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Measurement of force-sensitive protein dynamics in living cells using a combination of fluorescent techniques

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Corresponding Author:	Brenton Hoffman UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	brenton.hoffman@duke.edu
Order of Authors:	Katheryn Elaine Rothenberg
	Ishaan Puranam
	Brenton D Hoffman
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Duke | PRATT SCHOOL OF ENGINEERING

Department of Biomedical Engineering
1425 FCIEMAS, Box 90281
Durham, NC 27708

brenton.hoffman@duke.edu
Ph: (919) 660 5154
Fax: (919) 684 4488

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Dear Editor,

We are pleased to re-submit the manuscript entitled “Measurement of force-sensitive protein dynamics in living cells using a combination of fluorescent techniques” by Katheryn E. Rothenberg, Ishaan Puranam, and Brenton D. Hoffman.

We thank the editor and the reviewers for their detailed reading and thoughtful comments regarding our manuscript. We have done our best to address all comments and, as a result, the manuscript is significant improved. We hope it is now suitable for publication.

Sincerely,

Brenton D. Hoffman
Assistant Professor
Department of Biomedical Engineering
Duke University

TITLE:

Measurement of Force-Sensitive Protein Dynamics in Living Cells Using a Combination of Fluorescent Techniques

AUTHORS & AFFILIATIONS:
Katheryn E. Rothenberg, Ishaan Puranam, Brenton D. Hoffman
Department of Biomedical Engineering, Duke University, Durham, NC, USA

Corresponding Author:
Brenton D. Hoffman
brenton.hoffman@duke.edu
Tel: (919)-660-5154

Email Addresses of Co-authors:
Katheryn E. Rothenberg (katheryn.rothenberg@duke.edu)
Ishaan Puranam (Ishaan.puranam@duke.edu)

KEYWORDS:
Mechanobiology, mechanotransduction, biophysics, FRET-based tension sensors, FRAP, force-sensitive protein dynamics

SUMMARY:
Here, we present a protocol for the simultaneous use of Förster resonance energy transfer-based tension sensors to measure protein load and fluorescence recovery after photobleaching to measure protein dynamics enabling the measurement of force-sensitive protein dynamics within living cells.

ABSTRACT:
Cells sense and respond to physical cues in their environment by converting mechanical stimuli into biochemically-detectable signals in a process called mechanotransduction. A crucial step in mechanotransduction is the transmission of forces between the external and internal environments. To transmit forces, there must be a sustained, unbroken physical linkage created by a series of protein-protein interactions. For a given protein-protein interaction, mechanical load can either have no effect on the interaction, lead to faster disassociation of the interaction, or even stabilize the interaction. Understanding how molecular load dictates protein turnover in living cells can provide valuable information about the mechanical state of a protein, in turn elucidating its role in mechanotransduction. Existing techniques for measuring force-sensitive protein dynamics either lack direct measurements of protein load or rely on the measurements performed outside of the cellular context. Here, we describe a protocol for the Förster resonance energy transfer-fluorescence recovery after photobleaching (FRET-FRAP) technique, which enables the measurement of force-sensitive protein dynamics within living cells. This technique is potentially applicable to any FRET-based tension sensor, facilitating the study of force-sensitive protein dynamics in variety of subcellular structures and in different cell types.

INTRODUCTION:

The extracellular environment is a rich source of both biochemical and physical cues that dictate cell behavior. In particular, the physical nature of the microenvironment can mediate key cellular functions, including cell growth, migration, and differentiation[1-4]. Dysregulation of the mechanics of the microenvironment is a critical component to many diseases that do not yet have adequate treatments, such as cancer[5], atherosclerosis[6], and fibrosis[7]. A complete understanding of how cells convert physical stimuli into biochemically-detectable signals, a process termed mechanotransduction, requires the elucidation of the molecular mechanisms mediating force transmission, both into and out of the cells and within multiple subcellular structures.

Inside subcellular structures, proteins are constantly turning over; binding and unbinding based on the strength of their interactions with binding partners[8]. For forces to be successfully transmitted across a physical distance, there must be an unbroken chain of protein-protein interactions, meaning that a protein's turnover must be slow enough to sustain and transmit force to its binding partner[9]. While protein-protein interactions generally consist of several non-covalent bonds between the protein domains, the interaction is often conceptualized as a bound state that can transition to an unbound state under different conditions[10,11]. For a given protein-protein interaction, it is possible that force can have no effect on the lifetime of the interaction, known as an "ideal bond", reduce the lifetime of the interaction, known as a "slip bond", or increase the lifetime of the interaction, known as a "catch bond"[10]. Thus, there is an intricate relationship between protein load and protein dynamics, which we refer to as force-sensitive dynamics.

Towards understanding the effect of load on bond dynamics, a number of highly informative experiments have been performed on the single-molecule level. Using isolated proteins, or fragments of proteins and manipulation techniques such as magnetic tweezers, optical tweezers, and atomic force microscopy, these studies have demonstrated force-sensitive protein-protein interactions for several relevant proteins[11,12]. Both integrins[13] and cadherins[14], which are transmembrane proteins important for forming cell-matrix and cell-cell interactions, respectively, have demonstrated alterations in dynamics due to load. Within the cell, vinculin is recruited to both talin[15] and α-catenin[16] in a force-dependent manner and can form a catch bond with actin[17], indicating a crucial role for vinculin at both focal adhesions (FAs) and adherens junctions (AJs) under load. Single-molecule studies allow for the isolation of specific protein-protein interactions and yield unambiguous results, but they do not account for the complexity of the cellular environment.

Landmark experiments demonstrated that several subcellular structures, including FAs and AJs, are mechanosensitive, and exhibit enhanced assembly in response to internally-generated or externally-applied loads[18-22]. Additionally, several theoretical models have suggested that mechanosensitive assembly could be driven by force-sensitive protein dynamics[23-25]. To examine these force-sensitive dynamics within living cells, a few indirect approaches have been taken. FRAP and related techniques provide a relatively simple methodology for measuring protein dynamics in cells[26-29]. However, the measurement of protein load has been more limited. A typical approach is to compare protein dynamics in cells with and without the exposure to a

cytoskeletal inhibitor used to reduce overall cell contractility[8,30,31]. Conceptually, this is a comparison between a high load and low load state. However, there is no quantification of the load across the protein in either state, and there may be unintended biochemical effects of the inhibitor, such the loss of key binding sites along an F-actin filament. Another approach, specific to FAs, has been to measure total force exertion on the substrate by the FA using traction force microscopy to approximate molecular load and examine the relationship with the dynamics of a single protein within the FA[32]. While this approach allows for the quantification of total force, it does not provide molecularly specific information. FAs are made up of over 200 different proteins, many of which can bear load[33]. Thus, measuring the total force output of an FA potentially obscures the possibility of multiple force transmission pathways and does not reliably provide a measure of load on a specific protein.

Unlike previous approaches in mechanobiology, the advent of FRET-based tension sensors allows direct measurement of loads experienced by specific proteins inside living cells[34-36]. Here, we present a protocol that combines FRET-based tension sensors with FRAP-based measure of protein dynamics. We refer to this technique as FRET-FRAP. This approach enables the simultaneous measurement of protein load and protein dynamics, thus allowing the assessment of the force-sensitive protein dynamics in living cells (**Figure 1**). Already, the FRET-FRAP technique has been applied to the study of the force-sensitive dynamics of the mechanical linker protein vinculin[37]. Tension sensors have been developed for numerous proteins that are relevant in a variety of subcellular structures. For example, sensors have been developed for vinculin[34] and talin[38,39] in FAs, cadherins and catenins in AJs[40-42], nesprin in the nuclear LINC complex[43], α-actinin[44] and filamin[36] in the cytoskeleton, and MUC-1 in the glycocalyx[45], among others[46]. Similarly, FRAP is a commonly use technique has been used on mechanosensitive proteins within the focal adhesions[8,31], adherens junctions[47], actin cortex[26], and nucleus[48]. Moving forward, the FRET-FRAP technique should be broadly applicable to any of these existing sensors or newly developed sensors, allowing for the measurements of force-sensitive dynamics in a wide variety of subcellular structures and contexts. Towards this end, we provide a detailed, generalized protocol for implementing the FRET-FRAP technique applicable in these different systems. Hopefully, this will enable a wide variety of experiments elucidating the roles of various mechanosensitive proteins in regulating force transmission and in mediating cell behavior.

