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Application of Fluorescence Resonance Energy Transfer and Magnetic Twisting Cytometry to Quantitate Mechano-Chemical Signaling Activities in a Living Cell¹

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Abstract

Mechanotransduction is the process by which living cells sense mechanical forces and then convert them into biochemical signaling. Recently we showed that mechanical stress is transduced from the cell surface to remote cytoplasmic sites within 0.3 s, which is at least 40 to 50 times faster than soluble factor-induced signal transduction, and the sites of mechanotransduction colocalize with sites where mechanical stress causes microtubule displacement. These results suggest that mechanotransduction employs mechanisms different from those of soluble factor-induced signal transduction. Here we describe a protocol that utilizes fluorescence resonance energy transfer (FRET) and a magnetic twisting cytometry (MTC) device to capture rapid mechano-chemical signaling activities in living cells.

Introduction

Mechanotransduction is the process by which living cells sense mechanical forces and adhesive contacts then convert them into biochemical signals that elicit physiologic or pathologic responses in tissues, organs, or throughout the entire organism. Several mechanotransduction mechanisms have been proposed (1-4), the most straightforward of which is that mechano-chemical signaling is induced at the local force-membrane interface by conformational changes of membrane-bound proteins or their substrates, and then transduced deeper into the cytoplasm by a cascade of passive diffusion- or active translocation-based biochemical signaling components. The methods commonly used to elucidate the mechanisms of mechanotransduction include immunoblotting or immunostaining (5). However, these approaches are not sensitive enough to detect localized, rapid, transient signaling activities and thus have limited spatial and temporal resolution. To overcome these limitations, we employed a FRET (fluorescence resonance energy transfer) technique. FRET-based reporters are now available for a wide variety of proteins, including those involved in cell adhesion, migration, and mechanotransduction such as the tyrosine kinase Src (3), the guanosine triphosphatases Rho and Rac (6-8), and focal adhesion kinase (FAK; 9). Src is generally located at the endosomal membrane, which is physically connected with microtubules via motor proteins (10-12).

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In this protocol, we use a cytosolic Src reporter that consists of cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), an SH2 domain derived from Src, and a substrate domain derived from the c-Src substrate p130Cas (3). When Src is in its inactivate state, the CFP and YFP moieties of the reporter are close to one another (<10 nm), and most emissions from CFP transfer to YFP, thus FRET occurs and yellow light is emitted. When Src is activated at the endosomal membrane, it phosphorylates the substrate domain of the Src reporter, and this phosphorylated substrate binds to the reporter's SH2 domain, leading to a conformational change of the reporter protein. This conformational change in the Src reporter results in the separation of the CFP and YFP moieties thus decreasing FRET and emitting cyan light. Therefore, the relative intensity changes of CFP and YFP in images of cells transfected with this Src reporter can be used to measure Src activity in the cell. The focal plane of the cell can be varied by tuning the z-position control in the microscope. In order to visualize cytoplasmic enzymatic activity, rather than only the enzymatic activity at the plasma membrane, we choose the focal planes at ~1–1.5 μm above the basal surface of the cell throughout our experiments.

In this protocol, we describe the use of magnetic twisting cytometry (MTC), a technique which has been widely used to study the mechanical behavior of cells (13-16), to apply mechanical stress to cells. Ferromagnetic bead coated with an RGD (Arg-Gly-Asp) tripeptide are attached to cell surfaces and subjected to a magnetic field to deform the cell membrane locally. The MTC device consists of seven major components: (1) a high-voltage generator to provide the current to magnetize the beads, (2) one (for 1D MTC) or three separate (for 3D MTC) bipolar current sources for twisting the beads, (3) a computer for controlling the twisting apparatus, (4) an inverted microscope for observing the sample, (5) a CCD camera that uses software capable of synchronizing image capture with step function or oscillatory wave magnetic fields, (6) a device to maintain the correct temperature of the cultured cells, and (7) a microscope insert that holds the sample and contains either two pairs of coils (for 1D MTC) or three pairs of coils (for 3D MTC) that generate the alternating electric fields used to magnetize and twist the beads (available commercially from Eberhard). With the FRET technique and MTC, we showed, in contrast to the aforementioned proposed mechanotransduction mechanisms that require diffusion or translocation of biochemical signaling molecules, that mechanical force at the membrane can be transmitted through the cytoskeleton to remote cytoplasmic sites (14,17) by conformational changes of proteins that are physically linked to the cytoskeleton (16). This mechanical stress-induced signal transduction in deep cytoplasm occurs too rapidly (< 0.3 s) to be explained by a diffusion- or translocation-based mechanism.

