.
- 21 Add CO₂ independent medium to the dish to maintain the pH of the cell culture while imaging.
- 22 Place the dish in the 3D-MTC stage close to the coils. Then place the stage on the inverted microscope.
- 23 Find a single cell that shows high GFP-H2B expression (since transfection effectiveness varies from cell to cell, only the cells that express enough H2B-GFP (defined as fluorescence intensity is bright enough for viewing when 17% fluorescence excitation power is used) are good for quantitation of force-induced deformation).
- 24 Magnetize the magnetic beads with a strong (2500 Gauss at 0.5 ms) magnetic pulse.
- 25 Turn on a weak and long (1–75 Gauss for various durations) sinusoidal (or other waveforms such as a step-function or a ramp function, depending on particular biological questions to be addressed) twisting field in an orthogonal direction to the magnetizing field. These magnetic beads are ferromagnetic that maintain their magnetic moment after being magnetized, similar to a compass. When a twisting field is applied, this field interacts with the bead magnetic moment to generate a rotational movement to produce shear stresses between the bead and the cell surface (Fig. 2).

? TROUBLESHOOTING

- 26 Use the magnetic bead position tracking software (Beads Tracking) to track the bead positioning through a center-of-mass algorithm. This software also pre-sets a forcing frequency according to the experimental requirements (Fig. 3a, b). In addition, the twisting field amplitude and duration can also be pre-set. The software is able to pre-set many cycles of the twisting field at various frequencies in such a manner that the twisting magnetic field can go from low frequency (0.001 Hz) to high frequency (1000 Hz) and vice versa.
- 27 Capture the necessary bright-field or fluorescence images while the force is being applied.

Matlab program user manual for stiffness analysis TIMING 1 day

- 28 Run the “Bead_mito_finer.m” software in Matlab. Specify the address of your data: pathname = “location of your data”.

? TROUBLESHOOTING

- 29** Specify the parameters, including “bead_diameter” (bead size), c (magnetic constant of the bead), H (magnetic field), “gauss_per_volt” (3D-MTC parameter), “ $\mu\text{m\_per\_pixel}$ ”.

? TROUBLESHOOTING

- 30** Click “Run” tab and select “add to the path”, and load the images.
- 31** Select the beads you want in the image with red markers by clicking them with the left mouse key.
- 32** After selecting the beads, double click with the left mouse key somewhere randomly or the space bar and the program will run automatically.
- 33** Go to the folder of the original images and open the excel file named “Cell_XXX_select_beads”. This excel file contains three sheets, called G^* , G' , and G'' . Basically, it is $G^* = G' + iG''$. As defined here, G^* is the apparent complex modulus, G^* has the unit of Pa/nm, G' is storage modulus (the real part of G^*), and G'' is loss modulus (the imaginary part of G^*) (see Cell stiffness measurement in Step 37–44).
- 34** Multiply G^* , G' , or G'' by a conversion factor for quantifying the exact stress to get the complex shear modulus, storage shear modulus, or loss shear modulus: the conversion factor = $R/(6\beta)$, where R is the bead radius (μm), β depends on the bead embedment²⁹.
- 35** Through the measured conversion coefficient, each magnetic bead movement is converted into nanometer displacements. When the band-pass filter is taken, the cut-off frequencies is $0.44 f_T$ and $1.78 f_T$ (f_T is characteristic frequency of the band-pass filter). The band-pass filter can efficiently eliminate the noise of the microscope drift and the motion produced by the cell itself.

? TROUBLESHOOTING

- 36** Calculate the modulus measured with each magnetic bead at the specified twisting frequency.

Cell stiffness measurement TIMING 2 h

- 37** Immerse the beads in a viscous standard, apply a constant magnetic twisting field, and measure the bead rotation/displacement, in order to calibrate the bead magnetic moment constant. Calculate the applied stress (in Pa) from the applied twisting magnetic field (in Gauss). Multiply the bead constant (in Pa/Gauss) with the applied twisting field. For example, a 25 Gauss applies 15 Pa of stress to the cell if the bead constant is 0.6 Pa/Gauss.
- 38** Localize under the microscope a cell with a single bead bound to its apical surface.
- 39** Apply an oscillatory stress of 0.1 Hz using the 3D-MTC as shown in Fig. 2.