PROTOCOL:

1. Generate Samples for Imaging

1.1. Stably express tension sensor construct in desired cell type.

1.1.1. Clone tension sensor construct into pRRL vector or other viral expression plasmid.

Note: Several different molecular cloning tools are available to achieve this step including the use of restriction enzymes, overlap extension, and Gibson Assembly[35]. The pRRL vector is used in lenti viral transduction and enables a substantial degree of protein production through the use of the human cytomegalovirus (CMV) promoter. Different vectors may be needed for a particular context. For instance, the CMV promoter is silenced in some cell types[49]. Additionally, only FRET-

based sensors containing fluorescent protein that lack strong sequence homology, such as mTFP1 and Venus A206K, can be used to create stable cell lines. Sensors containing cyan fluorescent protein and yellow fluorescent protein will likely be subject to homologous recombination[50].

1.1.2. Generate lentivirus in Human Embryonic Kidney (HEK) 293T cells using psPax2 and pMD2.G packaging plasmids using standard virus production methods[51].

CAUTION: Lentivirus should only be handled by properly trained personnel in a biosafety level 2 laboratory environment.

Note: This combination of cells and packaging plasmids is appropriate for the use with pRRL. Other systems may be required with other vectors.

1.1.3. Transduce desired cells with virus using standard transduction protocols[52] and use flow cytometry to sort cells[53] selecting a homogenous population expressing each construct at approximately endogenous levels[37]. After the cell selection, experiments can be conducted immediately, or cells can be cryogenically frozen for later use. Do not exceed 2 freeze-thaw cycles for a given stable cell line.

Note: The use of cell lines deficient in the protein to be studied (*e.g.*, vinculin -/- MEFs for the use with the vinculin tension sensor) will increase the signal to noise ratio in FRET experiments as well as limit over-expression artifacts. Such stable mouse embryonic fibroblast (MEF) lines can be used for approximately 15 passages before significant loss of expression or degradation of sensors is apparent. If viral based methods are not desired, a plethora of commercial reagents can be used according to the manufacturer's protocol to transiently transfect a variety of cell types with tension sensors in an appropriate plasmid, such as pcDNA3.1. Optimal expression will be 24-48 h following transfection.

1.2. Prepare substrates for cell seeding.

1.2.1. Acquire 4, 35 mm glass-bottomed dishes.

1.2.2. Working in a cell culture hood, in a 15 mL canonical tube, make 4 mL of 10 µg/mL fibronectin in phosphate-buffered saline (PBS) solution using sterile PBS in the cell culture hood. Gently invert the tube once to mix and let the solution sit for 5 min in the cell culture hood.

Note: The concentration or type of ECM protein may have to be adjusted for other cell types. The conditions provided are suitable for MEFs.

1.2.3. Pipette 1 mL of fibronectin solution onto each glass-bottomed dish.

1.2.4. Leave the fibronectin solution on the dishes for 1 h at room temperature or overnight at 4 °C.

1.2.5. Aspirate the fibronectin solution, rinse once with PBS, and add 1 mL of PBS.

1.3. Seed the cells onto prepared substrates.

1.3.1. Start with the cells of interest at a confluence percentage appropriate for subcultivation in a 6 cm culture dish.

Note: Different cell types will require distinct cell culture conditions and subcultivation protocols. This section provides guidelines suitable for MEFs. Typically, MEFs are grown to 85% confluence before subcultivation.

1.3.2. Working within a cell culture hood, rinse the cells once with 3 mL of PBS. Add 1 mL of 0.05% Trypsin-EDTA and incubate for 5 min at 37 °C.

1.3.3. Add 3 mL of complete media to the 6 cm dish, collect the cells, and place into 15 mL conical tube.

Note: Composition for complete media will depend on the cell type being used. For MEFs, complete media is often defined as high glucose Dulbecco's Modified Eagle Medium with 10% fetal bovine serum, 1% Antibiotic-Antimycotic (containing Amphotericin B, Penicillin, and Streptomycin), and a 1% non-essential amino acid (NEAA) solution.

1.3.4. Spin the cells down at 1000 x g for 5 min.

1.3.5. Aspirate the media and resuspend the cell pellet in 1 mL of complete media.

1.3.6. Remove PBS from fibronectin-coated glass dishes. Count the cells and seed 30,000 cells onto each fibronectin-coated glass dish with the appropriate complete media for a final volume of 1.5 mL.

Note: This cell density is appropriate for MEFs and will lead to a population of cells that are not touching, but not exceedingly sparse. The exact cell number may need to be adjusted for other cell types or other imaging chamber.

1.3.7. Allow the cells to spread for 4 h following seeding. At 2 h of spreading, aspirate the growth media, and rinse once with imaging media, leaving 1.5 mL of imaging media.

Note: This spreading time is appropriate for MEFs but may need to be altered for other cells. However, incubation periods of longer than 6-8 h will lead to the significant deposition of ECM protein from serum in the complete media. Imaging media should contain the same additions as complete media but should be optically clear and not contain any compounds that fluoresce in imaging channels, such as flavins, or quench fluorescence, such as phenol red. A generally useful imaging media is DMEM-gfp Live Cell Visualization media supplemented with 10% FBS and 1% NEAA solution. If background autofluorescence is unacceptably high, then the amount of serum

can be reduced. If a media change is not possible after the initial plating, the cells can be directly resuspended in imaging media supplemented with a trypsin inhibitor.

2. Set up Microscope for Imaging

2.1. Turn on the microscope.

2.1.1. Turn on the arc lamp first.

Note: An arc lamp will release an electromagnetic pulse, which can damage other equipment that is already on.

2.1.2. Turn on the filter wheel controller, automated stage controller, microscope-computer interface, and camera.

2.1.3. Turn on the FRAP laser and laser position controllers.

CAUTION: High-powered lasers can be damaging to eyes if directly viewed. It is recommended to configure the microscope system to block laser excitation from being directed to the eye pieces, which can be accomplished by moving a mirror into the FRAP beam path during bleaching to reflect the laser toward the sample and prevent transmission to the eyepiece.

2.1.4. Turn on the computer and open microscope control software.

2.1.5. Allow 15 min for the arc lamp and FRAP laser to warm up.

2.2. Calibrate the FRAP laser.

2.2.1. Open the laser configuration window. Set **Illumination Setting** (during pulse) to the appropriate FRAP illumination settings for laser exposure to the sample. Set **Illumination Setting** (during imaging) to the illumination settings appropriate for imaging only the acceptor fluorophore.

2.2.2. Select the objective to calibrate under **Coordinate System Setting**. Uncheck **Manually Click Calibration Points** and check **Display images during calibration**.

2.2.3. Set the **Dwell time** to 10000 µs and the number of pulses to 100.

2.2.4. Place the calibration slide, made of ethidium bromide sealed between a glass slide and a coverslip, into the stage adaptor with the coverslip side down.

CAUTION: Ethidium bromide is a mutagen and should be handled using gloves. If the slide is compromised, dispose of according to the institution's guidelines.

2.2.5. Use the acceptor illumination settings to focus on the surface of the slide, identifiable as the focal plane with the brightest signal. Small defects in the coating will be visible to aid in focusing.

2.2.6. Move the slide to an area with uniform fluorescence across the imaging plane.

2.2.7. Click on **Create Setting**. The software will initialize the calibration process, automatically bleaching and detecting the position of the bleached point.

2.2.8. Ensure successful calibration by assessing the final image, which will be a 3 × 3 grid of bleached points that should be evenly distributed and in focus. Save the calibration image for future reference.

2.2.9. Remove the calibration slide and safely store. Calibration should be performed before beginning each experiment but does not need to be performed between samples.

3. Choose Parameters for FRET Imaging

3.1. Fix one of the generated samples of the cells expressing the tension sensor with 4% paraformaldehyde for 10 min. Paraformaldehyde solution should be methanol free, often referred to as EM-grade, to prevent denaturing of fluorescent proteins. Place in PBS after fixation.

CAUTION: Paraformaldehyde solutions are toxic. This step should be performed in a fume hood and the solution should be disposed of according to institutional policies.

Note: This optimization does not depend on protein dynamics, and a fixed sample allows for maximum imaging time without worrying about cell health.

3.2. Rinse the sample three times with PBS and leave in PBS.

Note: Use of most commercially-available mounting media will affect fluorophore properties, making the sample unsuitable for FRET imaging[54]. Ideally, the cells will be imaged immediately, but may be left overnight at 4 °C. Longer wait times will result in deterioration of the sample.

3.3. Place the sample into the microscope stage holder for imaging.

3.4. Open the Multi-Dimensional Acquisition (MDA) tool. Establish a sequential imaging of three channels: acceptor only excitation and emission (acceptor channel), donor excitation and acceptor emission (FRET channel), and donor only excitation and emission (donor channel).

Note: There are a variety of ways to image FRET samples. The three-channel or “three-cube” method of imaging paired with means of calibrating the system to measure FRET efficiency is recommended for FRET-based tension sensors[55,56]. This approach is fast, simple, nondestructive, requires only a standard fluorescence imaging microscope, and enables the comparison of

experiments across different days and imaging setups.