This protocol outlines the use of an MTC and FRET-based reporter of Src activity to visualize stress-induced Src activity inside living cells with high spatial and temporal resolution (Fig. 1). We also describe the use of a synchronous detection method to determine the colocalization of FRET with the microtubule marker mCherry-tubulin. With the exception of a few minor differences, which we have noted in the **Instructions**, the procedures for performing the two methods are the same up until analysis of the data. We thus provide two different sets of instructions for image analysis – one for experiments in which only the FRET reporter is used, and one for experiments in which both the FRET reporter and the microtubule marker mCherry-tubulin are used. Both methods require familiarity with Matlab.

Materials

Acetic acid ($\text{CH}_3\text{CO}_2\text{H}$)

Calcium chloride (CaCl_2)

CFP-YFP cytosolic Src reporter (from Dr. Y. Wang, University of Illinois, Urbana, IL)
carbon dioxide-independent medium (Invitrogen)

Collagen, type I from calf skin (powder, Sigma, Catalog #C9791)

Ferromagnetic beads (Fe_3O_4 ; 4.5 μm diameter; from Dr. W. Moller, Gauting, Germany or Dr. J. Fredberg, Boston, MA; magnetic beads with various surface properties are commercially available in an assortment of sizes from Spherotech, Inc, Lake Forest, IL)

Dimethyl sulfoxide, sterile-filtered (DMSO; Sigma)

Fetal bovine serum (FBS; HyClone)

Glass-bottom culture dishes (35 mm diameter; MatTek)

Hanks' balanced salt solution with calcium and magnesium (HBSS; Invitrogen) HEPES buffer (1 M, Hyclone)

Human airway smooth muscle cells (HASM cells; from Dr. R. Panettieri, University of Pennsylvania, Philadelphia, PA)

Insulin from bovine pancreas (Sigma)

L-Glutamine (100X) (Invitrogen)

Lipofectamine LTX (Invitrogen)

Lipofectamine PLUS (Invitrogen)

mCherry-tubulin expression construct (from Dr. R. Tsien, University of California, San Diego, CA)

Nutrient mixture F-12 HAM (Sigma)

Opti-MEM I medium (Invitrogen)

Penicillin-Streptomycin (Invitrogen or Sigma)

Phosphate-buffered saline (PBS; HyClone)

Sodium bicarbonate (NaHCO_3)

Sodium carbonate (Na_2CO_3)

Sodium hydroxide (NaOH)

Synthetic RGD-containing peptide (Ac-G(dR)GDSPASSKGGGGS(dR)LLLLLL(dR)- NH_2 , Peptide-2000 (Telios) (18) ; Peptides International, Inc., Louisville, Kentucky)

Transferrin (apo-) human (Sigma)

Equipment

Adobe Photoshop

40X 0.55 Numerical Aperture (N.A.) air and 63X 1.32 N.A. oil-immersion objectives (Leica)

CCD camera (Hamamatsu; model C4742-95-12ERG)

CFP/YFP Dual EX/EM (FRET) Filter sets for FRET experiments (Optical Insights): CFP: excitation, S430/25, emission, S470/30; YFP: excitation, S500/20, emission, S535/30. The emission filter set uses a 505-nm dichroic mirror.