- 40 Use the 3D-MTC software to track the displacement coordinates of the magnetic bead and saves them in a text file.
- 41 Calculate the cell complex shear modulus (cell stiffness) by quantifying the magnetic bead displacement and the bead-cell contact area. For equations of quantifying cell stiffness, see Supplementary Information.
- 42 Only select the beads whose displacement waves are synchronized to the input sinusoidal signals. This is to filter out spontaneous movements of the beads or microscope stage shifts.
- CRITICAL STEP Do not select the beads with displacements less than 5 nm (possibly due to the fact that the beads are not magnetized) and loosely bound beads for analysis. To increase the signal to noise ratio, average the peak amplitude of the displacement over 5–7 consecutive cycles for each cell.
- 43 Calculate the apparent complex shear modulus using the equation $G^* = T/d$. For each bead, calculate the elastic shear modulus G' (the real part of G^*) and the dissipative shear modulus G'' (the imaginary part of G^*) based on the phase lag. The measured apparent modulus has the units of torque per unit bead volume per unit bead displacement (Pa/nm).
- 44 Find the β value from a published report⁴⁶. The finite element analysis model is used to convert the apparent cell modulus (Pa/nm) to cell complex modulus (Pa) (or cell stiffness) based on the bead to cell surface contact area⁴⁶. The bead-cell contact area can be estimated by staining any focal adhesion protein (e.g., vinculin or talin). Then one can use the estimated bead embedment area to determinethe value of β from ref. 46 (Fig. 3).

Assessing the signal to noise ratio TIMING 1 h

- 45 Set twisting frequencies at 0.3, 0.5, 3, 10, 100, and 1000 Hz respectively; each frequency lasts 3–15 cycles.
- 46 Average the twist-induced synchronized signals at certain frequency equal to or greater than 3 cycles can increase the signal to noise ratios by averaging out non-synchronized noises. For example, a magnetic bead was stressed at 15 Pa and 0.1 Hz; the bead displacements were quantified after averaging the synchronized displacements over 7 cycles (Fig. 3c) and the cell stiffness (complex modulus) was computed (Fig. 3d); these data show that the cell appears to be stiffer along the long axis of the cell (Fig. 3d), consistent with the data from a published report that tensed actin bundles along the long axis of the cell are a major contributor to this behavior¹⁸.
- 47 Focus the objective lens on the magnetic bead and adjust the fine-tuning of Z-axis steps to increase the accuracy of the bead displacement quantitation.
- 48 Alter the twisting duration at a fixed frequency (e.g., at 0.1 Hz). Set the number of twisting cycles to 4, 8, 16, 32, or 64, in order to compare the signal to noise ratio at various numbers of cycles.

Displacement and strain maps analysis TIMING 12 hr

- 49 Record multiple fluorescent images of molecules (e.g., GFP-H2B) of a live cell at various time points during a cyclic force application.⁵⁰ Input the file names of the fluorescent images and compute the displacement maps with a software (file name: “Mito_amp_clean.m”) that uses an image correlation method⁴⁸.
- 51 Compute the strain (both bulk and shear strain) maps from the fluorescent images with a software (file name: “fttc_mito_NUC_arrowmap_gradient.m”) that compute strains using derivatives of displacements⁴⁹.

Box 1 (Optional) Super-resolution imaging.

All self-written C++ and Matlab software is freely available to all interested researchers (link: <https://pan.baidu.com/s/1hrJlgss>; <https://pan.baidu.com/s/1qXCGozy>). Two-tailed Student’s t-test was used for statistical analyses, with Bonferonni corrections when appropriate.

TROUBLESHOOTING

Troubleshooting tips can be found in Table 1.

TIMING

Steps 1–6, 3D-MTC interfaces with the confocal microscope (and STED, optional) system: 20 days

Steps 7–10, Coating Magnetic Beads with RGD (or other ligands): 1 day

Steps 11–16, Cell Culture and Transfections: 3 days

Steps 17–27, 3D-MTC experiment: 1 hour

Steps 28–36, Matlab program user manual for stiffness analysis: 1 day

Steps 37–44, Cell stiffness measurement: 2 hours

Steps 45–48, Determination of the signal to noise ratio: 1 hour

Steps 49–51, Determination of displacement and strain maps: 12 hours

Box 1 (Optional), Super-resolution imaging: 1 hour

ANTICIPATED RESULTS

Cell stiffness measurement

The 3D-MTC can be used to apply force stimulation via integrins at the cell surface and one can quantify intranuclear structural deformation. For example, a Arg-Gly-Asp (RGD)-peptide-coated magnetic bead was bound to cell adhesion molecule integrins on the cell surface and a stress of 15 Pa was applied at 0.1 Hz to a living cell that was transfected with histone 2B (H2B)-green fluorescent protein (GFP) as a marker for DNA (Fig. 4). When no stress was applied, the spontaneous displacement of GFP-H2B was very small (Fig. 4a; for a displacement map quantification method, see Supplementary Fig. 3). Displacement maps are generated by comparing the x-y coordinates of fluorescent signals of H2B in the two images