3.5. Scan the sample using an exposure time of 500 ms and a neutral density (ND) filter of 10%. Find a cell expressing the tension sensor with clear localization to a structure of interest.

3.6. Select an exposure time of 500 ms or the desired length for each imaging channel and an ND filter of 100% and acquire a FRET image sequence.

3.7. Estimate the average intensity of the sensor at the subcellular structures of interest in each imaging channel. Low signals may lead to inaccurate results due to improper correction estimates, non-linearities in detectors, or significant contribution of background signals. An approximate guideline is to aim for intensities above 10% of the dynamic range of the camera (*i.e.*, for a 16-bit camera, intensities should be above 6000).

Note: Identical optical settings (exposure times, filters, objectives, and other variables such as camera gain or binning) must be used for all FRET experiments that will be compared. Changing any of these settings will lead to an alteration in the amount FRET that is either generated and/or detected in the microscopy set-up. FRET efficiency measurements are independent of these setting, but the calibration factors used to determine FRET efficiency are not. In theory various sets of calibration, factors could be used to generate FRET efficiencies from different optical settings, but this is not recommended. Bleaching or phototoxicity can be different between the various settings, creating spurious results.

3.8. Acquire a second FRET image sequence of the same field of view. Estimate photobleaching between frames by comparing average intensity of the sensor in each imaging channel. Photobleaching should be kept to a minimum, preferably less than 1-5% loss of signal.

3.9. Adjust the imaging parameters to maximize the intensity while minimizing photobleaching. For coarse adjustments, change the ND filter being used during acquisition. For finer adjustments, change the exposure time in steps of 250 ms.

3.10. Repeat Steps 3.5 – 3.8 until adequate signal can be obtained while minimizing photobleaching.

Note: Typically, settings for the vinculin tension sensor in vinculin -/- MEFs are 1500 ms, 1500 ms, and 1000 ms for the donor, FRET, and acceptor channels respectively. The optimal values will vary with the type of illumination system, objective, filter sets, and sensor expression level.

4. Choose Parameters for FRAP Imaging

4.1. Optimize the laser settings to ensure complete bleaching of the region of interest (ROI) without bleaching the surrounding area or causing photodamage.

4.1.1. Fix one of the generated samples of the cells expressing the tension sensor with 4% paraformaldehyde for 10 min.

Note: This optimization does not depend on protein dynamics, and a fixed sample allows for maximum imaging time without worrying about cell health. This will also prevent the recovery of bleaching by mobile proteins, allowing for the isolation of the effect of bleaching, avoiding any effects from rapid, diffusion-mediated fluorescence recovery occurring between the incidence of bleaching and taking the first post-bleach image.

CAUTION: Paraformaldehyde solutions are toxic. This step should be performed in a fume hood and the solution should be disposed of according to institutional policies.

4.1.2. Rinse the sample 3 times with PBS and leave in PBS.

Note: The use of most commercially-available mounting media will affect fluorophore properties, making the sample unsuitable for FRAP imaging[54]. Ideally, the cells will be imaged immediately, but may be left overnight at 4 °C. Longer wait times will result in deterioration of the sample.

4.1.3. Place the sample into the microscope stage holder for imaging.

4.1.4. Open the laser configuration window. Start with setting a laser dwell time of 1000 µs and 10 pulses, meaning that each spot in the scan across the ROI will receive 10,000 µs of full-power laser

Note: A 500 mW, 515 nm laser was used for bleaching. This was chosen to selectively bleach Venus A206K, the acceptor in vinculin tension sensor, with maximal efficiency. If FRET-based tension sensors with other fluorescent proteins are used, another type of laser may have to be employed.

4.1.5. Find a cell expressing the tension sensor with clear localization to a structure of interest and acquire an image.

4.1.6. Draw a rectangular ROI outlining the area to bleach and store the ROI location. Pulse the laser. Snap another image of the sample.

Note: The box size should be approximately the size of the entire FA. Care should be taken that the box size does not vary drastically across experiments. The bleached area must be carefully monitored in proteins whose dynamics are affected by diffusion. This is a potential concern in transmembrane proteins, such as cadherins[47], or proteins that diffuse slowly[27,57].

4.1.7. Check the quality of photobleaching by checking that the entire ROI is bleached such that the intensity is near background levels. Additionally, make sure there is no bleaching outside of the ROI.

4.1.8. Adjust the laser settings as needed to achieve a significant amount of bleaching within the ROI without inducing significant bleaching outside the ROI. For coarse adjustments, raise and lower the dwell time in steps of 100 µs, and for fine adjustments, raise and lower the number of pulses in steps of 5 pulses.

4.1.9. Repeat Steps 4.1.5 – 4.1.8 until reaching the minimum settings at which the ROI is fully bleached without off-target photobleaching.

Note: Achieving a substantial initial bleaching value without inducing phototoxicity is a key aspect of FRAP analysis. Use the laser settings that result in a complete bleach in the fixed samples. In general, the minimal number of photons should be used to achieve the desired bleaching level. Also, the bleaching protocol should be kept relatively consistent during experiments, as alterations can affect measurements of protein dynamics[58].

4.2. Optimize time-lapse parameters to fully capture the dynamics of the protein of interest while minimizing photobleaching.

4.2.1. Prepare the microscopy set-up for live cell imaging, preferably with a heated stage and objective as well as CO_2 control. Allow to equilibrate for 20 min.

Note: To maintain the health of the imaged cells, temperature and pH must be maintained in the imaging vessel. A variety of heated stages and objective heaters can readily maintain the cell temperature at 37 °C. The control of pH for many media types can be accomplished by the use of a peristaltic pump to pass humidified 5% CO_2 over the sample at 15 mL/min. Alternatively, if CO_2 control is unavailable, live imaging media containing HEPES should be used to prevent large pH changes.

4.2.2. Place one of the generated samples of the cells expressing the tension sensor into the microscope stage holder for imaging. Allow to equilibrate for 10 min.

4.2.3. Using the MDA tool, set up a time-lapse to acquire 3-5 images pre-bleach, bleach the ROI, and continue taking 10-60 images.

Note: For vinculin at FAs, imaging every 5 s for 5 min post-bleach is sufficient to observe the dynamics without introducing excessive bleaching[31]. Useful starting points for imaging rate and duration for many other proteins can be found in the literature[47,48,59,60].

4.2.4. Use acceptor imaging settings that minimize the exposure of the sample to light, while maintaining a sufficient signal-to-noise ratio, to image the structure of interest. A good starting point is half of the ND filter and exposure time necessary for imaging of the acceptor during FRET.

4.2.5. Find a cell expressing the tension sensor with clear localization to a structure of interest and snap an image.

4.2.6. Draw a rectangular ROI to highlight where to bleach and store the ROI location. Initiate the time-lapse.

4.2.7. Examine the resulting set of images for potential issues.

4.2.7.1. If there are substantial jumps (greater than 10% of initial intensity) in fluorescence recovery between frames, reduce the time-step between frames.

4.2.7.2. If there is a significant global loss of fluorescence over time (greater than 5-10% of initial intensity), reduce the number of images taken post-bleach and/or change the imaging settings to reduce the exposure of the sample to light.

4.2.7.3. If fluorescence recovery has not plateaued by the end of the time-lapse, increase the total length of the time-lapse.

4.2.8. Adjust the time-lapse parameters accordingly and repeat Steps 4.2.5 – 4.2.7 until the fluorescence recovery is adequately captured without global photo-damage to the sample.

5. Acquire FRET-FRAP data

5.1. Prepare the microscopy set-up for live cell imaging, preferably with a heated stage and objective as well as CO_2 control. Allow to equilibrate for 20 min.

5.1.1. To ensure the health of the imaged cells, maintain the temperature at 37 °C in the imaging chamber. Use a peristaltic pump to pass humidified 5% CO_2 over the sample at 15 mL/min to maintain the pH.

5.1.2. Alternatively, if CO_2 control is unavailable, use live imaging media containing HEPES to prevent from large pH changes.

5.2. Open the MDA tool and set up with FRET imaging parameters, including the different filter sets.

5.3. Save this MDA to the experimental folder with the name of **MDA_FRET_Date**.

5.4. Set up another MDA with FRAP imaging parameters, including the different filter sets, the time-lapse settings, and the journal to pulse the laser after pre-bleach acquisition.

5.5. Save this MDA to the experimental folder with the name of **MDA_FRAP_Date**. Close the MDA window.

5.6. In the toolbar at the top of the screen, select **Journal | Start Recording**.

5.7. Open the MDA window, load the **MDA_FRET_Date** state and press **Acquire**. Then load the **MDA_FRAP_Date** state and press **Acquire**.

