Genetically Encoded FRET-Based Tension Sensors

Anna-Lena Cost,[1,2,3] Samira Khalaji,[1,2] and Carsten Grashoff[1,2,3]

[1]Group of Molecular Mechanotransduction, Max Planck Institute of Biochemistry, Martinsried, Germany
[2]Department of Quantitative Cell Biology, Institute of Molecular Cell Biology, University of Münster, Münster, Germany
[3]Corresponding authors: *alcost@biochem.mpg.de*; *grashoff@uni-muenster.de*

Genetically encoded Förster resonance energy transfer (FRET)-based tension sensors measure piconewton-scale forces across individual molecules in living cells or whole organisms. These biosensors show comparably high FRET efficiencies in the absence of tension, but FRET quickly decreases when forces are applied. In this article, we describe how such biosensors can be generated for a specific protein of interest, and we discuss controls to confirm that the observed differences in FRET efficiency reflect changes in molecular tension. These FRET efficiency changes can be related to mechanical forces as the FRET–force relationship of the employed tension sensor modules are calibrated. We provide information on construct generation, expression in cells, and image acquisition using live-cell fluorescence lifetime imaging microscopy (FLIM). Moreover, we describe how to analyze, statistically evaluate, and interpret the resulting data sets. Together, these protocols should enable the reader to plan, execute, and interpret FRET-based tension sensor experiments.

Keywords: biosensor • FLIM • FRET • mechanotransduction • tension sensor

How to cite this article:
Cost, A.-L., Khalaji, S., & Grashoff, C. (2019). Genetically encoded FRET-based tension sensors. *Current Protocols in Cell Biology*, e85. doi: 10.1002/cpcb.85

INTRODUCTION

Intracellular Force Transduction Occurs in the Piconewton Range

Even though cells can sense a wide range of mechanical stimuli, transduction of mechanical information inside cells seems to occur in a rather narrow window of low piconewton (pN) forces. Force propagation in biological systems is restricted to comparably low force magnitudes because most protein–protein interactions break, and many molecules denature when exposed to higher mechanical loads. The unfolding of proteins in the range of some tens to hundreds of pN was demonstrated using single-molecule atomic force microscopy (smAFM). For example, GFP-like molecules unfold around 35 to 40 pN (Dietz & Rief, 2004), and β-helical proteins denature at >40 pN (Alsteens, Martinez, Jamin, & Jacob-Dubuisson, 2013). Prominent structures like immunoglobulin domains (Rief, Gautel, Oesterhelt, Fernandez, & Gaub, 1997) or fibronectin type III repeats (Oberhauser, Badilla-Fernandez, Carrion-Vazquez, & Fernandez, 2002) unravel at forces of about 100 pN. Physiologically relevant force transduction, however, must occur below the threshold of protein denaturation to maintain the biological activity of the involved proteins. In line with this model, typical motor proteins—such as myosins

CURRENT PROTOCOLS A Wiley Brand
Current Protocols in Cell Biology e85
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpcb.85

(Finer, Simmons, & Spudich, 1994), kinesin (Svoboda, Schmidt, Schnapp, & Block, 1993), or dynein (Gennerich, Carter, Reck-Peterson, & Vale, 2007)—generate forces of around 5 pN per molecule, and such forces modulate, but do not abolish, protein activity. For example, application of low pN forces to the cell adhesion molecules talin and α-catenin leads to the exposure of cryptic binding sites and recruitment of their common binding partner vinculin (del Rio et al., 2009; Maki et al., 2016; Yao et al., 2016). Furthermore, enzymatic cleavage of von Willebrand factor is greatly facilitated by low pN forces (Zhang, Halvorsen, Zhang, Wong, & Springer, 2009), and certain receptor–ligand interactions, like the integrin–fibronectin bond, become stabilized at low mechanical loads (Kong, García, Mould, Humphries, & Zhu, 2009). Altogether, the available data indicate that important intracellular processes of mechanotransduction occur in the low pN range. Förster resonance energy transfer (FRET)-based tension sensors are developed to measure these forces.

Basic Working Principle of FRET-Based Tension Sensors

The basic design principle of FRET-based tension sensors is to integrate a tension sensor module (TSM) into the protein of interest (POI). TSMs comprise of two fluorophores that undergo efficient FRET and are connected with a mechanosensitive linker peptide; this linker element reversibly unfolds at low pN forces, which increases the separation distance between fluorophores thereby reducing the FRET efficiency. Thus, mechanical forces across the TSM cause a reduction in FRET efficiency, which can be quantified in live cells for example by fluorescence lifetime imaging microscopy (FLIM). As TSMs are genetically encoded, they can be inserted into intracellular and extracellular proteins to determine whether these molecules are exposed to mechanical tension (Cost, Ringer, Chrostek-Grashoff, & Grashoff, 2015).

To date, a range of proteins has been investigated with FRET-based tension sensors to quantify molecular tension in subcellular structures like cell adhesion complexes or the actin cortex (reviewed in Freikamp, Cost, & Grashoff, 2016; Gayrard & Borghi, 2016; Jurchenko & Salaita, 2015). If conducted properly, such experiments can provide unprecedented insights into the molecular mechanisms of cellular force transduction. However, careful planning and implementation of experimental procedures as well as proper data analysis are essential to obtain conclusive results and avoid data misinterpretation.

This article describes general considerations on how to develop and apply FRET-based tension sensor probes to investigate molecular tension within cells. We describe how to design tension sensor and control constructs and how to perform control experiments. In addition, we provide recommendations for cloning, preparation of cells, FRET experiments using FLIM, data analysis, and data interpretation to cover the complete workflow of a tension sensor experiment (Fig. 1).

STRATEGIC PLANNING

This section describes general considerations on the design of tension sensor constructs, suitable controls, and experimental planning.

Choice of the Tension-Sensitive Linker Peptide in TSM

At the beginning of a tension sensor project, it is usually unclear how much force a POI experiences in cells. The force sensitivity of a TSM is determined by the employed linker peptide, as unfolding of the linker increases fluorophore separation distance and reduces FRET (Fig. 2A). It is therefore often advantageous to generate a set of tension sensor constructs that are based on different TSMs, each sensitive to a distinct force regime (Table 1, Fig. 2B). Whenever choosing a particular TSM, keep in mind that a number of

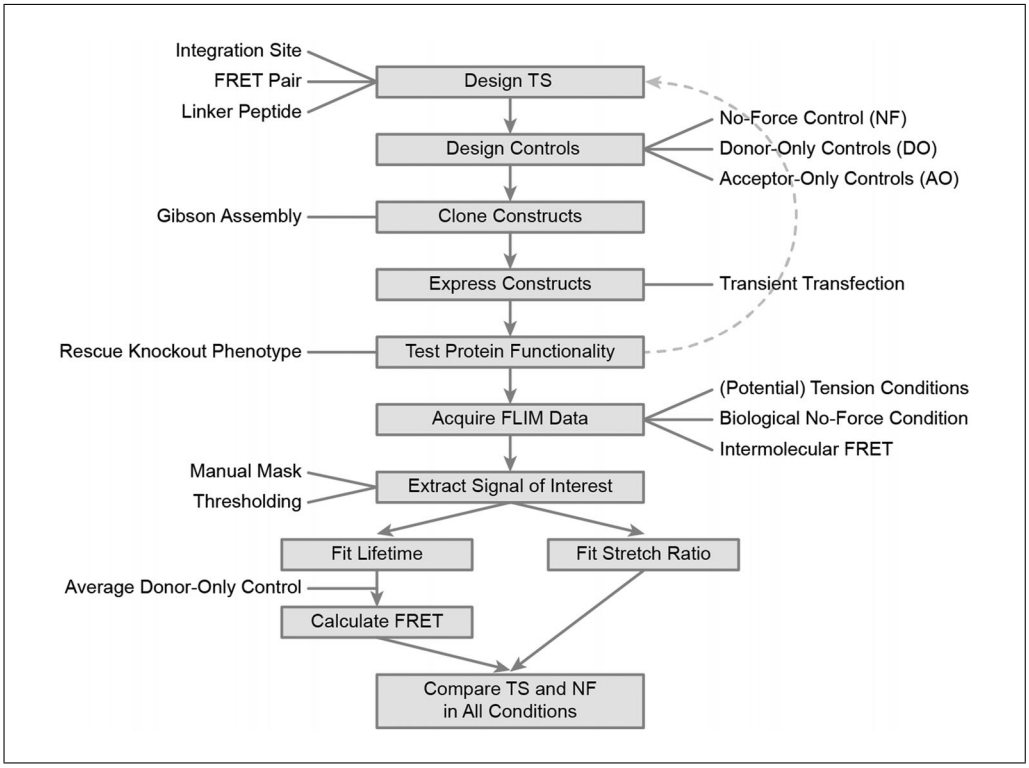

Figure 1 Workflow of a tension sensor experiment. First, tension sensor (TS) and matched no-force (NF), donor-only (DO), and acceptor-only (AO) controls are designed, cloned, and expressed in the model system of choice. Once an integration site has been identified that preserves protein functionality after TS module (TSM) integration, fluorescence lifetime imaging microscopy (FLIM) is performed under different conditions to compare tension sensor and no-force control. After signal extraction, either an average lifetime is determined to calculate Förster resonance energy transfer (FRET) efficiencies, or the ratio between stretched and non-stretched molecules is determined.

criteria have to be fulfilled to allow efficient force measurements. First, the linker peptide should not only extend under tension but also refold within a few milliseconds when forces dissipate. Second, the unfolding and refolding transition pathways of the linker peptide should be identical and thus free of hysteresis; this is important to be able to assign a given FRET efficiency to a distinct force value. Third, the force response should be insensitive to the speed at which mechanical forces are applied because it is usually unknown how quickly forces act on molecules in cells. To ensure that these specific biophysical requirements are fulfilled, TSMs should be calibrated, ideally with single-molecule force spectroscopy. Such experiments have demonstrated that F40 (Grashoff et al., 2010; Ringer et al., 2017), FL (Ringer et al., 2017), HP35 (Austen et al., 2015), and HP35st (Austen et al., 2015) quickly and reversibly fold and are free of hysteresis (Table 1). As these sensors were characterized in the presence of donors and acceptors, it was also confirmed that the employed fluorophores did not impair the force response characteristics of the sensor peptides. We therefore strongly recommend only using calibrated TSMs.