of the same nucleus at two different time points. When stress was applied along the long axis of the cell (magnetizing along Z-axis and twisting along Y-axis), a displacement map that was synchronous (i.e., the displacement signals have the same frequency and waveform as the input stress) with the input stress was obtained by averaging 7 cycles of the signals (Fig. 4b). In contrast, as the stress of the same amplitude and the same frequency was applied along the short axis of the same cell (magnetizing along Z-axis and twisting along X-axis), a different displacement map was generated (see the enlarged image that show displacements in different directions) (Fig. 4c). From these displacement maps, one could calculate strain maps of 2D bulk strains (tensile and compressive strains) and of shear strains (Fig. 5). When a force is applied to a cell, it can cause strains (computed from derivatives of displacements) on cellular structures. Some structures are stretched (tensile strains) and some are compressed (compressive strains), while some are sheared (shear strains). It appears that the peak bulk strains were 4 times higher than the peak shear strains (~8 % vs ~2 %) (note the scale in bulk strain maps is different from that in shear strain maps). From the bulk strain maps, one can also see that some parts of the chromatin are compressed (blue color) while some are stretched (red color). Importantly, when the stress angle was 90°, i.e., when the stress was applied along the short axis of the cell (90°), the GFP-H2B strains were higher than those when the stress was 0° (compare Fig 5a with Fig. 5b; note that 5b is more reddish and yellows than 5a, suggesting higher strains). These data suggest that the actin bundles along the long axis of the cell determine the anisotropic response of the cell¹⁸ and as a result the nuclear chromatin strains were smaller, consistent with what we found recently¹⁹. At a given applied stress amplitude, anisotropic displacement maps, cell stiffness, and strain maps of the nucleus were also quantified when the stress angles were varied at finer steps, i.e., at 0°, 36.9°, 45°, 53.1°, and 90° relative to the long axis of the same cell (Supplementary Fig. 4 and Fig. 5). These findings indicate the versatility of the 3D-MTC to provide mechanical stimulation at the cell surface in its ability to easily and precisely vary force directions.

Combining 3D-MTC with super-resolution imaging

To examine the utility of combining 3D-MTC in higher detail, we compared the displacement map of the GFP-H2B in the nucleus of the same living cell generated via 3D-MTC with confocal microscopy to STED nanoscopy. In addition to the increased GFP-H2B spatial resolution provided by STED nanoscopy, the displacement map of the same area in the same cell was also improved when the same stress was applied (compare Supplementary Fig 6a with Fig. 6b). We used the Full Width Half Maximum (FWHM) of the point spread function (PSF) to calculate the spatial resolution. The resulting spatial resolution of H2B using confocal microscopy is 251 nm, while it is increased to 118 nm for STED nanoscopy, slightly below the 70-nm resolution set by Leica for fix samples. A STED microscope employs the CW phase pattern and 592 nm excitation laser; scanning method is beam scanning. We used our STED nanoscope with a beam scanning speeds of 1000 Hz throughout the experiments. We obtained better spatial resolution at higher power of the depletion laser (90%) compared to lower powers (30%) (Supplementary Fig. 7), without observing detectable bleaching for short periods of exposure (Supplementary Fig. 8). However, continuous imaging for many minutes of the same region can result in bleaching and should be avoided. We also examined the potential impact of the high intensity-focused

STED depletion beam on the magnetized bead. In the absence of the magnetic twisting field, when the STED depletion laser beam was turned on, we observed no optical manipulation of the magnetized bead or displacements of the fluorescence H2B (Supplementary Fig. 9).

In summary, we anticipate that this interfaced system and its protocol will be useful to the fields of high-resolution imaging and mechanobiology where structural alterations and biochemical changes can be quantified at nanoscale resolution in response to physiologically relevant forces.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Youhua Tan, Mr. Shuang Zhang, and Miss Haiying Yi for technical assistance. This work was supported by NIH grant GM072744, Ministry of Science and Technology of China grant 2016YFA0101100, and funds from Huazhong University of Science and Technology.