5.8. At the end of the acquisition, in the toolbar at the top of the screen, select **Journal | Stop Recording**.

5.9. Save this journal to the experimental folder with the name of **FRETFRAP_Date** and add it to a toolbar for easy access. Close the MDA window.

5.10. Place one of the generated samples of the cells expressing the tension sensor into the microscope holder for imaging. Allow to equilibrate for 10 min.

5.11. Navigate the sample using the image acquisition under **Acquire | Acquire** with minimal exposure time and ND filter to identify the cells of interest.

5.12. Set continuous autofocus by navigating to **Devices | Focus**. Manually focus on the sample until reaching the correct imaging plane.

5.13. Click **Set Continuous Focus**, wait for the system to adjust, and click **Start Continuous Focusing**.

Note: This is not required, but significantly improves quality of FRAP recovery curves because it prevents the sample from drifting out-of-focus.

5.14. Find a cell expressing the tension sensor with clear localization to a structure of interest and snap an image.

5.15. Draw a rectangular ROI to highlight where to bleach. Store the ROI location.

5.16. Initialize the **FRETFRAP_Date** journal, which will begin the acquisition of FRET images followed by the initialization of the FRAP time-lapse.

5.17. Repeat Steps 5.14-5.16 until 10-15 image sets are acquired.

Note: Measurement cannot be repeated in the same cell. Once photobleaching occurs, FRET data is unreliable.

6. Analyze FRET-FRAP data

6.1. Analyze the FRET images using the software of choice.

Note: There are several ways to image and quantitate FRET[61], including ratiometric FRET[62] and FRET index[34,35]. However, it is highly recommended to use the estimates of FRET efficiency[55,63] for the interpretation of FRET-FRAP data. See the Discussion for further exploration of this topic.

For sensitized emission and calculation of FRET efficiency, custom software is available from the Hoffman Lab at https://gitlab.oit.duke.edu/HoffmanLab-Public.

6.2. Quantify relevant parameters for each subcellular structure that was bleached. This should include average FRET index/efficiency and average initial acceptor intensity (proportional to concentration) but could also include physical parameters such as ROI size.

6.3. Analyze the FRAP images using the software of choice.

Note: There are several ways to quantitate FRAP[26-29]. Key experimental concerns include accounting for bleaching during post-bleach imaging, changes in background intensity, and translocation of highly dynamic subcellular structures, such as focal adhesions. Bleaching corrections and variations in background illumination can be accomplished through the analysis of non-bleached and non-fluorescent regions of the images. Highly dynamic subcellular structures, particularly those showing excessive growth or disassembly dynamics are incompatible with standard FRAP analyses and should not be analyzed. Additionally, there are a variety of ways to normalize the data. The provided guidelines are for the simplest analysis.

6.4. Correct the recovery data for bleaching effects and then normalize to pre-bleach intensities. Quantify the half-time of recovery and the mobile fraction according to the following equation[28,34]:

$$MF - (MF - R_o)e^{-kt}$$

where MF is the mobile fraction, R_o is the initial recovery, and k is the recovery rate. The half-time of the recovery is determined by:

$\tau_{1/2} = \ln 2/k$.

Note: There are a variety of publicly-available software packages for completing these analyses[64] as well as a variety of ImageJ plugins. Custom software is available from the Hoffman Lab at https://gitlab.oit.duke.edu/HoffmanLab-Public. More analyses should be used for the situations where the diffusion affects the dynamics of the protein of interest, multiple time scales are apparent in the recovery, or non-standard bleaching geometries are used.

7. Interpret FRET-FRAP data

7.1 Compile relevant information for each ROI including: FRET index/efficiency, acceptor intensity, FRAP half-time, FRAP mobile fraction.

Note: FRET index or efficiency is used to determine average load across the protein within the ROI. Acceptor intensity measures the local concentration of the protein. The half time of recovery is a measure of protein dynamics. A smaller half-time indicates more rapid turnover. FRAP mobile fraction measures the amount of protein within the ROI that is actively turning over. A larger mobile fraction indicates that a larger percentage of the protein within the ROI is turning over.

7.2. To probe the effect of local concentration on protein turnover rate and amount, plot FRAP half-time and mobile fraction against initial acceptor intensity.

7.3. To probe the effect of protein load on protein turnover rate and amount, plot FRAP half-time and mobile fraction against FRET index/efficiency.

Note: Depending on the protein or structure, it may also be interesting to examine effects of physical parameters, such as structure size or eccentricity, on protein load or turnover.

REPRESENTATIVE RESULTS:

FRET-FRAP is made up of the combination of two fluorescent techniques, FRET and FRAP. As we focused on the effects of protein load, we used FRET-based tension sensors[34,46]. These sensors are often based on a tension sensing module consisting of two fluorescent proteins, such as mTFP1 and VenusA206K, connected by a flagelliform linker (**Figure 1A**). When the module is placed between the head and tail domains of a protein, it is possible to measure the load exerted across the protein. When analyzing FRET data, images taken in the acceptor channel are used to assess tension sensor localization and concentration, as this signal is independent of FRET (**Figure 1B**). After the calculation of FRET efficiency, protein load can be visualized on a colorimetric scale where a decrease in FRET efficiency toward cooler colors indicates an increase in protein load, and FRET efficiencies in the red range indicate low protein load (**Figure 1B**). FRAP imaging is conducted by using a laser to bleach the acceptor fluorophore in a single subcellular structure and monitor recovery over time (**Figure 1C**). The resulting normalized FRAP curve can be analyzed to extract parameters describing the protein dynamics, including the half-time of recovery and mobile fraction (**Figure 1D**). Because FRET and FRAP analyses were performed on the same cell, the average protein load and turnover in a subcellular structure can be plotted as a single point. Imaging multiple cells yields multiple points and an emerging trend can indicate whether a protein is destabilized (**Figure 1E**) or stabilized by molecular load (**Figure 1F**).

The vinculin tension sensor (VinTS) stably expressed in vinculin null MEFs very clearly localizes to FAs spread throughout the cell, as seen by looking at the acceptor channel image (**Figure 2A**). The acceptor channel image is used to create a segmentation mask that identifies each individual FA with a unique ID, visually designated by different colors (**Figure 2B**). The segmentation algorithm is based on the "water" method and labels the FAs approximately in order of brightness, as previously described[34,65]. The segmentation results are converted to a binary mask which is then applied to the FRET efficiency results (**Figure 2C**), and the average FRET efficiency within each unique FA is calculated (**Figure 2D**). Additional properties can be calculated for each FA in a similar manner, including average acceptor intensity, size, eccentricity, and location within the cell. This way, whichever FA is chosen for FRAP, can be matched to the unique FA ID and the associated properties.

FRAP imaging and analysis is sensitive to several factors that can be controlled, including laser and imaging parameters, and some factors that cannot be controlled, such as overall FA stability[26-29]. For example, too much exposure to light during the time-lapse imaging can lead to major issues in interpreting FRAP data. Although the analysis of control FAs that were not

bleached can be used to normalize for minor photobleaching over time, with too much exposure of the sample to light, the resulting FRAP curve shows an initial recovery followed by a dip in normalized intensity that cannot be accurately fit with an exponential function. If this effect is consistently observed in the data, it is necessary to re-optimize the imaging parameters to either decrease exposure time, increase the time-step between imaging frames, or decrease the length of the time-lapse to reduce the exposure of the sample to light.

Another example of FRAP data that is uninterpretable, is when the FA that was photobleached translocates rapidly during recovery[28]. A representative case of excessive translocation is shown in **Figure 3**. The initial image, where the ROIs are chosen, does not give an indication of FA stability (**Figure 3A**). Monitoring the bleached FA over time, it quickly moves away from the initial position and the automated tracking is unable to immediately follow due to the low fluorescent signal following photobleaching (**Figure 3A**). The resulting FRAP curve shows an initial phase of slight recovery with a jump when the fluorescence is recovered enough for the software to detect the FA and move the ROI (**Figure 3B**). This curve cannot be successfully fit by an exponential function. The rapid translocation of the FA also suggests that the FA structure is unstable. Thus, unstable FAs should not be included in the same FRET-FRAP analysis as stable FAs, due to both technical and biological issues.