If no information on the expected force range is available, we suggest starting with the FL-TSM because it responds with a marked FRET decrease at comparably low forces but benefits from a higher starting FRET efficiency as compared to F40-TSM (Ringer et al., 2017; Fig. 2B). To evaluate higher forces, HP35-TSM and HP35st-TSM should be utilized, which are most sensitive to forces of 6 to 8 and 9 to 11 pN, respectively (Austen et al., 2015; Fig. 2B).

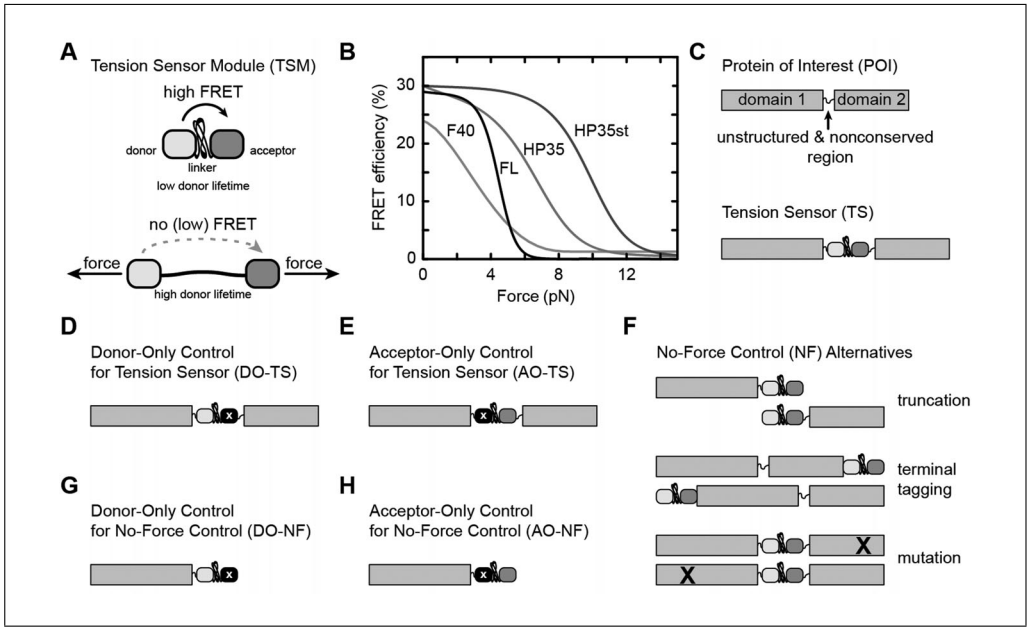

Figure 2 Tension sensor and control design. (**A**) The force-sensitive linker peptide in the tension sensor module (TSM) elongates in response to force, leading to an increase in the donor lifetime corresponding to a decrease in Förster resonance energy transfer (FRET) efficiency. (**B**) FRET–force calibrations by single-molecule force spectroscopy show the distinct force response of four different TSMs using the YPet/mCherry FRET pair (Ringer et al., 2017). (**C**) The TSM is preferably integrated in nonconserved, unstructured regions of the protein of interest (POI). (**D**) The donor-only control of the tension sensor construct (DO-TS) is required to determine the FRET efficiency. A single point-mutation in the acceptor fluorophore can be used to abolish intramolecular FRET. (**E**) An acceptor-only control (AO-TS) analogous to the donor-only control is required to determine intermolecular FRET. (**F**) A no-force control (NF) is crucial to distinguish tension-dependent from tension-independent FRET changes. To generate efficient NF constructs, several strategies can be followed: The POI can be truncated at the TSM integration site; the TSM, tagged terminal to the POI; or the POI, mutated to prevent force application. (**G**) Donor-only control matching the NF (DO-NF) is required for FRET efficiency calculation. (**H**) Together with DO-NF, an acceptor-only control matching the NF (AO-NF) is necessary to determine intermolecular FRET.

Table 1 Force Sensitivity of Linker Peptides

Force sensitive element		Force range (pN)	Calibration	Reference
F40	Flagelliform $(GPGGA)_8$	1-6		Grashoff et al., 2010
F25	Flagelliform $(GPGGA)_5$	2-11		Brenner et al., 2016
F50	Flagelliform $(GPGGA)_{10}$	1-5	Single-molecule force spectroscopy	Ringer et al., 2017
FL	Ferredoxin-like fold	3-5		Austen et al., 2015
HP35	Villin head piece peptide	6-8		
HP35st	Mutated villin head piece peptide	9-11		
	Synthetic $(GGSGGS)_5$	1-9		
	Synthetic $(GGSGGS)_7$	1-6	Modeling	LaCroix et al., 2018[a]
	Synthetic $(GGSGGS)_9$	1-5		
sstFRET	Spectrin repeats	5-7	DNA spring	Meng & Sachs, 2011
stFRET	α-helix	few		Meng, Suchyna, & Sachs, 2008

[a]Model predictions made for $(GGSGGS)_{4\text{-}20}$.

Choice of Fluorophores in TSM

The spectral properties of the TSM depend on the employed FRET pair and therefore can be adjusted to the available microscopy setup; the same general considerations as for other FRET experiments apply (Bajar, Wang, Zhang, Lin, & Chu, 2016; Day & Davidson, 2012; Sun, Rombola, Jyothikumar, & Periasamy, 2013). For FLIM-based FRET measurements, however, a donor fluorophore with a mono-exponential decay is highly preferable as it simplifies lifetime determination and reduces complexity during data analysis (Piston & Kremers, 2007). Moreover, fluorophores with fast maturation rates, high extinction coefficients, and sufficient photostability are advantageous. Tension sensor studies using FRET pairs with yellow/red (Austen et al., 2015; Price et al., 2018; Ringer et al., 2017), green/red (Kumar et al., 2016; LaCroix, Lynch, Berginski, & Hoffman, 2018), or blue/yellow (Borghi et al., 2012; Price et al., 2018) emission spectra have been published. For the special case of tension sensor multiplexing (i.e., the simultaneous measurement of two tension sensors) the two orthogonal pairs mTFP1/ShadowG and LSSmOrange/mKate2 are available (Demeautis et al., 2017; Ringer et al., 2017).

Choice of the TSM Integration Site

To perform tension sensor experiments, the TSM is genetically integrated into the POI. Two important aspects should be considered when choosing a TSM integration site. First, TSM insertion should not interfere with functionality of either the target protein or the TSM. Second, the TSM should be integrated at an intramolecular location that is suitable to report on the mechanical state of the target protein.

To identify an integration site that preserves the functionality of both the POI and the TSM, available information on domain structure and, ideally, conformation of the POI should be considered. In general, TSM integration into flexible, unstructured, and nonconserved linker domains is recommended (Fig. 2C), but nonessential domains or parts of unstructured regions may be also replaced with the TSM to maintain the overall length of the target protein. Regardless of the individual integration site, testing the functionality of the POI and the TSM is essential.

Typically, TSMs are integrated between two binding sites that mediate the engagement with mechanically active structures. In talin tension sensor constructs, for instance, the TSM is integrated into a flexible linker region between the integrin-binding head domain and the actin-binding rod domain of talin (Austen et al., 2015; Kumar et al., 2016). Note, however, forces are not necessarily constant across a molecule. Talin, for example, experiences an intramolecular tension gradient with high forces of more than 7 pN in the N-terminal region of the rod domain and significantly lower forces in more C-terminal areas (Ringer et al., 2017).

Design of Donor-Only and Acceptor-Only Controls

FRET efficiency (E) measurements by FLIM are based on the reduction in donor lifetime caused by FRET in the presence of the acceptor (τ_{DA}) as compared to the average donor lifetime without acceptor ($\overline{\tau_D}$), according to

$$E = 1 - \frac{\tau_{DA}}{\overline{\tau_D}}.$$

An efficient way to determine the average lifetime of the donor in the absence of an acceptor is to measure a so-called *donor-only control*. A straightforward method to generate such a construct is to render the acceptor in the tension sensor nonfluorescent by point mutagenesis (Fig. 2D). In such a construct, the donor fluorophore experiences the same microenvironment as in the tension sensor, but FRET does not occur. The *acceptor-only control*, which is required for intermolecular FRET measurements (Basic

Protocol 3), should be designed analogously with a point mutation in the donor fluorophore to generate a FRET-deficient tension sensor-like molecule (Fig. 2E). Other applied donor-only controls are constructs in which only the donor fluorophore is inserted into the POI. This control is easier to generate but does not mimic as precisely the microenvironment that the donor fluorophore is exposed to in the tension sensor construct (Price et al., 2018).