References

1. Discher DE, Mooney DJ, Zandstra PW. Growth factors, matrices, and forces combine and control stem cells. *Science*. 2009; 324:1673–1677. [PubMed: 19556500]
2. Wang N, Tytell JD, Ingber DE. Mechanotransduction at a distance: mechanically coupling the extracellular matrix with the nucleus. *Nat. Rev. Mol. Cell. Bio.* 2009; 1:75–82.
3. Wozniak MA, Chen CS. Mechanotransduction in development: a growing role for contractility. *Nat. Rev. Mol. Cell. Bio.* 2009; 1:34–43.
4. Humphrey JD, Dufresne ER, Schwartz MA. Mechanotransduction and extracellular matrix homeostasis. *Nat. Rev. Mol. Cell. Bio.* 2014; 12:802–812.
5. Evans EA, Hochmuth RM. Membrane viscoelasticity. *Biophys. J.* 1976; 1:1–11.
6. Henderson E, Haydon PG, Sakaguchi DS. Actin filament dynamics in living glial cells imaged by atomic force microscopy. *Science*. 1992; 257:1944–1946.
7. Hoh JH, Schoenenberger CA. Surface morphology and mechanical properties of MDCK monolayers by atomic force microscopy. *J. Cell Sci.* 1994; Pt 5:1105–1114.
8. Matzke R, Jacobson K, Radmacher M. Direct, high-resolution measurement of furrow stiffening during division of adherent cells. *Nat. Cell. Biol.* 2001; 6:607–610.
9. Ashkin A, Dziedzic JM, Yamane T. Optical trapping and manipulation of single cells using infrared laser beams. *Nature*. 1987; 326:769–771.
10. Block SM, Goldstein LS, Schnapp BJ. Bead movement by single kinesin molecules studied with optical tweezers. *Nature*. 1990; 348:352–355.
11. Dai J, Sheetz MP. Mechanical properties of neuronal growth cone membranes studied by tether formation with laser optical tweezers. *Biophys. J.* 1995; 3:988–996.
12. Guck J, et al. The optical stretcher: a novel laser tool to micromanipulate cells. *Biophys. J.* 2001; 2:767–784.
13. Bausch AR, Ziemann F, Boulbitch AA, Jacobson K, Sackmann E. Local measurements of viscoelastic parameters of adherent cell surfaces by magnetic bead microrheometry. *Biophys. J.* 1998; 4:2038–2049.
14. Wang N, Butler JP, Ingber DE. Mechanotransduction across the cell surface and through the cytoskeleton. *Science*. 1993; 260:1124–1127.
15. Wang N, Ingber DE. Control of cytoskeletal mechanics by extracellular matrix, cell shape, and mechanical tension. *Biophys. J.* 1994; 66:1281–1289.