With satisfactory FRET and FRAP data, the next step is completing the FRET-FRAP analysis by simultaneously assessing protein load and dynamics. **Figure 4A** shows the FRET efficiency maps of three vinculin null MEFs stably expressing VinTS. The FAs outlined in white were chosen for FRAP analysis, and the acceptor intensities are shown over time. These three FAs have vinculin under different amounts of load and display a different vinculin recovery profile. Quantifying these properties by calculating the half-time of recovery and plotting against the average FRET efficiency in each FA demonstrates the overall trend of vinculin being stabilized by increased load (**Figure 4B**). However, the mobile fraction plotted against FRET efficiency shows no trend, suggesting that mobile fraction is not regulated by molecular load (**Figure 4C**). Introducing a point mutation into the VinTS at amino acid 50 (A50I) has been shown to prevent vinculin binding to a major binding partner within FAs, talin[66]. The alteration of this protein-protein interaction affects vinculin force-sensitive dynamics. Vinculin null MEFs stably expressing VinTS A50I have different cell and FA morphologies, different vinculin loading profiles, and different vinculin dynamics (**Figure 4D**). Quantifying the half-times of recovery and FRET efficiencies and plotting shows that when the vinculin-talin interaction is disturbed, vinculin at FAs is destabilized by increased load (**Figure 4E**) while mobile fraction shows no trend (**Figure 4F**).

FIGURE AND TABLE LEGENDS:

Figure 1. Principles of FRET-FRAP technique. (A) Schematic of the FRET-based tension sensor module (TSMod) inserted into a protein of interest and the effect of tension on the FRET signal. (B) To quantify FRET using sensitized emission, images are taken to capture donor signal (not shown), acceptor signal, and FRET signal. With appropriate corrections, the FRET image can be assigned a colorimetric scale to visualize how much tension is being applied to the sensor. (C) FRAP is conducted using the acceptor signal, which is directly proportional to the concentration. (D) FRAP imaging analysis produces curves of fluorescence intensity over time that can be fit

using mathematical models to determine protein dynamics. (E, F) When FRET and FRAP are combined, force and turnover in a single FA can be measured. Measuring multiple FAs in multiple cells yields a relationship between protein load and protein turnover. In this analysis, a relationship in which increased load correlates with increased turnover is referred to as a force-destabilized state (E). In this analysis, a relationship in which increased load correlates with decrease turnover is referred to as a force-stabilized state (F). This figure has been modified from Rothenberg *et al.*[37].

Figure 2. FA identification and FRET analysis. (A) A vinculin null MEF expressing the VinTS visualized in the acceptor channel, where the intensity indicates local concentration of vinculin. Scale bar = 30 µm. (B) FAs are segmented based on the acceptor channel to create a FA ID mask where each FA is assigned a unique ID, here shown as different colors, approximately in order of brightness. (C) The FA ID mask is converted to a binary mask and applied to the FRET efficiency image to show the FRET efficiency values only at FAs. (D) The FRET efficiency within each FA is averaged to obtain a single value for each FA, which is associated with the FA ID in the output data table.

Figure 3. Example of a translocating FA. (A) A vinculin null MEF expressing the VinTS A50I mutant sensor is visualized in the acceptor channel, with the color table inverted for clarity. Scale bar = 30 µm. The FA outlined in black was selected for bleaching. Zoomed-in images show the FA progression over time with the red outline indicating where the software identified the FA. Scale bar = 2 µm. (B) The resulting normalized FRAP curve from data in (A). There is an approximately 5% jump in intensity following point 3 resulting from the FA translocating quickly before sufficient recovery for the software to detect the change in FA location.

Figure 4. Representative FRET-FRAP results. (A) Vinculin null MEFs expressing the VinTS displayed as average FRET efficiency images of the entire cell (scale bar = 30 µm) with zoomed-in inverted acceptor channel images showing FRAP recovery progression (scale bar = 2 µm). (B) FRAP half-time of recovery plotted against FRET efficiency for 32 cells, with the points representing cells in (A) highlighted in red. (C) FRAP mobile fraction plotted against FRET efficiency for the same cells in (B). (D) Vinculin null MEFs expressing the VinTS A50I mutant sensor displayed as average FRET efficiency images of the entire cell (scale bar = 30 µm) with zoomed-in inverted acceptor channel images showing FRAP recovery progression (scale bar = 2 µm). (E) FRAP half-time of recovery plotted against FRET efficiency for 21 cells, with the points representing cells in (D) highlighted in red. (F) FRAP mobile fraction plotted against FRET efficiency for the same cells in (E). Data were originally published in Rothenberg *et al.*[37] and are visualized here in a new format.

DISCUSSION:

The FRET-FRAP method allows for direct measurement of force-sensitive protein dynamics, a property that has been difficult to directly probe inside living cells. The sensitivity of protein dynamics to molecular load is critical to the protein's function as a force transmitter or transducer. Loading is required for the transmission of both internally-generated and externally-applied forces, called mechanotransmission, and for the conversion of those forces into

biochemically-detectable signals, called mechanotransduction. However, the alterations in load can affect the duration a protein stays bound, thus, the less time a protein spends bearing load, the less chance the force has to be transmitted to other proteins or transduced into a biochemically-detectable signal and sensed. The FRET-FRAP method bridges the gap between the molecular and cellular level by allowing molecular-scale measurements of force-sensitive dynamics to be accessed within a broader cellular context. Furthermore, it allows for these measurements to be taken while perturbing the intracellular or extracellular environment either biochemically or mechanically. This technique should be applicable to any FRET-based tension sensor, allowing for the investigation of protein mechanical state in a variety of subcellular structures and extracellular contexts.

Critical steps in ensuring that the desired FRET-FRAP measurements are obtained involve optimizing the imaging parameters and performing data analysis and interpretation. Optimizing the imaging parameters, as described within the protocol, is necessary to limit the photodamage to the sample, while allowing for the desired structures and dynamics to be distinguished and for sufficient signal strength for the calculation of FRET. Establishing these imaging parameters for a particular cell line and protein of interest early on will facilitate direct comparison between different experimental groups. It is worth noting that alterations to the system, such as mutating the protein of interest or introducing inhibitors, can lead to changes in protein localization (thereby altering signal intensity) and dynamics. The optimized parameters should enable clear, accurate measurements across all experimental conditions. Therefore, it is recommended to choose the parameters that are not at the extreme end of being useful, for example, being able to barely distinguish signal from noise.

While the imaging in this protocol was described for an epifluorescence microscope and attached FRAP laser module, FRET-FRAP is applicable to other imaging systems. For example, this technique can be adapted to line-scanning confocal microscopes as well as spinning-disk confocal microscopes with an attached photobleaching module. Imaging settings should be optimized in an analogous fashion to achieve adequate signal-to-noise without causing photodamage or excessive photobleaching. Particularly concerning FRET imaging, high quantum efficiency detectors are required to obtain sufficient signal for successful FRET calculation without inducing fluorophore damage. There are a number of publications describing separate FRET or FRAP imaging using a confocal microscope[67-69], which can be used to guide optimization for FRET-FRAP imaging.

Following the experiment, data analysis should be treated carefully and performed in a reproducible, preferably automated, manner. Due to the inability to bleach more than 2-3 subcellular regions in a single cell before bleaching too much of the available pool of protein, the throughput of this technique is relatively limited. Thus, data sets are often combined across multiple days of imaging, requiring consistent treatment of data. Both FRET and FRAP offer challenges with data analysis. FRET index and FRET efficiency measurements allow for a quantification of protein load. FRET index is a relative measure that is highly dependent on microscope settings, while FRET efficiency measurements are absolute and independent of microscope settings[55,70]. We have recently shown that a previously developed method using

"three-cube" imaging can be used to determine FRET efficiency from measurements of sensitized emission that are typically quantified with FRET Index when using FRET-based tension sensors[56]. The measurements of FRET efficiency are required if the measurements of the absolute forces experience by the tension sensors are to be calculated[34]. The cells expressing FRET-based tension sensors, especially stable cells at high passage numbers, may recombine or degrade the sensors, leading to unusable FRET data[50]. This is easily identified when calculating donor-to-acceptor ratios during the calculation of FRET efficiency[37,56] but may be harder to detect using FRET index. When starting with a FRET-based tension sensor, it can be helpful to obtain a large data set (>50 cells) of only FRET data for the constructs of interest to identify the expected range of FRET efficiencies. Additionally, FRAP data may be difficult to extract from structures that are very mobile, such as FAs that are rapidly sliding or disassembling. Selecting a subpopulation of structures or optimizing cell plating conditions to mitigate this effect can help to minimize this issue.

In concept, FRET-FRAP can be applied to any FRET-based sensor in any subcellular region, with proper optimization. In practice, it may be difficult to capture force-sensitive dynamics of proteins that are not under substantial mechanical load or that have half-times of recovery on the very short timescale of a few seconds or on the long timescale of tens of minutes. Results from single-molecule studies can point to the proteins that may demonstrate force-sensitive dynamics within living cells. Thus, far this includes many FA and AJ proteins[13-16] as well as some cytoskeletal elements[71-73]. Fortuitously, FRET-based sensors have been designed for many of these proteins[46]. These results can guide the selection of a protein of interest; however, it should not be expected that FRET-FRAP data will exactly mirror the results from these single-molecule studies. In fact, biochemical regulation, interactions with other proteins, and local cytoskeletal structure may obscure, or alter, the effects of forces on protein-protein interactions. The ability to observe these complexities is a unique strength of the FRET-FRAP approach.