Dual-View imaging system (Optical Insights)

Inverted Microscope (Leica)

Matlab (Mathworks; Version 7.2)

MTC device (Commercially available via special order from EOL Eberhard, Obervil, Switzerland)

Recipes

Recipe 1: Magnetic Beads

Beads arrive as powder. The beads are resuspended in 95% ethanol to a concentration of 5 mg/ml for sterilization and are stored at 4 °C.

Recipe 2: Carbonate Buffer

Na ₂ CO ₃	159 mg
NaHCO ₃	293 mg
Distilled water	80 ml

Adjust pH to 9.4 with acetic acid. Add distilled water to a final volume of 100 ml.

Sterilize by passing through a 0.2 µm filter, and store at 4 °C.

Recipe 3: RGD solution (5 mg/ml)

RGD	0.5 mg
DMSO	100 µl

Mix well in a sterile hood and store at 4 °C.

Recipe 4: Culture Medium for HASM cells

Nutrient mixture F-12 HAM medium (Sigma) supplemented with	
FBS	10% (v/v)
Penicillin	100 U/ml
Streptomycin	100 µg/ml
L-Glutamine	2 mM
NaOH, sterile-filtered	12 mM
CaCl ₂ , sterile-filtered	1.7 µM
HEPES	25 mM

Mix well in a sterile hood and store at 4 °C.

Recipe 5: Collagen solution (3 mg/ml)

Collagen	3 mg
Acetic acid	20 mM
Distilled water	1 ml

Mix well, Sterilize by passing through a 0.2 µm filter, and store at 4 °C.

Before use, dilute this stock solution into 20 µg/ml with PBS.

Recipe 6: Transfection Medium for HASM cells

Nutrient mixture F-12 HAM medium (Sigma) supplemented with

L-Glutamine	2 mM
NaOH, sterile-filtered	9 mM
CaCl ₂ , sterile-filtered	1.6 μ M
HEPES	25 mM

Mix well in a sterile hood and store at 4 °C.

Recipe 7: HEPES-buffered HBSS

HBSS supplemented with

HEPES	20 mM
D-glucose, anhydrous	2 g/l

Sterilize by passing through a 0.2 μ m filter, and store at 4 °C.

Instructions

Coating Magnetic Beads with RGD

Steps 1–3 are performed in a sterile hood.

1. Transfer 200 μ l of magnetic beads (Recipe 1) into a standard 1.5 ml microfuge tube.
2. Add 1.5 ml PBS to beads, and centrifuge at 1200 rpm for 1.5 min in a microfuge. Discard the supernatant.
3. Add 1 ml Carbonate Buffer (Recipe 2) and 10 μ l RGD peptide solution (Recipe 3). The final concentration of RGD will be 50 μ g/ml, which is enough to saturate the beads ($\sim$ 1 RGD-peptide per 3 nm² bead surface area).
4. Mix overnight on a rotator or nutator at 4 °C. The beads may be used immediately or stored at 4°C for up to two weeks before use.

Cell Culture and Transfections

HASM cells should be maintained in the medium described in Recipe 4 as per instructions described in Se Hu *et al.* (14). Passages 3 to 8 should be used for these experiments. For lipotransfection, we use the manufacturer's protocol (Invitrogen).

1. Two days before transfection, add 200 μ l type I collagen solution (20 μ g/ml, Recipe 5) to the center (glass surface, 14 mm diameter) of the glass-bottom dish (35 mm diameter) and allow the collagen to be absorbed to the surface overnight at 4 °C.
2. One day before transfection, remove excess collagen solution from the coated glass surface of the dish and wash the surface 3 times with 2 ml PBS.
3. Transfer HASM cells ($\sim$ 300,000 cells) into the collagen-coated glass-bottom culture dish (35-mm dish) with Transfection Medium (Recipe 6) so that the cells are 60 to 80% confluent at the time of transfection ($\sim$ 50,000 cells on the 14-mm glass bottom).
4. On the day of transfection, dilute 1 μ g of the CFP-YFP cytosolic Src reporter plasmid in 200 μ l Opti-MEM I medium in a 1.5-ml microfuge tube and mix thoroughly.