16. Wang N, Ingber DE. Probing transmembrane mechanical coupling and cytomchanics using magnetic twisting cytometry. *Biochem. Cell Biol.* 1995; 73:327–335. [PubMed: 8703406]
17. Fabry B. Signal transduction in smooth muscle selected contribution: Time course and heterogeneity of contractile responses in cultured human airway mouse melanoma B16 cells. *J. Appl Physiol.* 2001; 2:986–994.
18. Hu S, et al. Mechanical anisotropy of adherent cells probed by a three dimensional magnetic twisting device. *Am. J. Physiol. Cell Physiol.* 2004; 287:C1184–C1191. [PubMed: 15213058]
19. Tajik A, et al. Transcription upregulation via force-induced direct stretching of chromatin. *Nat. Mater.* 2016; 15:1287–1296. [PubMed: 27548707]
20. Hell SW, Wichmann J. Breaking the diffraction resolution limit by stimulated emission: stimulated emission depletion microscopy. *Opt. Lett.* 1994; 19:780–782. [PubMed: 19844443]
21. Klar TA, Jakobs S, Dyba M, Egner A, Hell SW. Fluorescence microscopy with diffraction resolution barrier broken by stimulated emission. *Proc. Natl. Acad. Sci. USA.* 2000; 97:8206–8210. [PubMed: 10899992]
22. Betzig E, et al. Imaging intracellular fluorescent proteins at nanometer resolution. *Science.* 2006; 313:1642–1645. [PubMed: 16902090]
23. Rust MJ, Bates M, Zhuang X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat Methods.* 2006; 3:793–796. [PubMed: 16896339]
24. Sahl SJ, Leutenegger M, Hilbert M, Hell SW, Eggeling C. Fast molecular tracking maps nanoscale dynamics of plasma membrane lipids. *Proc. Natl. Acad. Sci. USA.* 2010; 107:6829–6834. [PubMed: 20351247]
25. Patterson G, Davidson M, Manley S, Lippincott-Schwartz J. Superresolution imaging using single-molecule localization. *Annu. Rev. Phys. Chem.* 2010; 61:345–367. [PubMed: 20055680]
26. Kittel RJ, et al. Bruchpilot promotes active zone assembly, Ca²⁺ channel clustering, and vesicle release. *Science.* 2006; 312:1051–1054. [PubMed: 16614170]
27. Balzarotti F, Eilers Y, Gwosch KC, Gynnå AH, Westphal V, Stefani FD, Elf J, Hell SW. Nanometer resolution imaging and tracking of fluorescent molecules with minimal photon fluxes. *Science.* 2017; 355:606–612. DOI: 10.1126/science.aak9913 [PubMed: 28008086]
28. Fabry B, et al. Scaling the microrheology of living cells. *Phys. Rev. Lett.* 2001; 87:148102. [PubMed: 11580676]
29. Chowdhury F, et al. Is cell rheology governed by nonequilibrium-to-equilibrium transition of noncovalent bonds? *Biophys. J.* 2008; 95:5719–5727. [PubMed: 18835892]
30. Heller I, et al. STED nanoscopy combined with optical tweezers reveals protein dynamics on densely covered DNA. *Nat. Methods.* 2013; 10:910–916. [PubMed: 23934077]
31. Neuman KC, Block SM. Optical trapping. *Rev Sci Instrum.* 2004; 75:2787–809. [PubMed: 16878180]
32. Gutiérrez-Medina B, Andreasson JO, Greenleaf WJ, Laporta A, Block SM. An optical apparatus for rotation and trapping. *Methods Enzymol.* 2010; 475:377–404. [PubMed: 20627165]
33. Shroff SG, Saner DR, Lal R. Dynamic micromechanical properties of cultured rat atrial myocytes measured by atomic force microscopy. *Am. J. Physiol.* 1995; 269:C286–C292. [PubMed: 7631757]
34. Raman A, et al. Mapping nanomechanical properties of live cells using multi-harmonic atomic force microscopy. *Nat. Nanotechnol.* 2011; 6:809–814. [PubMed: 22081213]
35. Cartagena A, Raman A. Local viscoelastic properties of live cells investigated using dynamic and quasi-static atomic force microscopy methods. *Biophys. J.* 2014; 106:1033–1043. [PubMed: 24606928]
36. Hubmayr RD, et al. Pharmacological activation changes stiffness of cultured human airway smooth muscle cells. *Am. J. Physiol. Cell Physiol.* 1996; 271:C1660–C1668.
37. Pourati J, et al. Is cytoskeletal tension a major determinant of cell deformability in adherent endothelial cells? *Am. J. Physiol. Cell Physiol.* 1998; 274:C1283–C1289.
38. Wang N, et al. Cell prestress. I. Stiffness and prestress are closely associated in adherent contractile cells. *Am. J. Physiol. Cell Physiol.* 2002; 282:C606–C616. [PubMed: 11832346]