Planning Suitable Control Constructs and Control Experiments

Powerful control constructs are key to efficient tension sensor measurements. They are necessary because FRET is not exclusively sensitive to the fluorophore separation distance but is also affected by the relative fluorophore orientation, the local pH, and temperature, among other parameters (Lakowicz, 2006). Thus, controls are required to distinguish tension-dependent from tension-independent effects. The *no-force control*, therefore, mimics a tension sensor construct that does not respond to changes in molecular tension. Three different approaches can be taken to develop to such a no-force control (Fig. 2F). First, a TSM can be integrated into the POI that is truncated after the TSM integration site. This approach is useful if the truncation does not impair the subcellular localization of the POI. The second approach is terminal tagging of the POI with the TSM. In many cases, N- or C-terminal addition of the TSM does not impair subcellular localization, and the POI is fully functional. Moreover, the POI will experience potential mechanical tension, but forces cannot be transduced across the TSM. It should be noted, however, that the TSM in such constructs is exposed to a different microenvironment owing to the distinct integration site. The third and most elegant no-force control uses a tension sensor protein in which point mutations or small deletions in the POI abolish force transmission. The advantage of this control is that the TSM is exposed to the same microenvironment as in the tension sensor protein but cannot be mechanically loaded. However, this type of no-force control requires detailed understanding of the POI.

In any case, a donor-only control matching the no-force control construct needs to be generated (Fig. 2G) because the donor lifetime depends on the microenvironment. In addition, an acceptor-only control matching the no-force control construct is required (Fig. 2H) to compare the levels of intermolecular FRET in cells expression tension sensors and no-force controls.

Equally important for the interpretation of FRET data is the definition of *biological no-force conditions*, in which mechanical tension across the FRET probes can be prevented or acutely released. The expected result of such an experiment is that FRET efficiencies in cells expressing the tension sensor increase (indicating loss of tension) whereas transfer rates in cells expressing the no-force control remain unaffected. The design of no-force conditions strongly depends on the POI and often includes the treatment of cells with chemical compounds (e.g., motor protein inhibitors).

Testing Protein Functionality After TSM Integration

Integrating a TSM into a POI is likely to affect protein functionality. Therefore, already at the planning stage of a tension sensor experiment, experiments should be designed to evaluate protein functionality after TSM integration. The specific functionality assays depend on the POI but often involve the rescue of knockout cells with the tension sensor protein. Proper subcellular localization of the tension sensor can provide first indications of protein functionality, but such assays should be complemented with more quantitative experiments. These may include the analysis of protein turnover by fluorescence recovery after photobleaching (FRAP; Austen et al., 2015; Grashoff et al., 2010) or the generation and analysis of a model organism, in which the tension sensor construct replaces the endogenous protein (Lagendijk et al., 2017; Lemke, Weidemann, Cost, Grashoff, & Schnorrer, 2018; Meng, Suchyna, Lazakovitch, Gronostajski, & Sachs, 2011).

CLONING USING GIBSON ASSEMBLY

This protocol describes the generation of an expression construct, in which the TSM is integrated into a POI and inserted into a vector backbone. Readers with cloning experience can directly continue with Basic Protocol 2 (transient transfection) or Basic Protocol 3 (FRET experiment).

In the Gibson assembly protocol, multiple DNA fragments are assembled simultaneously in a plasmid backbone; the advantage of the technique is that it does not require compatible restriction sites and allows seamless ligation of multiple DNA fragments in one step (Gibson et al., 2009). The method uses the NEBuilder® HiFi DNA Assembly Master Mix that initiates DNA 5′-end chew back creating single-stranded 3′ overhangs, which facilitate annealing of DNA fragments with complementary sequences. Incorporating nucleotides to fill in the gaps in the annealed DNA fragments then leads to a seamless assembled DNA molecule.

Materials

DNA purification kit for:
- Gel and PCR purification (e.g., NucleoSpin® Gel and PCR Clean-up kit; Macherey-Nagel, 740609.250)
- Bacterial culture, few ml volume (e.g., NucleoSpin® Plasmid EasyPure; Macherey-Nagel, 740727.250)
- Bacterial culture, 250 ml volume (e.g., NucleoBond® Xtra Midi; Macherey-Nagel, 740412.50)

Appropriate restriction enzymes for DNA sequence of interest
Restriction enzyme buffer
Dephosphorylation kit (e.g., QuickCIP; New England Biolabs, M0508S)
Gibson assembly mix (e.g., NEBuilder HiFi DNA Assembly Master Mix; New England Biolabs, E2621)
OmniMAX™ 2T1^R Chemically Competent *Escherichia coli* (e.g., Thermo Fisher Scientific, C854003)
LB agar plates (see recipe)
LB medium (see recipe)
Appropriate antibiotics (e.g., ampicillin)
Glycerol

37°C and 80°C incubator, with shaking capabilities for tubes and flasks
1.5- and 2-ml microcentrifuge tubes
14-ml round-bottom polypropylene tubes (e.g., Fisher Scientific, 352059)
Centrifuge and microcentrifuge
Spectrophotometer (e.g., NanoDrop)
DNA sequencing kit (e.g., Mix2Seq Kit OVERNIGHT; Eurofins Genomics)
1-liter Erlenmeyer flask, sterile
Aluminum foil
250-ml centrifuge tubes

Additional reagents and equipment for plasmid and primer design (see Support Protocol 1), PCR (see Internet Resources), agarose gel electrophoresis (see Internet Resources), and bacterial transformation (see Support Protocols 2 and 3)

Assemble DNA

1. Design plasmid and PCR primers with homologous overlap regions of adjacent DNA fragments in silico (see Support Protocol 1; Fig. 3).
2. Amplify DNA inserts using PCR to generate DNA fragments with homologous overlaps (see Internet Resources for PCR protocol).

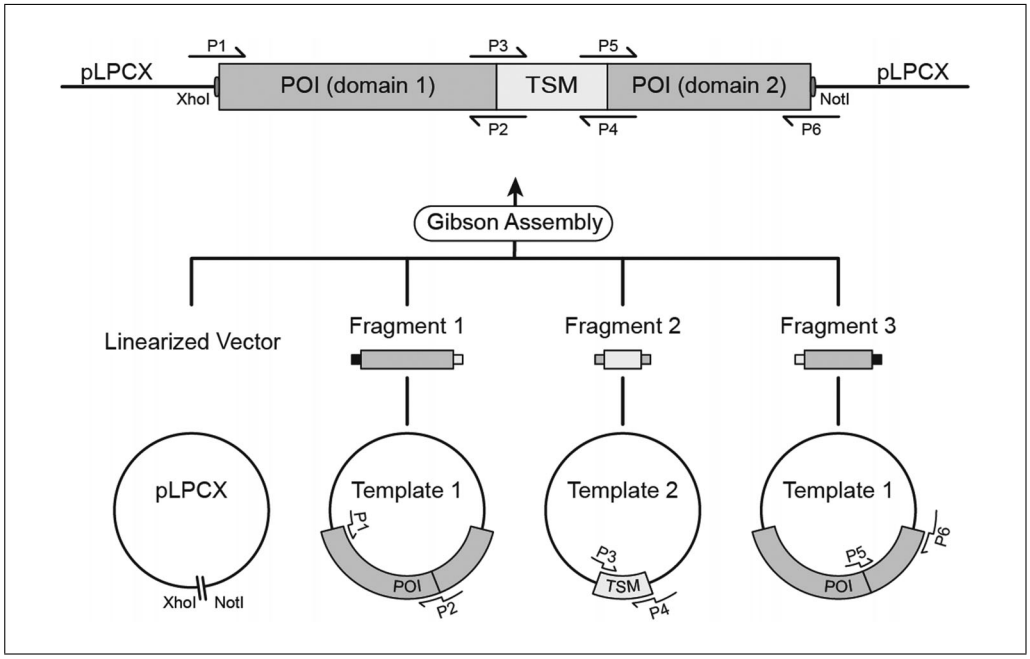

Figure 3 Primer design for Gibson assembly. Design a virtual map for the tension sensor, which can be used as a template to design PCR primers for each fragment with overlapping sequences. The Gibson assembly contains the linearized vector and PCR fragments comprising domains of the protein of interest (POI) or the tension sensor module (TSM) and overlaps with neighboring fragments.

3. After PCR amplification, separate DNA products on an agarose gel (see Internet Resources for agarose gel electrophoresis). Isolate bands of the correct size, and purify DNA with a gel purification kit following the manufacturer's instructions.

   To more easily identify correctly sized bands, simulate the expected sizes of the amplified fragments with in silico tools (e.g., the "Simulate Agarose Gel" tool from the SnapGene software).

4. Linearize 1.5 µg plasmid backbone (e.g., pLPCX) with restriction enzymes. Use 1 µl of each restriction enzyme and 3 µl restriction enzyme buffer, and add deionized, distilled water up to 30 µl. For most enzymes, digest at 37°C for 1 hr (see Internet Resources for restriction enzyme digest).

   Some DNA will be lost during the following gel purification step, and therefore sufficient starting material is required.

   Use software (e.g., SnapGene) to identify singular restriction sites for linearization.

   For enzymes from New England Biolabs, the online double-digest finder (see Internet Resources) is a useful tool to select the appropriate restriction enzyme buffer.

5. Add 1 µl QuickCIP per 1 pmol DNA ends. Incubate at 37°C for 10 min, and then inactivate the phosphatase by incubation at 80°C for 2 min.