(Note: If you are using the synchronous detection method, add 1 μg each of the CFP-YFP reporter and the mCherry-tubulin expression construct, and increase the volume of Opti-MEM I medium to 300 μl)

5. Add 1 μl Lipofectamine PLUS to the diluted DNA solution, mix gently, and incubate for 5 min at room temperature.

(Note: If you are using the synchronous detection method, increase the volume of Lipofectamine PLUS to 2 μl .)

6. Add 2.5 μl Lipofectamine LTX to the diluted DNA solution, mix gently, and incubate for 30 min at room temperature.

(Note: If you are using the synchronous detection method, increase the volume of Lipofectamine LTX to 5 μl .)

7. Add 200 μl Lipofectamine-DNA complex solution to the glass-bottom dish of HASM cells.

(Note: If you are using the synchronous detection method, add 300 μl of DNA solution to the cells.)

8. Incubate the cells at 37 $^{\circ}\text{C}$ in 0.5% CO_2 for 24 to 48 hrs prior to imaging.

MTC and FRET Microscopy

Magnetic twisting cytometry (MTC) is a technique used to exert mechanical stresses on living cells by first magnetizing and then rotating ferromagnetic beads that are bound to the surface of or internalized within cells. Before starting the experiment, it is important to determine the parameters for applying the magnetic field and for collecting the FRET data. For MTC, the magnetic twisting field can be varied from 0 to 75 Gauss (G) either as a step function (a constant magnetic field) or as a sinusoidal oscillatory wave of varying frequencies. Either a constant (step function) or an oscillatory magnetic field can be applied to a cell to measure stress-induced biochemical activities in the cell, whereas an oscillatory field can be applied in order to precisely determine the magnitude of the cytoskeletal deformation by using the synchronous detection method. The stress applied to the cell (in piconewton per μm^2 ($\text{pN}/\mu\text{m}^2$), i.e., Pascal (Pa)) is defined as the ratio of the applied torque (in $\text{pN}\mu\text{m}$) to six times the bead volume (in μm^3). In practice, the applied stress (in Pa) can be converted from the applied twisting magnetic field (in G) by using the bead constant (in Pa/G) times the applied twisting magnetic field (in G). The bead constant reflects the magnetic property of the bead and differs for each batch of beads, and thus must be calibrated. By changing the magnitudes of the magnetic twisting field (in G), one can obtain the bead constant (in Pa/G) when the magnetic beads are immersed in a medium with known viscosity (10). This step is important for converting applied magnetic fields to applied stresses on the cell surface (13). For example, a 50 G step function applies 17.5 Pa of stress to the cell if the bead constant is 0.35 Pa/G (16). A dish of cells bound to beads may only be magnetized once. Since the magnetic field is applied to the entire dish, only one biochemical activity assay may be performed per dish.

1. On the day of experiment, centrifuge the tube containing the RGD-coated magnetic beads (1 mg/ml in Carbonate buffer) at 1200 rpm for 1.5 min at room temperature. Discard the supernatant.
2. Resuspend the beads in 1 ml PBS and microfuge at 1200 rpm for 1.5 minutes. Discard the supernatant.
3. Add 1 ml of PBS to the RGD-coated beads to make a solution of 1 mg/ml.