39. Puig-de-Morales M, et al. Cytoskeletal mechanics in adherent human airway smooth muscle cells: probe specificity and scaling of protein-protein dynamics. *Am. J. Physiol. Cell Physiol.* 2004; 287:C643–C654. [PubMed: 15175221]
40. An SS, Fabry B, Treppe X, Wang N, Fredberg JJ. Do biophysical properties of the airway smooth muscle in culture predict airway hyperresponsiveness? *Am. J. Respir. Cell Mol. Biol.* 2006; 35:55–64. [PubMed: 16484685]
41. Berntsen P, et al. Biomechanical effects of environmental and engineered particles on human airway smooth muscle cells. *J. R. Soc. Interface.* 2010; 7:S331–S340. [PubMed: 20356875]
42. Na S, et al. Rapid signal transduction in living cells is a unique feature of mechanotransduction. *Proc. Natl. Acad. Sci. USA.* 2008; 105:6626–6631. [PubMed: 18456839]
43. Na S, Wang N. Application of fluorescence resonance energy transfer and magnetic twisting cytometry to quantify mechanochemical signaling activities in a living cell. *Sci. Signal.* 2008; 1:11.
44. Poh YC, et al. Rapid activation of Rac GTPase in living cells by force is independent of Src. *PLoS. ONE.* 2009; 4:e7886. [PubMed: 19924282]
45. Poh YC, et al. Dynamic force-induced direct dissociation of protein complexes in a nuclear body in living cells. *Nat. Commun.* 2012; 3:866. [PubMed: 22643893]
46. Mijailovich SM, Kojic M, Zivkovic M, Fabry B, Fredberg JJ. A finite element model of cell deformation during magnetic bead twisting. *J. Appl. Physiol.* 2002; 93:1429–1436. [PubMed: 12235044]
47. Craig WS, Cheng S, Mullen DG, Blevitt J, Pierschbacher MD. Concept and progress in the development of RGD-containing peptide pharmaceuticals. *Biopolymers.* 1995; 37:157–175. [PubMed: 7534489]
48. Toli-Nørrelykke IM, Butler JP, Chen J, Wang N. Spatial and temporal traction response in human airway smooth muscle cells. *Am. J. Physiol. Cell Physiol.* 2002; 283:C1254–C1266. [PubMed: 12225988]
49. Hu S, Chen J, Butler JP, Wang N. Prestress mediates force propagation into the nucleus. *Biochem. Biophys. Res. Commun.* 2005; 329:423–428. [PubMed: 15737604]

Box 1**Interfacing 3D-MTC with STED****Optional: Super-resolution imaging using STED nanoscopy**

1. Excite the GFP-H2B with the 488 nm laser at 40 μ W (microwatt) laser-power and detect the emission fluorescence using the HyD detector between 498–560 nm.
2. Use a 592 nm STED laser at 810 mW (milliWatt) laser power to stimulate emission depletion. The actual depletion laser power at the objective lens is ~64.8 mW. Use a 100 $\times$ oil immersion objective for imaging (the useable field-of-view is 152 μ m $\times$ 152 μ m).

CRITICAL STEP We use 100 $\times$ to obtain high resolution time-lapse images of cytoplasmic structures or nuclear structures before, during, and after force application to determine force-induced displacements (and the computed strains).

Figure 1. Schematic of the 3D-MTC and confocal fluorescence microscope interface
(a) Schematic of the three dimensional magnetic twisting cytometer (3D-MTC) that produces homogeneous magnetic fields along the x-, y-, and z-axis independently (arrows represent bead rotation directions) (see Supplementary Fig. 2 for detailed dimensions). The cell is at the center and located in the focal plane of the microscope (Leica SP8 confocal microscope). Scale bar, 5 μm . **(b)** The 3D-MTC PC communicates with the confocal PC to manage image acquisition by the DAQCard-6713. The 3D-MTC specifies the frequency and amplitude of the sinusoidal wave signal from the connecting box SCB-68-A to drive the twisting coils through the voltage control current source and to generate the requested signals into the multifunction input DAQCard-6052E-A in order to synchronize image acquisition by means of an external trigger. The coils of the 3D-MTC are positioned on the microscope stage. The current flowing in the coils is simultaneously fed back output from the connecting box SCB-68-B and acquired by the DAQCard-6062E-B. For fluorescence, Leica SP8 confocal system supplies excitation beams 458 nm (cyan), 476 nm (light blue), 488 nm (dark green), 496 nm (light green) and 514 nm (yellow), filtered from an argon laser head using an acousto-optical tunable filter (AOTF). Dashed boxes are the STED laser head (592 nm, orange) (Optional). These beams are fiber-coupled and delivered to the confocal tip-tilt piezo mirror (M1) after being combined by dichronic mirror D1. A STED doughnut is formed by a binary phase plate (PP). The scanned fluorescence signal is collected by the Hybrid Detector (**HyD**). **CL**, condenser lens, Leica S28, 0.55; **OL**, oil-immersion objective lens, Leica HCX PL APO 100 $\times$, NA = 1.40. **D1–D12**, dichronic mirrors.