   The amount of DNA ends in pmol can be estimated using the average molecular weight of a single nucleotide pair (660 g/mol), the amount of DNA X in µg, and the number of nucleotides N in kilobase pairs (kb):

$$\text{DNA ends } [\text{pmol}] = 2\,\frac{\text{X}}{660\ \text{g/mol}}\,\frac{1000}{\text{N}}$$

   Avoid prolonged incubation with phosphatase because unspecific activity leads to the removal of 3′ phosphates.

The dephosphorylation step may be skipped if ligation overhangs do not overlap and thus prevent religation.

6. To check successful restriction and to separate the dephosphorylated plasmid backbone from enzymes, perform agarose gel electrophoresis, and isolate the respective band. Purify DNA using a gel purification kit.

7. Prepare Gibson assembly mix in a 1.5-ml microcentrifuge tube on ice according to the manufacturer's user manual. Briefly, use 10 µl assembly master mix, and add a total DNA amount of 0.02 to 0.5 pmol for 2 to 3 fragments and 0.2 to 1 pmol for 4 to 6 fragments. Use a 2- to 3-fold molar excess of inserts to vector (see Internet Resources for how to calculate molar ratios). Add deionized, distilled water up to 15 µl, and incubate the mixture in a thermomixer at 50°C for 1 hr.

   The manufacturer's user manual recommends a total volume of 20 µl. In the authors' experience, however, best transformation efficiencies are achieved when the assembly volume is 15 µl and the entire assembly volume is used for bacterial transformation (see Support Protocol 2).

   If only a few colonies are observed after transformation, test different vector:insert ratios ranging from 1:1 to 1:5.

Amplify DNA

8. Transform DNA into bacteria (see Support Protocols 2 and 3).

   Tension sensor expression constructs can span many kb, and the large plasmid size reduces transformation efficiency. In the authors' experience, transformation of chemically competent cells was successful for constructs of up to 17 kb if bacteria were sufficiently competent and if the entire Gibson assembly mix was used for transformation.

   Competence of bacteria strongly depends on the details of the preparation protocol (see Support Protocol 3).

9. Grow bacterial colonies on LB agar plates containing the appropriate antibiotic by overnight incubation at 37°C.

10. Pick a single colony from the LB agar plate with a sterile pipette tip. Transfer the pipette tip into a round-bottom tube containing 4 ml antibiotic-supplemented LB medium to grow a mini culture. Pick at least 4 to 6 colonies.

    The number of required colonies depends on the size and complexity of the assembled DNA constructs. Some constructs might require screening of additional colonies to identify positive clones.

11. Incubate mini cultures at 37°C while shaking vigorously (250 rpm) for 6 to 16 hr.

    Incubation of bacterial cultures for 6 hr using a high-copy-number plasmid, like pBluescript, yields roughly 0.4 to 0.6 µg/µl plasmid DNA. Low-copy-number plasmids (e.g., pLPCX) require longer incubation times of up to 16 hr and yield about 100 to 200 ng/µl purified plasmid DNA. Adjust the bacterial incubation times accordingly.

12. Pour 2 ml mini culture into a 2-ml microcentrifuge tube, and centrifuge 3 min at 4500 × *g*, room temperature, to pellet the bacteria. Discard supernatant and collect another 1.75 ml bacterial culture in the same microcentrifuge tube.

13. Keep the remaining 250 µl mini culture inside the round-bottom tube. Close cap tightly, and store at 4°C for potential midi culture preparation.

Check correct DNA assembly

14. Isolate plasmid DNA from the pelleted bacteria of the mini cultures with a DNA isolation kit following the manufacturer's user manual.

15. Determine DNA concentration of the plasmid preparations. Keep DNA samples on ice for short-term storage and at −20°C for long-term storage.

16. Verify correct plasmid assembly by restriction enzyme digest and subsequent analysis by agarose gel electrophoresis.

    Choose restriction enzymes such that empty backbone and the correctly assembled plasmid are easy to distinguish. Using software tools like SnapGene's "Simulate Agarose Gel" might be helpful to find the appropriate restriction enzymes.

17. Confirm correct DNA sequence by DNA sequencing.

    Sequencing the entire insert region is crucial because unwanted DNA mutations can be introduced during PCR or Gibson assembly.

Amplify DNA

18. Once the correct DNA sequence is confirmed, start a midi culture in 250 ml antibiotic-containing LB medium using a 1-liter Erlenmeyer flask. Loosely cover flask with aluminum foil, and incubate at 37°C while shaking vigorously (250 rpm) for ∼16 hr. After bacterial growth, prepare a glycerol stock by mixing 600 µl bacteria culture with 400 µl autoclaved glycerol.

    The glycerol stock can be stored at −80°C and used to start future midi cultures.

19. Pellet bacteria in a 250-ml centrifuge tube 15 min at 4500 × *g*, room temperature, and isolate plasmid DNA with the appropriate kit following the manufacturer's user manual.

20. Determine DNA concentration, and confirm DNA sequence by DNA sequencing. Store plasmid at −20°C.

    DNA quality suffers from repeated freeze-thaw cycles resulting in reduced transfection efficiencies. Therefore, aliquot DNA samples for one or two transfections.

    Typical DNA concentrations range between 0.4 to 1.0 µg/µl.

SUPPORT PROTOCOL 1

GIBSON PRIMER DESIGN

This protocol describes how to design oligonucleotides as PCR primers to assemble one or multiple DNA fragments in a vector backbone using Gibson assembly. Correct primer design is essential for the success of the Gibson assembly.

Materials

Computer running sequence analysis program (e.g., SnapGene Software)

1. Assemble DNA fragments in silico with a sequence analysis program such as SnapGene Software.

   The virtual sequence displays both DNA strands that can be used as a template to design overlapping primers.

2. Adjust the gene-specific part of a primer to a length of about 18 to 22 base pairs (bp).

   The melting temperature (Tm) of the primer should range between 50°C and 60°C. The 3′-terminus of the priming part should contain a G or C nucleotide.

3. Adjust the nonpriming overlap sequences of the primer (homologous to the 5′-terminus of the adjacent fragment) to a length of about 20 to 25 bp with a Tm of around 50°C to 60°C.

   To amplify the POI (domain 1) fragment (Fig. 3), for example, the overlap sequence in the forward primer P1 is identical to the 20 terminal bp of the forward strand of the pLPCX left-arm (5′ to 3′ direction). The gene-specific part of P1 is identical to the 6 bp of XhoI and the 19 first bp on the forward strand of the POI (domain 1) left-arm.

4. Design the reverse primer analogously.

   Note that the reverse primer has nonpriming overlap sequences with another fragment.

   For example, the POI (domain 1) reverse PCR primer (Fig. 3, P2) overlaps with the 20 terminal bp on the bottom strand of the TSM right-arm (5′ to 3′ direction). The gene-specific part of P2 is identical to the 25 terminal bp on the bottom strand of the POI (domain 1) right-arm.

   The priming gene-specific parts of forward and reverse pair primers should have a Tm within 5°C of each other.

   To calculate the precise Tm of each primer, online tools such as Tm Calculator from Thermo Fisher Scientific (see Internet Resources) may be used.

   Additional bp can be inserted into the DNA sequence (e.g., to restore or introduce restriction sites). Note that Gibson assembly does not require restriction sites, and ligations can be seamless.

TRANSFORMATION OF COMPETENT BACTERIA

SUPPORT PROTOCOL 2

This protocol describes the DNA transformation of chemically competent bacteria with the assembled plasmids using a heat-shock procedure.

Materials

LB medium (see recipe), with and without antibiotics
LB agar plates containing antibiotics (see recipe)
OmniMAX 2T1^{R} Chemically Competent *E. coli* (e.g., Thermo Fisher Scientific, C854003; see Support Protocol 3)
Gibson assembly mixture (see Basic Protocol 1)

42°C water bath
Thermomixer
37°C incubator, with shaking capabilities
Centrifuge
Delta cell spreader
Parafilm

1. Prewarm water bath to 42°C, and prewarm thermomixer, LB medium, and LB agar plates to 37°C.
2. Thaw 100 µl heat shock competent bacteria (stored as aliquots at −80°C; see Support Protocol 3) on ice.
3. Add 2 to 15 µl Gibson assembly mixture to the bacteria and mix carefully.

   The manufacturer's user manual recommends the use of 2 to 5 µl Gibson assembly mixture. In the authors' experience, adding the entire Gibson assembly mix (15 µl) increases transformation efficiency. This may be desired when working with large plasmids of >10 kb.

4. Incubate DNA/bacteria mixture on ice for 30 min.

5. Heat shock bacteria by placing the tube in the 42°C water bath for 60 to 75 sec.
6. Transfer DNA/bacteria mixture immediately on ice, and incubate for 3 min.
7. Add 900 µl prewarmed antibiotic-free LB medium, and incubate at 37°C with shaking at 250 rpm in a thermomixer for 1 hr.
8. Centrifuge mixture 3 min at 4500 × *g*, room temperature, to pellet the bacteria. Discard LB medium, and resuspend pellet in 100 µl LB medium.
9. Using a cell spreader, plate bacteria onto prewarmed LB plates. Incubate LB plates overnight at 37°C.

   Make sure the LB plates contain antibiotics at the appropriate concentration, typically 50 to 100 µg/ml, for clone selection.

   Even distribution of the bacteria on the agar plate is important to obtain isolated, single colonies.

   Avoid prolonged incubation times of LB plates, as this will lead to the fusion of large colonies or the appearance of satellite colonies.
10. The next day, pick single colonies, and grow in LB medium containing antibiotics for plasmid amplification. For storage, seal plate containing bacterial colonies with Parafilm, and store at 4°C.