4. Remove the dish of transfected HSAM cells from the incubator. Remove the culture medium from the cells except for those covering the glass region at the center of the dish.
5. Add 30 to 40 μ l of beads (equivalent to 30 to 40 μ g) to the cells adhering to the glass portion of the dish.
6. Return the cells to the 37 °C, 0.5% CO₂ incubator for 15 min to allow integrin clustering and formation of focal adhesions with the beads.
7. Remove the cells from the incubator and rinse them twice with 2 ml PBS. Add the PBS gently to the side of the dish and rinse cells by gently tilting the to avoid disturbing the cells.
8. Add CO₂-independent medium or HEPES-buffered HBSS (Recipe 7) to the cells carefully to avoid disturbing the cells.
9. Place the dish on the stage of the inverted microscope and locate a transfected cell that is attached to a magnetic bead. Use brightfield illumination to locate a cell to which a single magnetic bead is attached; exclude cells that are attached to more than one bead. Use fluorescence illumination to determine whether that cell has the fluorescent reporter(s).
10. Before beginning the experiment, magnetize the bead by applying a very strong magnetic pulse ($\sim$ 1000 G, <0.5 ms). For biochemical activity assays, apply the force only once.
11. Collect brightfield and phase-contrast images of the cell. If you are using the synchronous method, also collect red fluorescence images of the cell.
12. Collect two or three cyan and yellow fluorescence images before applying the magnetic field as the baseline images before stress application. If you are using the synchronous detection method, also collect red fluorescence baseline images. We use the Dual-View imaging system to simultaneously capture both CFP (1344×512 pixels) and YFP (1344×512 pixels) images on the same screen.
13. Choose parameters for MTC and FRET microscopy. Parameters for a typical experiment are listed below:

Applied stress: 17.5 Pa (50 G step function)

Exposure time for each image: 80–273 ms.

Duration of imaging: $\sim$ 3 s or longer.

(Note: For a synchronous detection experiment, an oscillatory rather than a step-function field should be applied.).
14. Collect cyan and yellow fluorescence images for 3 s or longer while applying constant magnetic field (50 G) to the cells. For a synchronous detection experiment, collect brightfield and fluorescence (mCherry tubulin) images during oscillatory stress application.

Image Analysis for FRET Reporter

We used Matlab to create a customized program to calculate the CFP/YFP emission ratio of the Src reporter in living cells. For details, refer to the Matlab manual. Other commercially available programs, such as MetaMorph (Molecular Devices), can be used as well. The image file (1344×1024 pixels, if you are using the Hamamatsu C4742–95–12ERG camera) for each time point consists of both CFP and YFP images (1344×512 pixels each). All procedures in

this section are carried out using Matlab with the Image Processing Toolbox (Mathworks). All these steps are automated in the Matlab.

The CFP/YFP emission ratio increases over time due to photobleaching of the acceptor (YFP). Because there is no Src kinase in the nucleus, the intensity values of pixels within the nucleus do not change during mechanical stimulation. Therefore, choose a region of the nucleus to use as a reference for determining the bleaching kinetics for each experiment. Instructions for processing individual images are described below.

1. Split each image into its constituent CFP and YFP images and make separate files for each by cropping from the original image and saving each channel's image as separate files.
2. Subtract background signals. Choose a region in the field of view that does not contain any fluorescent cells in either the CFP or YFP image and measure the average intensity in this region. Subtract this average background intensity from each of both CFP and YFP images, respectively, in Matlab.
3. Align the background-subtracted CFP and YFP images with one another pixel-by-pixel by maximizing the normalized cross-correlation coefficient of CFP and YFP images. We use Matlab's "normxcorr2" function to cross-correlate CFP and YFP images.
4. Since the YFP image is brighter than the CFP image and yields better cell contours, one needs to use the YFP image to make a binary mask. Determine the intensity threshold and generate a binary mask image by using Matlab's "graythresh" function based on Otsu's method (19). Set the pixel value inside the cell to one and the pixel value outside the cell to zero.
5. Multiply the background-subtracted CFP image by the binary mask image such that the region outside the cell contours of the CFP image is set to zero.
6. Normalize the aligned CFP/YFP emission ratios to the lower emission ratio and display the CFP/YFP emission ratio image as a linear pseudocolored image. For example, if the range of the CFP/YFP emission ratio of an HASM cell transfected with cytosolic Src reporter is between 0.1 and 0.4, divide the ratio values by the minimum emission ratio, which is 0.1 in this case. The result is a normalized emission ratio, the range of which is between 1 and 4. One can use Matlab's default "jet" colorbar or make a custom colorbar to represent FRET ratio image of the cell by inputting CFP and YFP image file names in our custom-made Matlab software, FRET_DualView.m, and running the software to obtain all the FRET images automatically.
7. To correct for photobleaching, measure the average intensity values in the nucleus at each time point, and normalize them to the average intensity values at time zero (the time when the first image was collected). Subtract these values from the normalized CFP/YFP emission ratios of the corresponding cell generated in step 5 to obtain corrected final CFP/YFP emission ratio images.