Figure 2. Schematic of applying stress with 3D-MTC

The ligand-coated bead (black dot) is bound to the cell (orange color, the nucleus is colored green), and a strong magnetic pulse (2500 Gauss) was applied in y (top left panel), z (middle left panel), or x (bottom left panel) to magnetize the ferromagnetic bead (note that two-pulse signals inside the coils in each panel to illustrate the fact that the pulses are everywhere inside the coil pair), then the weak sinusoidal magnetic field was applied in different directions, generating shear stresses to rotate the bead in the desired direction. **(a)** The bead rotates in the Y-Z plane (torque is in the x direction); **(b)** The bead rotates in the Z-X plane (torque is in the y direction). Note that to simulate complex loading patterns in a living tissue, complex magnetic twisting fields and hence very complex stress patterns can be generated after the magnetic bead is magnetized in certain direction. More than one twisting fields (e.g., 2 or even 3 twisting fields) can be turned on simultaneously at different magnitudes and different frequencies to force the bead to rotate spirally in x-y-z dimensions¹⁸.

Figure 3. Calculation of cell stiffness from magnetic bead displacements

(a) The brightfield image of an adherent and elongated B16-F1 melanoma cell. A 4 μm RGD-coated magnetic bead (white arrow) was bound to the cell surface via integrins. (b) The Bead Tracking software puts a “+” sign at the center of the bead mass and tracks its movement in response to applied stresses. (c) The traces of bead displacements as function of sinusoidal stress inputs. The displacements are an average of 7 cycles of the sinusoidal force to filter out noises. Note the displacements were synchronous with the input stress and there was little phase lag between the input stress and the displacements, suggesting that this cell was mostly elastic. (d) Computed cell stiffness (complex modulus). Mean ± s.e.m.; n = 6 cells; *** $P < 0.001$.

Figure 4. Nuclear H2B displacements under stress

From left to right: bright-field images of the cell, GFP-Histone 2B fluorescence images, and displacement map images of the same murine melanoma B16 cell. The nucleus is outlined with a dashed line. The thick white arrows point to the bead, the red arrows indicate direction of displacement, the color bar indicates the displacement magnitude. **(a)** No applied stress (non-magnetized beads); **(b)** After magnetizing in the Z axis and apply a 15 Pa torque (0.1 Hz) in Y axis bright-field images; **(c)** After magnetizing in Z axis and apply a 15 Pa torque (0.1 Hz) in X axis bright-field images; The white square in each displacement map is enlarged and presented on the right (the 4th image in each row) to show the differences in displacement between each stress angle **(b and c)**. It is apparent that stress angles (loading directions) are important in generating major differences in GFP-H2B displacement. The thin white arrow in right panel of **(a)** (150 nm) provides a scale bar for the magnitude of the red arrows in right panels of **(b)** and **(c)**. Image acquisition time is 1 second per image. Scale bars, 5 μm .

Figure 5. Anisotropic strains of H2B depend on loading directions

The same cell as in Fig. 4 is shown. The bulk (2D dilatational and compressive) strains or shear strains were computed from the displacement maps in Fig. 4 using the method described previously⁴⁹. The stress was applied at 15 Pa at 0.1 Hz for ~100 sec at 0° stress angle and ~100 sec at 90° stress angle. **(a)** The strain maps when the stress angle was 0° (relative to the cell long axis). **(b)** The strain maps when the stress angle was 90°. The pink arrow represents the direction of bead center displacement.

Table 1

Troubleshooting table

Step	Problem	Possible reason	Solution
1	System no response	The system is not compatible	32-bit operating system, support C++
1,2	The system is unable to initialize	The incorrect driver versions have been installed	Make sure that the driver versions match the operating system.
5	The object lens cannot be fit into the coils	The coil inner diameter is too small	Fit the lens to the center of the coils; the coils should be large enough (>5 cm in diameter) to allow the microscope lens to fit in and to move freely
6	Magnetizing no response	The system is not compatible	Run the system on a 32-bit operating system which supports C++
20	The beads do not adhere to the cells	The beads are not properly coated with cell receptor ligands	The magnetic beads should be immersed in 75 % alcohol for 8 hours before use to allow proper coating
26	The magnetic bead has no displacements	The bead is not magnetized and/or the signal amplitude of twisting waveform is too low.	Magnetize the bead first and/or set the proper signal amplitude of twisting waveform
29	Cannot find file (an error message in Matlab)	The path is incorrect	Pathname is absolute address path
30	Cannot track the beads (an error message in Matlab)	The bad parameters are set incorrectly	Set the type of beads according to the actual situation
35	The drift is too large	Anti-vibration table is not working well (no air in the tank or air leaks out)	Fix the anti-vibration table. Redo the experiments