SUPPORT PROTOCOL 3

GENERATION OF CHEMICALLY COMPETENT BACTERIA

Heat shock competent bacteria need special preparation to be susceptible to DNA transformation. Following the individual steps of this protocol is essential to obtain sufficiently competent bacteria for the transformation of large tension sensor constructs. The protocol is specific to OmniMax bacteria. Note that alternative bacterial strains are available.

Materials

OmniMAX 2T1^{R} Chemically Competent *E. coli* (e.g., Thermo Fisher Scientific, C854003)
LB agar plate with 10 µg/ml tetracycline (see recipe)
LB medium with and without tetracycline (see recipe)
Transformation and storage solution (see recipe)
87% glycerol

37°C incubator, with shaking capabilities
100-ml Erlenmayer flask
Spectrophotometer
10-ml or greater centrifuge tubes
Centrifuge, capable of cooling to 4°C
1.5-ml microcentrifuge tubes

Additional reagents and equipment for transformation (see Support Protocol 2)

1. Streak 1 vial bacteria on an LB agar plate containing 10 µg/ml tetracycline, and grow cells overnight at 37°C.
2. Pick a single colony, and incubate in 10 ml LB medium with tetracycline overnight at 37°C in a sterile 100-ml Erlenmeyer flask with shaking at ∼140 rpm.

   Incubate the bacterial culture for a maximum of 16 hr to avoid cell overgrowth.
3. Inoculate 100 ml antibiotic-free, prewarmed LB medium with 2 ml preculture, and shake at 37°C for ∼1.5 to 2.5 hr until the optical density (OD) reaches $OD_{550} = 0.5$.

Stopping the bacterial culture at the correct OD is important to obtain highly competent cells. Note that these bacteria double every ~20 min during exponential growth, and the OD increases rapidly.

4. Cool bacterial culture on ice for 10 min.

   Competence of the bacteria increases if temperature is kept at 4°C; we recommend working in a cold room.

5. Transfer bacteria into precooled, sterile centrifuge tubes. Pellet bacteria in precooled centrifuge 15 min at 1100 × *g*, 4°C.

6. Resuspend bacteria from all centrifuge tubes in a total volume of 10 ml ice-cold transformation and storage solution.

7. Add 2.5 ml sterile 87% glycerol.

   Working with sterile reagents and equipment is essential as a contaminant in the bacterial stock would be transferred to all subsequent steps. Therefore, glycerol and tubes should be autoclaved before the experiment.

8. Aliquot 100 µl resuspended bacteria into sterile 1.5-ml microcentrifuge tubes resulting in ~125 aliquots.

9. Snap freeze in liquid nitrogen (i.e., throw samples into liquid nitrogen, stir, and collect), and store at –80°C.

   Aliquoting and snap freezing the samples should be performed as quickly as possible. Therefore, prepare tubes in advance, and work with two people: one person to aliquot the bacteria and the other person to close tubes and snap freeze.

   Keep bacterial aliquots on dry ice after the snap freezing.

10. Check competence of bacteria by performing an established transformation protocol; compare competence of old and freshly prepared cells.

    Bacteria lose competence over time. For most constructs, bacteria can be used for up to 6 months, but large constructs require freshly prepared competent bacteria that should not be older than 6 to 8 weeks.

TRANSIENT TRANSFECTION OF CELL LINES WITH TENSION SENSOR CONSTRUCTS

BASIC PROTOCOL 2

This protocol describes transient DNA transfection to introduce tension sensor expression constructs into mammalian target cells such as mouse fibroblasts. Readers using different model systems or with established transfection protocols can directly continue with Basic Protocol 3.

Materials

Target cells of interest (e.g., mouse fibroblast)
Opti-MEM™ I Reduced Serum Medium, GlutaMAX™ Supplement (e.g., Thermo Fisher Scientific, 51985026)
Lipofectamine™ 3000 Transfection Reagent (e.g., Thermo Fisher Scientific, L3000015)
Plasmid DNA
P3000™ Enhancer Reagent (e.g., Thermo Fisher Scientific, L3000015)
Dulbecco's modified Eagle medium (DMEM), high glucose, GlutaMAX, pyruvate, antibiotic-free (e.g., Thermo Fisher Scientific, 31966047)
Fetal bovine serum (FBS; e.g., Gibco, 10270-106)
1 mg/ml fibronectin from bovine plasma (e.g., Merck Chemicals GmbH, 341631-5MG), in sterile phosphate-buffered saline (PBS)

Dulbecco's PBS (DPBS; e.g., Sigma-Aldrich, D8537)
0.5% trypsin-EDTA, without phenol red (e.g., Thermo Fisher Scientific, 15400054)
Imaging medium: HEPES-buffered DMEM containing 4.5 g/liter D-glucose (e.g., Thermo Fisher Scientific, 21063029)

6-well plate
1.5-ml microcentrifuge tubes
37°C incubator
35-mm glass-bottom imaging dish (ibidi, 151126/1)

Day 1

1. Seed target cells into a 6-well plate. Adjust seeding density to achieve 70% to 90% confluence at the time of transfection.

Day 2

2. Prepare transfection reagent according to manufacturer's instructions. Briefly, prepare 2 separate tubes with 125 µl Opti-MEM. Add 7.5 µl Lipofectamine 3000 into the first tube and 2.5 µg DNA with 5 µl P3000 into the second tube. Mix tubes separately, and then add DNA-containing solution to Lipofectamine. Mix tube by inversion, and incubate transfection mixture at room temperature for 10 to 15 min.

   Opti-MEM can be replaced with the normal cell culture medium if the target cell line cannot tolerate Opti-MEM.

   The use of antibiotics during transfection increases cytotoxicity. Thus, use antibiotic-free medium.

   If transfection efficiency is low, use a fresh aliquot of DNA because repeated freeze-thaw cycles impair transfection efficiency. Alternatively, adjust the amount of DNA.

   Do not vortex the master mix solution.

3. Exchange medium in the 6-well plate with 1 ml prewarmed, antibiotic-free DMEM supplemented with 10% (v/v) FBS.

4. Add the entire 250 µl transfection mixture dropwise onto cells. Incubate cells at 37°C for at least 6 hr or overnight.

   When using Opti-MEM, dropwise addition of the Lipofectamine reagent turns the medium transparent. Gently move the plate to evenly distribute the transfection mixture.

   The incubation time of cells with the transfection mixture can be adjusted. Prolonging the incubation time often increases transfection efficiency but can also increase cytotoxicity. Incubation times of up to 24 hr are usually tolerated, but sensitive cell lines such as primary cells may only tolerate 6 hr of incubation.

Day 3

5. Coat imaging dish with 400 µl of 1 mg/ml fibronectin by incubating overnight at 4°C. Subsequently, wash dish with 1 ml DPBS.

   Coating can be shortened to 1 hr by incubating at 37°C.

   Depending on the experiment, fibronectin coating may not be required. It will help the adhesion and spreading of cells expressing fibronectin receptors.

6. To analyze transfected cells, detach cells with 200 µl trypsin-EDTA. Then dilute cell suspension in 3.8 ml DMEM supplemented with 10% (v/v) FBS.

7. Plate 500 µl cell suspension in the imaging dish containing 2 ml DMEM.

   Depending on the initial cell density, volumes may be adjusted to achieve the desired cell density during imaging.

Seeding time as well as the time after transfection might influence forces transduced across the POI. Therefore, standardize these times to reduce biological variability.

8. Before imaging, replace DMEM with 2 ml prewarmed imaging medium.

   Imaging medium does not contain phenol red and therefore reduces background fluorescence during imaging.

ANALYZING TENSION SENSOR FRET EFFICIENCY USING LIVE-CELL FLIM

BASIC PROTOCOL 3

In this protocol, we describe the measurement and FRET efficiency determination of tension sensor and control samples using time-correlated single-photon counting (TCSPC)-FLIM. For a detailed description of TCSPC, see Wahl (2014). As microscopy protocols strongly depend on the available microscopy setup, we focus on general considerations of microscopy and data analysis procedures.

Materials

Tension sensor–expressing cells (see Basic Protocol 2)
Cells expressing no-force control (see Basic Protocol 2)
Cells expressing donor-only controls matching the tension sensor and no-force control (see Strategic Planning and Basic Protocol 2)

Confocal laser scanning microscope with appropriate lasers, objectives, and filters
Computer running data analysis software (e.g., MATLAB)

Acquire FLIM data

This section gives recommendations on how to collect FLIM data.

1. Prepare tension sensor–expressing and control-expressing cells with the same protocol (e.g., according to Basic Protocol 2).

   Once optimized, the procedure for how cells are prepared should be standardized to minimize variability.

2. Check the instrument response function (IRF) of the microscope setup using a fluorophore or organic dye with a known and preferably short fluorescence lifetime (see Internet Resources for an IRF detection protocol).

   The IRF should be characterized by a single peak and a rapid fluorescence decay.

   If the IRF displays additional peaks, determine their source or contact microscopy support. Additional peaks in the IRF indicate suboptimal instrument performance.

3. Acquire FLIM data with constant laser intensity and defined acquisition time (i.e., scan speed and number of repeats). Avoid using maximal photon count or similar settings as endpoint for measurements.

   The amount of photons that can be extracted from images depends the experimental condition and POI. As a rule of thumb, acquiring 500,000 photons per image is a good starting point. Note that the fit quality during lifetime determination improves with increasing photon counts. Higher photon counts can also be used to increase the spatial resolution.