Image Analysis for mCherry-Tubulin (the Synchronous Detection Method)

We recently demonstrated that sites of stress-induced Src activation colocalize with sites of microtubule deformation (16) by simultaneously measuring Src activity and microtubule displacement. A synchronous detection method (20) was coupled with FRET analysis to visualize and quantify both Src activity and microtubule displacement in response to mechanical stress.

Setting up and performing the experiment for the synchronous detection requires additional step, and these modifications are noted in the text of the **Cell Culture and Transfections** and **MTC and FRET Microscopy** sections. Of particular important are modifications in steps 13 and 14 in the **MTC and FRET Microscopy** section. Note that an oscillatory rather than a step-function magnetic field should be applied, and be sure to collect brightfield and fluorescence images during each oscillatory stress application 6–10 times per cycle. The synchronous detection method detects displacement or deformations of microtubules at a resolution of 4 to 5 nm (14,20), and correlates them with the force application. This technique requires familiarity with Matlab and the data analysis methodology outlined below. All these steps are automated in the Matlab except for step 4, which is done by using the Adobe Photoshop. For detailed protocols and steps, see (16).

1. One can use “imread” function to change the image file to matrix form, suitable for use in the Matlab. To reduce the noise caused by spontaneous movements of the microtubules, average the images taken during the same twisting phase (if you take 10 images per cycle, there are 10 twisting phases) over 10 cycles to generate one complete cycle of 10 averaged images by using Matlab’s “mean” function.
2. To identify the local displacement of an image, divide the fluorescence image into small arrays of 11×11 pixels that overlap by five pixels by inputting corresponding column and row values of the matrix.
3. Obtain the displacement field of the microtubules by comparing corresponding arrays at the same location (11×11 pixels) between two images taken at different phases during the twisting cycle and by shifting the arrays of the second image by subpixel increments ($1/25$ pixels) in the Fourier domain until the mean square differences of the pixel intensities between the shifted array and the corresponding array from the first image reach a minimum. This step requires familiarity with the Fourier transform (20).
4. To assay colocalization, open both the Src activity image generated in the **Image Analysis for FRET Reporter** section and microtubule displacement image generated in this section for the same cell by using the Adobe Photoshop and overlay the two images to determine whether the Src activation sites and microtubule displacement sites overlap.

Troubleshooting

If you turn on the twisting magnetic field but no stress is applied to the cells, determine whether or not the bead is magnetized. A magnetized bead on the dish will move when the twisting field is turned on. If the bead is not magnetized, repeat step 10 from the **MTC and FRET Microscopy Instructions**.

If a cell rounds up after the mechanical stress is applied, make sure that the cell is firmly adhered to the substrate. Apply a different form of mechanical stress, such as AFM (atomic force microscopy), optical tweezers, a magnetic gradient, parallel plates, or a glass micropipette, for example, and observe the cell. If the cell also rounds up after applying mechanical stress in one of these different forms, then the cells are not adequately adhered to the substrate. Be careful to insure that the magnitude of these applied stresses is within the physiologic range to avoid stress-induced cell injury or apoptosis, especially when the duration of stress application is long (minutes to hours).