A combination of manipulations to the cell and the protein of interest can be used to elucidate the important factors in regulating protein dynamics. For example, it can be helpful to have a sensor that is force-insensitive, either through the deletion or mutation of a force-binding domain[35,74] as there should be no dependence of the protein turnover dynamics on the force reported by the sensor. Additionally, the mutations of other critical binding sites or phosphorylation sites in the protein can provide a more complete picture of how the protein of interest is being regulated. Making global changes to the cell or the environment through cytoskeletal inhibitors or by changing the substrate properties (ex. extracellular matrix or stiffness), respectively, can help determine how the force-sensitive dynamics of the protein respond to mechanical perturbations. Combining the information on protein load and force-sensitive dynamics with other biophysical properties of the protein can help to establish the mechanical state of the protein of interest. This can include the localization and local protein-protein interactions within a subcellular structure[75,76]. Additionally, the protein could reside in different conformation states, even within the same subcellular structure, depending on context[76,77]. Protein load, dynamics, localization, and conformation can all be simultaneously affected by internally-generated and externally-applied forces[37,76,78,79], dictating a protein's role in force transmission and mechanotransduction. The versatility of the FRET-FRAP method and its

potential compatibility with a variety of proteins and manipulations should enable the elucidation of the interaction between bulk mechanics, protein dynamics, and mechanosensitive signaling.

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The authors have nothing to disclose.

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78 Kim, T. J. *et al.* Dynamic visualization of alpha-catenin reveals rapid, reversible conformation switching between tension states. *Current Biology.* **25** (2), 218-224, (2015).
79 Yao, M. *et al.* Mechanical activation of vinculin binding to talin locks talin in an unfolded conformation. *Scientific reports.* **4** 4610, (2014).

Figure 1

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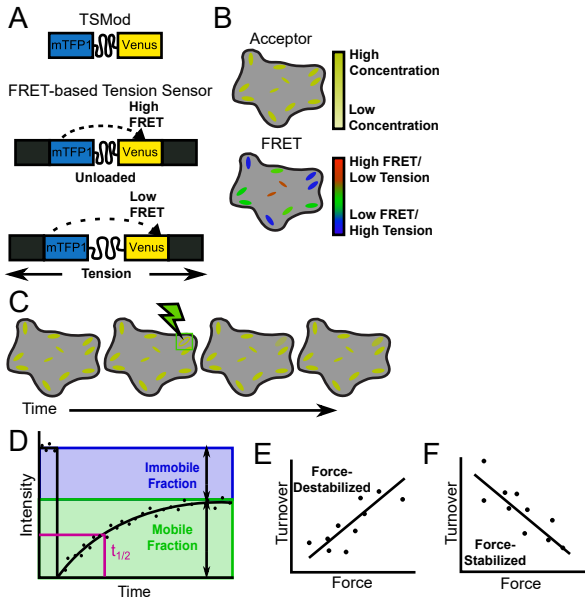

Figure 2

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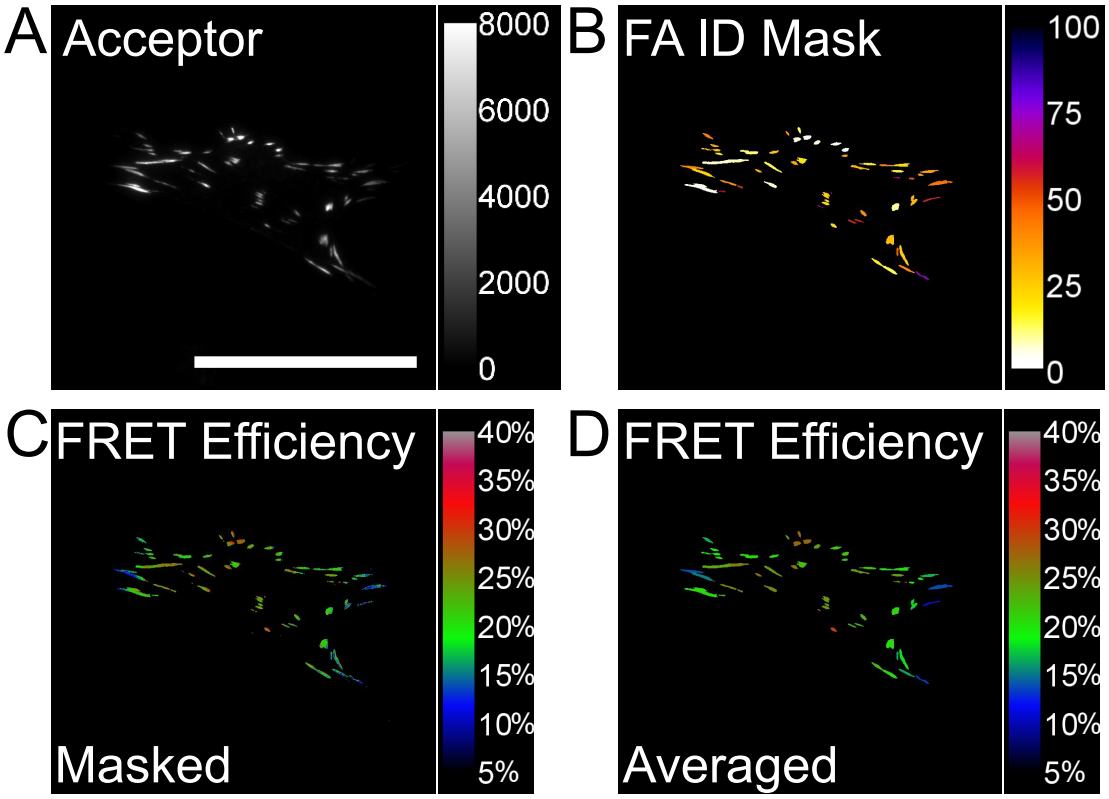

Figure 3

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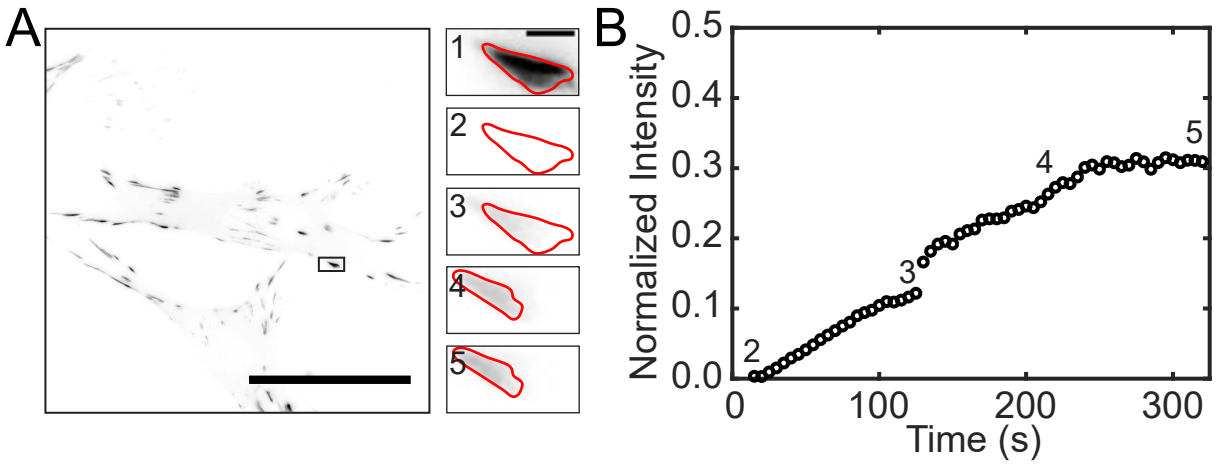