   The here-mentioned photon count is determined with the authors' setup, which is a confocal laser scanning microscope (Leica TCS SP5X) equipped with pulsed white light laser (NKT Photonics), FLIM X16 TCSPC detector (LaVision Biotech), 63× water-immersion objective (HCX PL APO CS), and a band-pass filter (Chroma, 545/30 nm). Constructs based on the YPet/mCherry FRET pair are measured at 508-nm excitation wavelength with a repetition rate of 80 MHz and 70% laser intensity. The line scan speed is 400 Hz covering 512 × 512 pixels, which corresponds to ~60 × 60 μm^2 or 120 × 120 μm^2

depending on the zoom. A total of 20 or 100 repeats of the same image are summed. Note that these settings are specific to the biological sample and instrument used.

To allow a comparison of tension sensor and control samples, keep the instrument settings constant throughout the experiment.

Confirm that detector-dead times are short enough to collect all photons at the chosen count rate. Bright samples and high laser intensities cause high count rates.

Higher photon counts can be achieved by using a slower scan speed or by increasing the number of repeats; increasing repeat number is often preferable as the measurement is less affected by photobleaching.

Photobleaching of the donor fluorophore reduces photon counts but does not affect the resulting lifetime because bleached fluorophores do not contribute to the detected signal. Photobleaching of the acceptor, however, changes the resulting average donor lifetime because FRET is abolished but not the donor emission. Thus, a tension sensor with a photobleached acceptor contributes with the lifetime of a fully elongated linker irrespective of its stretch state. For that reason, it is essential to use the very same imaging conditions for tension sensor and no-force control. As the lifetime of donor-only controls is unaffected by photobleaching, these samples might be measured with less repeats as compared to tension sensor and no-force control.

Keep the laser repetition rate unchanged between experiments. For most fluorophores, repetition rates of 40 or 20 MHz are suitable.

4. Acquire FLIM data of tension sensor, no-force control, and donor-only controls in the same session. Vary the measurement order of these constructs over the course of the experimental days to average uncontrolled effects like variations in laser intensity. Repeat the experiment in 4 to 5 independent sessions using independently prepared samples.

Determine fluorescence lifetime within a region of interest

This section describes how to isolate the signal of interest and fit the fluorescence lifetime.

5. Import data for subsequent analysis.

   The authors use a custom-written MATLAB program for FLIM data analysis (Austen et al., 2015; Price et al., 2018; Ringer et al., 2017). FLIM data should contain both the pixel location of photons (Fig. 4A) and the photon arrival time after excitation pulse (Fig. 4B).

6. Use spatial information to determine regions of interest (ROIs). To this end, sum all temporal information to a spatial-resolved image, and then manually draw masks around the ROIs (e.g., focal adhesions or cell–cell contacts; Fig. 4C).

   This manual step allows exclusion of unwanted background signal and definition of multiple ROIs in one image.

7. Extract the signal from structures of interest with an automated signal extraction procedure (Fig. 4D).

   The basic principle of all thresholding algorithms is to generate a binary mask covering the structure of interest.

   The simplest possible threshold is a fixed-intensity value. In practice, an intensity threshold is often insufficient because expression levels and hence intensities vary. Moreover, the subcellular structures of interest might not be the only bright signal in the image. Thresholding with more elaborate algorithms may be used to separate intensity classes (e.g., by Multi-Otsu thresholding; Austen et al., 2015; Lemke et al., 2018; Ringer et al., 2017) or to identify structures based on their shape and size (Price et al., 2018).

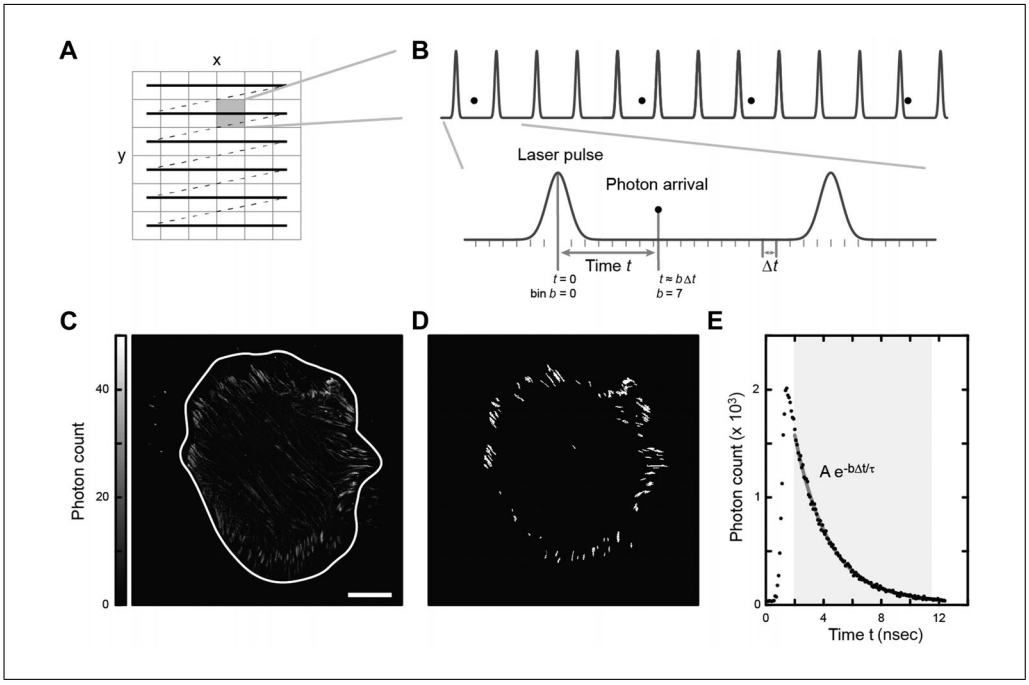

Figure 4 Fluorescence lifetime imaging microscopy (FLIM) data collection and analysis. (**A**) Scanning individual pixels provides spatial resolution. (**B**) Pulsed laser beams excite with a specific frequency. The time of photon arrival t (black dots) is related to the corresponding excitation pulse to achieve temporal resolution of the image. Photon arrival times are binned in time bins b of width Δt. (**C**) An intensity image is generated by summing the temporal information. A manual mask is drawn around the structure of interest. Scale bar: 20 μm. (**D**) The signal of interest is extracted using automated thresholding. (**E**) To generate a photon count histogram, all signal inside the mask is plotted over the photon arrival time. A tail fit (gray line; fit range indicated by gray shading) is used to determine the fluorescence lifetime τ.

Depending on the ROIs and structure size, masks may only cover 0.5% to 5% of all pixels, which contain only 5% to 20% of the overall photon count. Therefore, masking greatly increases the signal-to-noise ratio by limiting the analysis to structures of interest.

To achieve spatial resolution, divide signal into individual substructures. To this end, pixels may be assigned to substructures based on either shape or local intensity maxima (Price et al., 2018).

8. Fit the fluorescence lifetime from the isolated structure of interest. Sum the spatial information of the signal within one mask, but preserve temporal information N_b (i.e., the number of photons arrived in time bin b with time width Δt). A lifetime τ can be fitted from the histogram of photon arrival times by deconvolution with the IRF or tail fitting (Fig. 4E) of a mono-exponential function:

$$N_b = A\ \mathrm{e}^{-b\Delta t/\tau} + N_0,$$

where A is the amplitude and N_0 the background photon count. Mono-exponential tail fitting is a relatively simple and robust way to determine the average fluorescence lifetime from a fluorescence decay.

The fluorescence lifetime of the donor fluorophore should be well described by a mono-exponential decay function. In a tension sensor experiment, at least two lifetimes are expected, corresponding to the non-stretched tension sensor at zero force and the stretched tension sensor under tension. Ideally, such data sets are described by bi-exponential fits. However, in experiments with high repetition rates (80 MHz) meaning short traces, the robust mono-exponential tail fit may be used because it has fewer free fit parameters than multi-exponential fits. Significant changes in the fluorescence lifetime of tension sensor

and control samples are reflected in the mean lifetime obtained by a mono-exponential fitting procedure.

If the time resolution used for fitting is reduced (i.e., time bins have a broader width) fewer photons are required for a robust fit result. The authors use a time bin width of 80 psec.

For tail fits, the tail region of the photon count has to be determined in a standardized fashion. As the IRF might shift between experimental days, the bin with the maximal photon count may be determined by averaging all lifetime decays from one experiment. The start point of the tail fit is then chosen at a specific number of time bins after the maximum. The endpoint of the tail fit should be set at a defined number of time bins after the start point to receive a constant time trace length (Fig. 4E). The authors use 7 time bins after the maximum and fit for 120 bins (i.e., 9.6 nsec).

As a fit routine, the authors use MATLAB's 'fmincon' with a maximum-likelihood cost function based on Poisson statistic (Price et al., 2018; Ringer et al., 2017), which becomes important if spatial resolution is required and photon count rates per mask are low. Otherwise, standard least-square methods can also be used.

9. Check data for sufficient fit quality, and exclude data sets with poor fit quality from further analysis.

   The number of photons in a fit might be used as a first selection criterion, but fit quality is better assessed from the fit function's confidence interval. The confidence interval width can be normalized to the lifetime to determine a relative error. Regardless of how fit quality is determined, apply the same exclusion criteria for tension sensor and control samples.

Average donor-only lifetime for FRET efficiency calculation

This section describes considerations on how to determine the average donor lifetime in the absence of the acceptor.

10. Determine whether FRET efficiency calculations for tension sensor and no-force control can be based on the same donor-only lifetime value. If donor-only lifetimes of constructs matching tension sensor and no-force control (Fig. 2D, G) are comparable, merge lifetime measurements of both constructs to determine an average donor-only lifetime. Otherwise, use separate donor-only lifetimes for FRET efficiency calculations.