Notes and Remarks

Applying stress to a particular part of a cell

If one wants to study how different regions of the cell periphery, such as filopodia or lamellapodia, respond to stresses, one must assess the effect of the size of the bead-cell contact area on the actual magnitude of the stress that is applied to the cell. Of concern when applying mechanical stress to a thin cellular projection is the effect of the underlying rigid surface of the dish. If the cell is plated on a dish coated with a very thin layer of extracellular matrix proteins, most of the applied force will be balanced by the rigid substrate just underneath the cell. If one coats the rigid dish with a thick layer of matrix proteins such as type-1 collagen or other flexible substrates, then one needs to determine the effect of the matrix stiffness on cell biological behavior and on the cell's response to mechanical forces (see a recent review in (21); 16).

Using different sized beads

One can use beads of different sizes for these experiments. Beads smaller than 0.5 μm in diameter, may be quickly endocytosed. In this case, it is important to visually determine the subcellular location of the bead when the mechanical stress is applied. Using a combination of small beads and markers of specific subcellular structures, it might be possible to apply stresses to specific intracellular membranes such as phagosomes, lysosomes, mitochondria, and the nucleus. Large beads, greater than 20 μm in diameter, may induce global cellular deformations, thus it would be difficult or impossible to determine the local molecular and structural changes in response to the force. To apply the force only to the plasma membrane, use a bead greater than 1 μm in diameter to prevent quick endocytosis by the cell.

Density of Ligand on the bead

Different concentrations of ligand used to coat the beads might alter biochemical activities in the cytoplasm for a given applied stress. We have found that lower coating densities ($<5 \mu\text{g}/\text{ml}$ RGD per mg bead) will lead to less mechanical response from the cell (i.e., cellular stiffness is less) for the same applied stress. Presumably higher ligand concentrations stimulate a greater density or increase the strength of focal adhesions, and thus a greater response.

Multiple beads and force transmission from one cell to another

In principle, one could assay biochemical changes of a cell when more than one bead is attached to determine the potential additive effects of forces. One may also place a bead on the surface of one cell and observe microtubule deformations and biochemical changes transduced to a neighboring cell (which does not have a bead) through adhesive contacts.

Biochemical activities other than Src

In principle, this method could be applied to assay any biochemical activities in the cytoplasm or in the nucleus, as long as a biosensor or a reporter is delivered inside the cell for reporting the specific biochemical activity.

Simultaneous image acquisition

In order to capture rapid mechano-chemical signaling in a living cell using a FRET-based reporter, simultaneous acquisition of fluorescence images via separate channels is essential. In this protocol, therefore, we use a Dual-View system (Optical Insights) to visualize individual fluorescence images (e.g., CFP and YFP images) simultaneously. This requirement of capturing the CFP and YFP images simultaneously does introduce some limitations to the method. When using a Dual-View imaging system with a FRET-based CFP-YFP reporter, the intensity of the emission signal from the sample is divided in half in order to capture the CFP

and YFP images side-by-side. Splitting the emission signal thus raises the lower limit of the level of fluorescence that can be detected. To overcome this limitation, one might use a low-light, highly sensitive CCD camera.

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Fig. 1. Schematic of the protocol

Live adherent cells are transfected with the CFP-YFP Src reporter or cotransfected with the Src reporter and mCherry-tubulin by lipofection. 24 to 48 hrs after transfection, an RGD-coated ferromagnetic bead is attached to the apical surface of the cell. The dish containing the cells is mounted on an inverted microscope so that Src activity in response to local mechanical force induced by twisting the magnetic bead can be observed. For colocalization analysis, a synchronous detection method is used to visualize displacements of microtubules marked with mCherry-tubulin in the same cell in response to a local mechanical force.