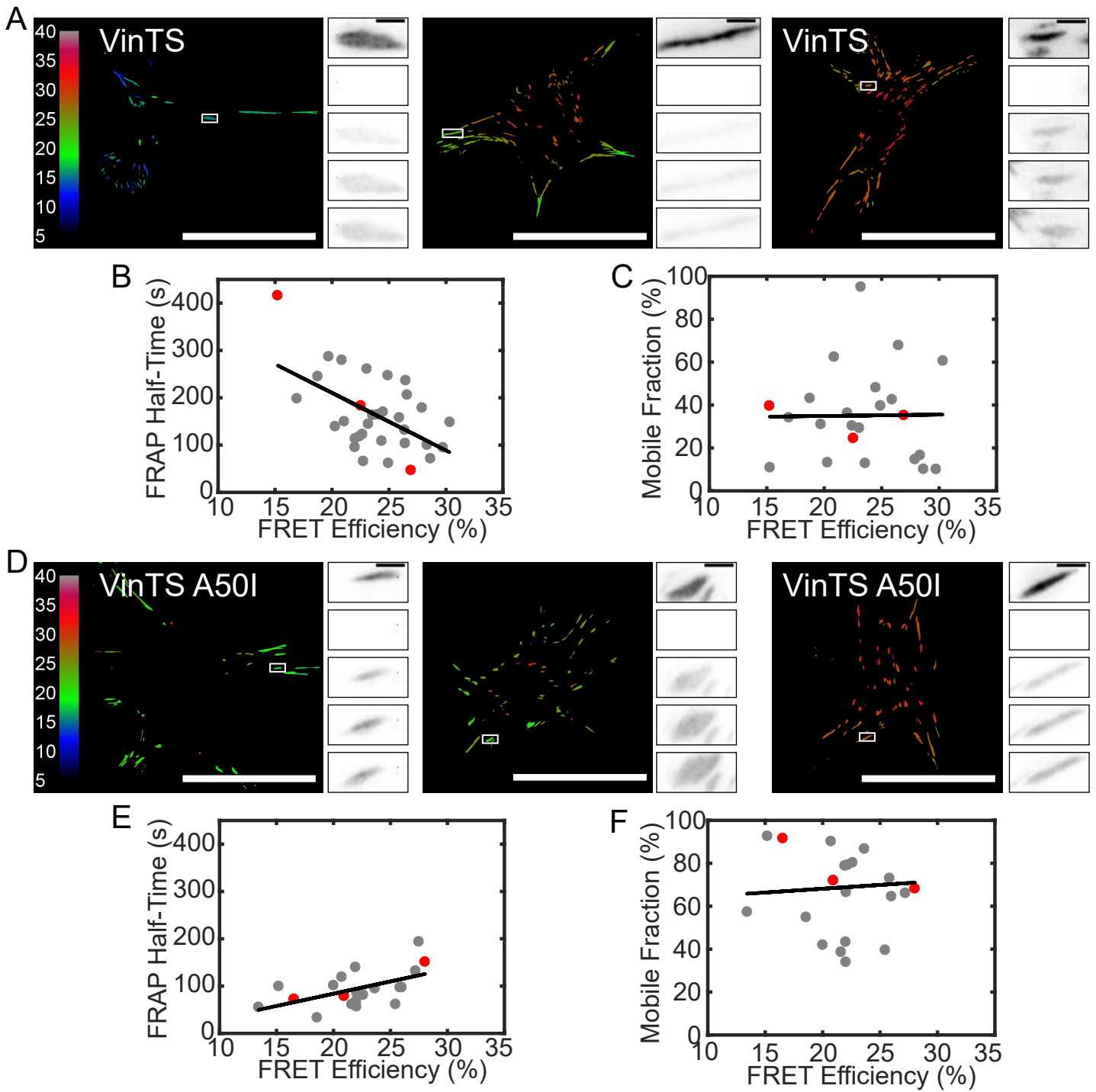

Table of Materials

Click here to download Table of Materials JoVE_Materials_Final.xls

Name of Material/ Equipment	Company	Catalog Number
0.05% Trypsin-EDTA	Thermo Fisher	25300062
16% Paraformaldehyde	Electron Microscopy Sciences	30525-89-4
60x Objective NA1.35	Olympus	UPLSAPO 60XO
Antibiotic-Antimycotic Solution (100x)	Gibco	15240-062
Automated Stage	Prior Scientific	H117EIX3
Custom Dichroic Mirror	Chroma Technology Corp	
Custom mTFP1 Emission Filter	Chroma Technology Corp	
Custom mTFP1 Excitation Filter	Chroma Technology Corp	
Custom Venus Excitation Filter	Chroma Technology Corp	
DMEM-gfp Live Cell Visualization Medium	Sapphire	MC102
Dulbecco's Modified Eagle's Medium	Sigma Aldrich	D5796
Fetal Bovine Serum	HyClone	SH30396.03
Fibronectin, Human	Corning	47743-654
FRAPPA Calibration Slide	Andor	
FRAPPA System with 515 nm Laser	Andor	
Glass-bottomed Fluoro Dishes	World Precision Instruments	FD35
HEK293-T Cells	ATCC	CRL-3216
Hexadimethrine Bromide, Polybrene	Sigma Aldrich	H9268-5G
High-glucose Dulbecco's Modified Eagle's Medium	Sigma Aldrich	D6429
Inverted Fluorescent Microscope	Olympus	IX83
JMP Pro Software	SAS	
Lambda 10-3 Motorized Filter Wheels	Sutter Instruments	LB10-NW
LambdaLS Arc Lamp with 300W Ozone-Free Xenon Bulb	Sutter Instruments	LS/OF30
Lipofectamine 2000	Invitrogen	11668-027
MATLAB Software	Mathworks	
MEM Non-Essential Amino Acids	Thermo Fisher	11140050
MetaMorph for Olympus	Olympus	
Micro-Humidification System	Bioptechs	130708
MoFlo Astrios EQ Cell Sorter	Beckman Coulter	B25982
Objective Heater Medium	Bioptechs	150819-13
OptiMEM	Thermo Fisher	31985070

Phosphate Buffered Saline	Sigma Aldrich	D8537
pMD2.G Envelope Plasmid	Addgene	12259
pRRL Vector		
psPax2 Packaging Plasmid	Addgene	12260
sCMOS ORCA-Flash4.0 V2 Camera	Hamamatsu Photonics	C11440-22CU
Sorvall Legend XT/XF Centrifuge	Thermo Fisher	75004505
Stable Z Stage Warmer	Bioptechs	403-1926
Venus Emission Filter	Semrock	FF01-571/72

Comments/Description

T450/514rpc
ET485/20m
ET450/30x
ET514/10x

with L-glutamine and sodium bicarbonate

provided along with FRAPPA unit

gift from Dr. Kam Leong (Columbia University)

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Author(s): Katheryn E. Rothenberg, Brenton D. Hoffman

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CORRESPONDING AUTHOR:

Name:	Brenton D. Hoffman
Department:	Biomedical Engineering
Institution:	Duke University
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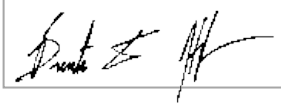
Signature:	 Date: 6/13/2018

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Re: Article Number JoVE58619
Title: "Measurement of force-sensitive protein dynamics in living cells using a combination of fluorescent techniques"

Response to Editor and Reviewer Comments:

We thank the editor and the reviewers for their detailed reading and thoughtful comments regarding our manuscript. We have done our best to address all comments and, as a result, the manuscript is significant improved.

Editorial comments:
Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have thoroughly proofread the manuscript and believe there are not errors.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

The copyright for the figures was obtained before the first submission and not significant changes have been made.

3. Please provide an email address for each author.

This has been done.

4. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

The short abstract has been re-written:

Here, we present a protocol for the simultaneous use of Förster Resonance Energy Transfer-based tension sensors to measure protein load and Fluorescence Recovery After Photobleaching to measure protein dynamics enabling the measurement of force-sensitive protein dynamics within living cells.

5. Please rephrase the Long Abstract to more clearly state the goal of the protocol.

This point has been addressed through the addition of the following sentence to the Long abstract.

"Here we describe a protocol for the Förster Resonance Energy Transfer-Fluorescence Recovery After Photobleaching (FRET-FRAP) technique, which enables the measurement of force-sensitive protein dynamics within living cells."

6. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

We had edited the Introduction to clearly state the overall goal of the method. Especially the following sentence in the final paragraph:

“Here we present a protocol that combines FRET-based tension sensors with FRAP-based measure of protein dynamics. We refer to this technique as FRET-FRAP. This approach enables the simultaneous measurement of protein load and protein dynamics, thus enabling assessment of the force-sensitive dynamics of proteins in living cells (Figure 1).”

7. Please define all abbreviations before use.

We believe we have properly defined all abbreviations.

8. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

We believe we have used SI abbreviations for all units

9. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.
We believe we have put space after all number.

10. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Metamorph, MATLAB, etc.

We believe we have removed these. A search of Metamorph and MATLAB revels no occurrences.

11. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Searching for these terms, reveals no more occurrences of personal pronouns in the protocol.

12. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.

We believe that the protocol only contains action items directing the reader to do something and that these items are written in the imperative tense.

13. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

We believe we have added sufficient detail to any potentially confusing steps. We also believe we have answered the “how” question and referenced published material when necessary.

14. 1.1.1: Please describe how this is done.

We have provided references to protocols describing this procedure

15. 1.3.3: Please provide composition of the complete media.

Composition for complete media has been added.

16. 1.3.7: Please provide composition of the imaging media. Please also specify the volume of imaging media used.

The composition of the imaging media has been specified in a note as in contains a commercial production. The volume of imaging medium has been added.

17. 5.1: Please mention how CO2 is controlled.

CO2 control and more details regarding live imaging have been added.

18. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted less than 2.75 pages that identify the essential steps of the protocol for the video.

19. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

The highlighted portion is written in complete sentences with actions in the imperative tense.

20. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We believe that sufficient detail to preform the highlighted steps is written.

21. References: Please do not abbreviate journal titles.

References have been edited to display the full journal titles.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This work by Rothenberg and Hoffman describes a new methodology based on the simultaneous combination of FRET and FRAP techniques for the measurement of protein dynamics within living cells. The authors applied this methodology to measure the response of focal adhesions due to molecular load, and in particular on vinculin. This new methodology is interesting to measure the mechanotransductive properties of force-sensitive proteins, and could be applied to many other types of mechanosensitive proteins and cellular structures. Even though the described technique is highly complex and some practixe will be required to master it, in the opinion of this referee the manuscript provides interesting results based on the original publication of the same authors. However, there are minor problems that the authors need to address before being accepted for publication. See below other comments and concerns.

We thank the reviewer the detailed reading of the manuscript and positive outlook on the usefulness of this technique.

Major Concerns:
In general, more clarification and caution notes are missing below each point of the protocol. These notes should provide further information about security issues, alternative methods, or tips, among other informations. Authors are therefore encouraged to add more notes in this regard.

We agree with the reviewer and added substantial more notes. Unfortunately, the additions are too numerous to include in the letter.

For clarity, please consider adding a brief paragraph about future improvements of the described techniques at the end of the manuscript.

We feel that it will confuse readers to have a description of other possible techniques at the end of this protocol paper, and therefore have chosen not to complete this suggestion.

Minor Concerns:
INTRODUCTION SECTION

1) Page 3, Paragraphs 3-4: The mechanoresponsive properties of focal adhesions and cadherins must be described by commenting on the pioneering works from Sasha Bershadsky and Daniel Riveline. The following works need to be included in the Introduction section:
-Riveline et al, Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism. J Cell Biol. 2001, 153(6):1175-86.
-Brevier et al, Force-extension relationship of cell-cell contacts. Phys Rev Lett. 2007, 98(26):268101.

Citations for both works have been added.

2) For clarity, a last paragraph should be included at the end of this section. It should briefly describe the protocol content and field of application. It should clearly state the cellular structure to be studied by means of the FRET-FRAP methodology (i.e. vinculin). Finally, to differentiate from the original publication, the authors must clarify that directed cell migration will not be addressed in this protocol, but only the FRET-FRAP technique.

We agree that the last paragraph of the introduction needed more refinement. It has been edited with the reviewer's comments in mind.

Unlike previous approaches in mechanobiology, the advent of FRET-based tension sensors allows direct measurement of loads experienced by specific proteins inside living cells. Here we present a protocol that combines FRET-based tension sensors with FRAP-based measure of protein dynamics. We refer to this technique as FRET-FRAP. This approach enables the simultaneous measurement of protein load and protein dynamics, thus allowing assessment of the force-sensitive dynamics of proteins in living cells (Figure 1). Already, the FRET-FRAP technique has been applied to the study of the force-sensitive dynamics of the mechanical linker protein vinculin. Tension sensors have been developed for numerous proteins that are relevant in a variety of subcellular structures. For example, sensors have been developed for vinculin and talin in FAs, cadherins and catenins in AJs, nesprin in the nuclear LINC complex, α-actinin and filamin in the cytoskeleton, and MUC-1 in the glycocalyx, among others. Similarly, FRAP is a commonly use technique has been used on mechanosensitive proteins within the focal adhesions, adherens junctions, actin cortex, and nucleus. Moving forward, the FRET-FRAP technique should be broadly applicable to any of these existing sensors or newly developed sensors, allowing for measurements of force-sensitive dynamics in a wide variety of cellular structures and contexts. Towards this end, we provide a detailed, generalized protocol for implementing the FRET-FRAP technique applicable in these different systems. Hopefully, this will enable a wide variety of experiments elucidating the roles of various mechanosensitive proteins in regulating force transmission and in mediating cell behavior.

Given the title of the manuscript, as well as alterations to the summary and introduction, we do not feel it is necessary to explicitly state the protocols regarding the study of directed cell migration are not addressed in the manuscript.

PROTOCOL
1) Page 4, point 1.1.3: Full details about the MEFs cells should be provided.

The protocol has been changed to focus on a "desired cell type". As there is no reason to suspect that FRET-FRAP will only work in one cell type, we believe the level of detailed now provided is appropriate. If researchers seek to reproduce the exact results of the Biophysical Journal paper, the details of the MEFs are provided there.

2) Page 4, point 1.1.4: This should be included as a note below point 1.1.3.

This change has been made.

3) Page 4, section 1.2.1: Hyphen in "35-mm". Consider removing to be consistent with 1.3.1.

This change has been made

4) Page 5, section 1.2.2: "invert" what? Include PBS 1x instead of just PBS (this is valid for the entire manuscript; please, correct).

The specification of the use of canonical tube has been specified in section 1.2.2. As we only discuss standard PBS, we feel the nomenclature of 1x PBS is redundant.

5) Page 5, section 1.2.3: Replace "solution" by "fibronectin solution"; Replace "hr" by "h" (the same in 1.3.7; Please, check the entire manuscript).

Both corrections have been made. We have searched and all use of h for hour have been removed.

6) Page 5, section 1.3.1. Give full details about the culturing conditions, i.e. type of media, serum, etc. Define the "complete media" in this section.

Details regarding culture conditions was added.

7) Page 6, section 2.2: Please, provide the actual experimental values used in all the subsections 2.2.x.

This section regards the calibration of the laser used the FRAP, and the pertinent value are listed. We think the reviewer is referencing Section 3 and have provided the pertinent parameters.

8) Page 8, section 4.1.4: Provide full technical details about the laser for FRAP experiments.

Details regarding the laser for FRAP experiments have been added.

9) Page 8, section 4.2.1: Provide full details about cell culture conditions: temperature (i.e. 37ºC?) and CO_2 level (5%?). Idem in section 5.1.

Pertinent details have been added to both sections. Additionally, the specific live cell imaging chamber we use is listed in associated spreadsheet.

10) Page 8, section 4.2.3. Provide references.

Appropriate references have been added. .

FIGURES

1) Figure 4A, 4D: Provide color bars next to the figures.

The requested change to the figure has been made.

2) Figure 4C and 4F: Remove fitting. The points show no trend.

We believe that indicating the lack of trend by a flat line is suitable.

REFERENCES
1) Short name in some publications is missing (e.g. see ref. 16, 23,...).

This issue has been fixed for all references.

MATERIAL/EQUIPMENT
1) For clarity, re-order the different items in alphabetical order.

Items have been alphabetized.

Reviewer #2:
Manuscript Summary:
Overall this is a timely protocol article on the FRET-FRAP technique, which was recently published by the Hoffman group. Two existing techniques FRAP and FRET (which by themselves are widely used) are being combined to understand the relationship between tensile forces and protein turnover.

We thank for the reviewer for their positive assessment of the manuscript.

Major Concerns:
No major concerns are noted.

Minor Concerns:
Some of the text is highlighted in yellow. This seems to be an obvious oversight, perhaps a draft copy was uploaded?

Highlighted text in yellow represents text that will be used for the video portion, a formatting requested by JOVE.

There is a bit of discussion on FRET efficiency vs index measurements. Could the authors link to a JOVE or other protocols paper that discusses FRET and how to apply efficiency measurements? As written it is not clear to a new user how one would go about measuring FRET and different approaches that could be taken.

We have added an extended discussion of FRET and several key citations throughout the manuscript. The key paragraph from the Discussion section is pasted below for convenience. The citations have been removed for clarity.

"FRET index and FRET efficiency measurements allow for a quantification of protein load. FRET index is a relative measure that is highly dependent on microscope settings, while FRET efficiency measurements are absolute and independent of microscope settings. We have recently shown that a previously developed method using "three-cube" imaging can be used to determine FRET efficiency from measurements of sensitized emission that are typically quantified with FRET Index when using FRET-based tension sensors. Measurements of FRET efficiency are required if measurements of the absolute forces experience by the tension sensors are to be calculated.