11. Compare donor-only lifetimes across individual experimental days of one experiment to judge whether changes in donor-only lifetime reflect day-to-day variability and whether similar differences in the corresponding tension sensor and no-force control samples are expected.

    This should help to decide whether to use the day-matched donor-only lifetime or to employ an average donor-only lifetime that is based on all experimental days.

12. Compare donor-only lifetimes in different experiments to determine whether individual lifetimes match the specific experimental condition. If donor-only lifetimes across different experiments are comparable, use an average lifetime (across all experiments).

Determine intermolecular FRET

This section describes how intermolecular FRET between neighboring molecules can be determined.

13. Generate cells expressing two constructs that are incapable of undergoing intramolecular FRET but potentially capable of undergoing intermolecular FRET (see Strategic Planning; Fig. 2D, E, G, H).

Simultaneous transient transfection of both expression plasmids (using equimolar amounts of both constructs and the same total amount of DNA as for a single transfection; Basic Protocol 2) usually leads to co-expression of both constructs with comparable expression levels per cell. For some proteins, however, co-expression may require the combination of stable transduction and transient transfection.

14. Acquire FLIM data in cells co-expressing donor-only and acceptor-only constructs at the same level. Take intensity images of donor and acceptor channel after the FLIM image to document relative intensities.

    In most cases, intermolecular FRET is low compared to the transfer rates observed in tension sensor constructs. Consequently, more photons per donor fluorophore are emitted.

15. Compare intermolecular FRET efficiencies for tension sensor and no-force control.

    If these values are similar, intermolecular FRET is unlikely to account for potential changes observed between tension sensor and no-force control. If values are different, it should be tested to what degree the observed differences account for potential FRET differences between tension sensor and no-force control samples.

DETERMINATION OF MOLECULAR STRETCH RATIO

ALTERNATE PROTOCOL

Tension sensor measurements not only allow determination of FRET efficiencies (Basic Protocol 3) but can also be used to estimate how many tension sensor molecules of a given population are in stretched (high force) or non-stretched (no force) conformation (Lemke et al., 2018; Ringer et al., 2017). This protocol describes considerations regarding the determination of this mechanical engagement ratio. For measurement and signal extraction, the same procedures as described in Basic Protocol 3 apply.

1. Determination of the molecular stretch ratio is based on the assumption that all sensors of a recorded population are either in the stretched or non-stretched state. This is a reasonable assumption for sensors that are based on FL-TSM, which displays an almost digital force–FRET response.

   Mechanical stretch ratios can also be calculated for other TSMs under the assumption that tension is either below or above the force required to fully elongate the linker peptide.

2. The lifetimes of stretched and non-stretched tension sensors are not directly additive. Instead, decay functions sum according to:

$$N_b = A_{\text{stretched}}\, \mathrm{e}^{-b\Delta t/\tau_{\text{stretched}}} + A_{\text{non-stretched}}\, \mathrm{e}^{-b\Delta t/\tau_{\text{non-stretched}}} + N_0,$$

   where $A_{\text{stretched}}$ and $A_{\text{non-stretched}}$ are the amplitudes of the stretched and non-stretched states, respectively; $\tau_{\text{stretched}}$ and $\tau_{\text{non-stretched}}$, the lifetimes of stretched and non-stretched states, respectively; b, the time bin; Δt, the time bin width; and N_0, the background photon count.

3. The number of emitted photons is decreased when the donor fluorophore undergoes FRET. Consequently, the ratio of photons emitted by TSMs in the stretched and non-stretched state underestimates the amount of non-stretched sensors. The ratio (r) of the amplitudes, however, scales with the number of molecules in the stretched and non-stretched states:

$$N_b = A_{\text{total}}\left[r\ \mathrm{e}^{-b\Delta t/\tau_{\text{stretched}}} + (1-r)\ \mathrm{e}^{-b\Delta t/\tau_{\text{non-stretched}}}\right] + N_0,$$

   where $A_{\text{total}} = A_{\text{stretched}} + A_{\text{non-stretched}}$ and $r = A_{\text{stretched}}/A_{\text{total}}$.

4. Bi-exponential fits use five free parameters (A_{total}, r, $\tau_{\text{stretched}}$, $\tau_{\text{non-stretched}}$, N_0), while mono-exponential fits have only three free parameters (A, τ, N_0). The

increased number of free parameters reduces fit stability and requires higher photon counts and longer time traces to obtain robust fits.

To increase fit robustness, reduce the number of free fit parameters in bi-exponential fitting.

5. To reduce the number of free parameters, the fluorescence lifetimes of stretched and non-stretched tension sensor can be experimentally determined by mono-exponential lifetime fits of donor-only ($\tau_{\text{stretched}}$) and no-force control ($\tau_{\text{non-stretched}}$) samples. The bi-exponential fit to determine the relative amount of stretched molecules r is then performed while having $\tau_{\text{stretched}}$ and $\tau_{\text{non-stretched}}$ fixed with three free parameters (A_{total}, r, N_0).

6. Compare the stretch ratio of the tension sensor at different conditions.

   The ratio r is limited between one (all molecules stretched) and zero (no molecules stretched). Therefore, statistical tests that use the distribution of data (e.g., Student's t test and Kolmogorov-Smirnov test) are only applicable if data points do not reach these boundaries. In all other cases, ranked tests like the Wilcoxon-Mann-Whitney U test need to be used instead.

   The no-force control should not be stretched, and therefore a ratio close to zero is expected.

   The ratio determination can be further improved if the incomplete maturation of the acceptor chromophore is taken into account (see Support Protocol 4).

SUPPORT PROTOCOL 4

MOLECULAR STRETCH RATIO WITH INCOMPLETE ACCEPTOR CHROMOPHORE MATURATION

The ratio determination described in the Alternate Protocol can be further improved by taking incomplete maturation of the acceptor chromophore into account. Please note that not all fluorophores, being complex proteins, can be expected to be fully matured.

1. Perform a bi-exponential fit of the no-force control data with a fixed value for the donor-only lifetime ($\tau_{\text{donor without FRET}}$); leave the remaining four parameters free (A_{total}, r_{noFRET}, $\tau_{\text{donor with FRET}}$, N_0) to determine the lifetime of the donor undergoing FRET ($\tau_{\text{donor with FRET}}$) using:

$$N_b = A_{\text{total}} \left[r_{\text{noFRET}}\ \mathrm{e}^{-b\Delta t/\tau_{\text{donor without FRET}}} + (1 - r_{\text{noFRET}})\ \mathrm{e}^{-b\Delta t/\tau_{\text{donor with FRET}}} \right] + N_0,$$

   where A_{total} is the amplitude, $r_{\text{noFRET}} = A_{\text{donor without FRET}}/A_{\text{total}}$, b the time bin, Δt the time bin width, and N_0 the background photon count.

   The authors routinely use YPet/mCherry as FRET pair; mCherry is known by in vitro measurements to be fluorescently active to about 40% (Hendrix, Flors, Dedecker, Hofkens, & Engelborghs, 2008). This value is recaptured using the bi-exponential fit with four free parameters on the no-force control.

2. Perform a bi-exponential fit for the tension sensor samples with fixed lifetimes, $\tau_{\text{donor without FRET}}$ and $\tau_{\text{donor with FRET}}$. The resulting relative amount of molecules without FRET (r_{noFRET}) is then a composite of tension sensor molecules with non-fluorescent acceptor (also present in the no-force control) and of stretched tension sensor molecules, where the increased distance between donor and acceptor fluorophore prevents FRET.

3. Perform a bi-exponential fit for the no-force control samples with fixed lifetimes, $\tau_{\text{donor without FRET}}$ and $\tau_{\text{donor with FRET}}$. Then, rescale the tension sensor ratio r_{noFRET} to the no-force control level. This rescaled ratio r reflects the amount of stretched molecules.

In the no-force control, the amount of donor without FRET is caused exclusively by nonfluorescent acceptor because no molecules are stretched. Therefore, to calculate the ratio of stretched tension sensor molecules r, rescale with zero at the median of the no-force control.

The ratio of molecules without FRET is more accurate than the direct determination of the ratio of stretched molecules as described in the Alternate Protocol because the underlying decays are reflected more accurately. In the authors' experience, however, the (rescaled) ratios of stretched and non-stretched molecules are comparable with both methods.

The major advantage of taking the incomplete chromophore formation of the acceptor into account is that resulting ratios are not close to boundary conditions (one is lifetime of donor-only and zero is all molecules undergoing FRET). One consequence is, for example, that often the Kolmogorov-Smirnov test can be used instead of the ranked Wilcoxon-Mann-Whitney U test.

REAGENTS AND SOLUTIONS

LB agar plate

In a sterile 500-ml bottle, mix 16 g LB agar granulate (Luria/Miller, BROTH, 6675.2) in deionized, distilled water (up to 400 ml; 4% [w/v] final). Briefly swirl to mix, and mark with autoclave tape. Loosely close cap, and autoclave. Cool at room temperature until touchable with bare hands, and if necessary add antibiotics. Pour 12 ml into sterile 100 mm × 15 mm petri plates, and quickly swirl plates for even agar distribution. Keep at room temperature until solidified, and store at 4°C for up to 8 weeks.

Store plates upside down to avoid vapor condensation on the agar surface.

LB medium

To a sterile 500-ml bottle add:
10 g LB granulate (Luria/Miller, BROTH, X968.2; 2.5% [w/v] final)
400 ml deionized, distilled water
Sterilize by autoclaving
Store at room temperature for up to 8 weeks
If LB medium contains antibiotics, store at room temperature for up to 2 weeks

Transformation and storage solution

5 g tryptone-peptone (1% [w/v] final)
2.5 g yeast extract (0.5% [w/v] final)
2.5 g NaCl (100 mM final)
2.5 g polyethylene glycol (MW 3000/3500; 10% [v/v] final)
25 ml dimethyl sulfoxide (5% [v/v] final)
25 ml of 1 M $MgCl_2$ (50 mM final)
Bring to 500 ml with deionized, distilled water
Adjust pH to 6.5 with HCl
Filter sterilize with a 0.22-µm filter
Store at 4°C for up to 1 year

COMMENTARY

Background Information

Genetically encoded FRET-based tension sensors are developed to analyze mechanical tension across individual proteins in living cells and organisms. This is different from traction force microscopy, which is used to determine forces at the cellular level using specialized gels or fabricated micropillars (Polacheck & Chen, 2016). To analyze forces across individual molecules in vitro mostly atomic force microscopy or optical and magnetic tweezers are employed

(Freikamp, Mehlich, Klingner, & Grashoff, 2017). Forces across cell surface receptors are accessible with chemically modified surfaces using polyethylene glycol-, protein-, or DNA-based force sensors (Liu, Galior, Ma, & Salaita, 2017; Morimatsu, Mekhdjian, Adhikari, & Dunn, 2013; Zhang, Ge, Zhu, & Salaita, 2014). To target proteins in living cells, fluorescence-based approaches have been developed (Cost et al., 2015), of which the single-molecule calibrated, FRET-based tension sensors described here are most widely used.

As FRET-based tension sensors measure forces across individual proteins, experiments provide insights into force transduction across a particular POI. They do not measure force transduction across entire macromolecular complexes. Thus, the absence of tension across a specific POI does not necessarily indicate the absence of force transduction across the entire subcellular structure.

Typical live-cell FRET measurements are ensemble recordings in which many tension sensor molecules are measured simultaneously. As a result, tension sensor–based measurements provide an average force value, and it is usually unclear how mechanical forces are distributed across the population of analyzed molecules. Often, a given change in FRET efficiency can be explained by at least two scenarios: (1) all molecules experience the same amount of tension or (2) some molecules experience a lot of tension while others bear no tension at all. To be able to investigate molecular forces more precisely, multiple TSMs responding to different force levels can be used (Table 1).

In this article, we describe FRET measurements using TCSPC-FLIM. In principle, FRET can be determined by many different methods (Day & Davidson, 2012; Padilla-Parra & Tramier, 2012; Piston & Kremers, 2007; Sun et al., 2013; Zeug, Woehler, Neher, & Ponimaskin, 2012). However, we strongly recommend the use of quantitative techniques that allow the calculation of FRET efficiencies, which are insensitive to the technical setup and unaffected by experimental specifics such as fluctuations in laser intensity. The major drawbacks of FLIM are the expensive equipment and comparably long acquisition times. However, highly sensitive FLIM systems are now available, and extended acquisition times are no longer a limiting factor in these systems.

Critical Parameters

Controls

FRET-based tension sensor experiments require extensive controlling. As outlined above, these controls evaluate the functionality of the target protein (and the TSM) after TSM integration. As FRET is sensitive to many factors (not only fluorophore separation distance), data interpretation relies on the comparison of the tension sensor construct with a no-force control and the evaluation using biological no-force conditions. For FRET efficiency measurements, appropriate donor-only controls need to be utilized as well. Performing these control experiments is clearly key to reliable interpretation of tension sensor measurements.

Microscopy

To reduce biological variability, establish a workflow, and maintain this workflow for sample preparation and image acquisition. Importantly, preparation and measurement procedures need to be consistent for tension sensor and no-force control. Furthermore, the IRF should be characterized by a single peak; additional peaks in the IRF indicate suboptimal instrument performance and influence lifetime determination.

Data analysis

Data analysis procedures should be mostly automated to reduce the potential for biased analyses and ensure reproducibility. Particularly, the lifetime fit has to be performed in a standardized fashion.

Troubleshooting

Troubleshooting guides are provided for cloning (Table 2), transient transfection (Table 3), and FRET experiments (Table 4).

Anticipated Results

Statistical analysis

Live-cell FRET measurements are noisy and therefore require data collected from multiple images in different experiments. We recommend about 20 images per condition, construct, and experimental day and at least 4 to 5 independent experimental days before statistical evaluation of the data. To compare data sets, a two-sided Kolmogorov-Smirnov test is recommended. This test requires the same distribution of compared data sets but, in contrast to the more widely used Student's *t* test, no normality of the data. If the data

Table 2 Troubleshooting Guide for Cloning[a]

Problem	Possible cause	Solution
Few or no colonies after transformation	LB plates with wrong antibiotic	Check antibiotic resistance of the plasmid, and ensure that LB plates contain the appropriate antibiotic at the correct concentration
	LB plates too old	Prepare fresh plates
	Gibson assembly mixture lost activity	Assemble positive control provided by the kit to test whether the reagents of the kit are still biologically active and the transformation protocol works
	Bacteria lost competency	Prepare fresh competent cells (large constructs require higher competence than small constructs)
Too many colonies after transformation	High transformation efficiency	Reduce amount of transformed DNA
Low concentration of gel-purified products	Poor gel purification	Check troubleshooting guide in the gel purification kit manual
	Insufficient starting material	Repeat digestion with 2-3 times more original material
	Wrong PCR protocol	Adjust cycling parameters based on melting temperature (Tm) of the primers
Colonies contain incorrectly sized fragments or sequencing shows DNA deletions or rearrangements	Wrong or contaminated PCR products	Repeat PCR with new starting material
	Insufficient Gibson assembly	Ensure each fragment has appropriate overlapping sequences with adjacent fragments or the plasmid backbone
		Increase DNA molar ratio (e.g., 5× for smaller fragments)
	Incorrect primer design	Primers should have 20-bp sequence homology with PCR template
		Avoid primers that dimerize or have the potential to form hairpins
		Tm for a pair of primers should not be more than 5°C different
Digestion of backbone plasmid shows multiple smaller bands on agarose gel	Restriction enzyme recognizes different restriction sites in the backbone sequences	Check for star activity of the enzyme and adjust digestion protocol accordingly or choose alternative restriction sites to linearize the vector

[a]Also refer to troubleshooting guides in the manufacturer's user manuals.

are normally distributed, the Student's *t* test may be applied, but the normality assumption should be checked using the Lilliefors test or other similar test. If images are spatially resolved and data points correspond to individual substructures, statistical tests should consider the interdependence of data originating from the same image. For these types of analysis, the authors recommend more elaborate linear-mixed effects models.

Lifetimes

FLIM-based FRET measurements provide information on the fluorescence lifetime of tension sensor and control constructs. The lifetime of the donor fluorophore is shortened in

Table 3 Troubleshooting Guide for Transient Transfection[a]

Problem	Possible cause	Solution
Cells die during transfection	Toxic effect of transfection reagent	Shorten transfection time
	Toxic effect of antibiotics	Ensure that all medium used during transfection is antibiotic-free
Insufficient construct expression	Insufficient transfection	Increase amount of DNA (and adjust transfection reagents accordingly)
		Prolong transfection time up to 1 day
	Insufficient expression	Increase time between transient transfection and measurement to allow for construct expression and fluorophore maturation
		Reduce time between transient transfection and measurement to avoid dilution of plasmid DNA during cell division

[a]Also refer to the troubleshooting guide in the manufacturer's user manual.

the presence of the acceptor as long as the linker peptide is not elongated by mechanical forces. Therefore, the lifetime of the tension sensor is expected to be longer than the lifetime of the no-force control but shorter than the donor-only controls (Fig. 5A). The lifetime determination of the donor fluorophore is usually more reliable, so the variance of the donor-only lifetime is expected to be smaller in comparison to tension sensor and no-force control (Fig. 5A). Similarly, the changes in median and spread between different experiments are typically smallest for donor-only controls and largest for the tension sensor construct itself (Fig. 5B). This is caused by the underlying population of tension sensor molecules that may be exposed to a range of forces resulting in a more widely spread lifetime distribution.

FRET efficiencies

The determined fluorescence lifetimes allow the calculation of FRET efficiencies ($E = 1 - \tau_{DA}/\overline{\tau_D}$). Keep in mind that fluorescence lifetime values and FRET efficiencies are anti-correlated: a shorter lifetime corresponds to a higher FRET efficiency. Therefore, mechanical tension is indicated by a decrease in FRET efficiency as compared to the no-force control (Fig. 5C). If forces across the POI are reduced, the FRET efficiency of the tension sensor increases, while the FRET efficiency of the no-force control should remain unaffected (Fig. 5C).

Intermolecular FRET (i.e., FRET between neighboring molecules) is expected to be significantly lower than the FRET efficiency of the no-force control. The co-expression of donor-only and acceptor-only constructs corresponding to tension sensor and no-force control can be used to determine intermolecular FRET, which should be similar for both constructs (Fig. 5D).

Forces

In principle, forces can be calculated from the observed FRET efficiency differences between tension sensor and control samples if calibrated TSMs are used. However, the force-response characteristic of the employed linker peptide will determine the detectable force range (Fig. 2B). The FL linker, for example, displays an almost digital unfolding response at 3 to 5 pN, while the F40 linker extends gradually at 1 to 6 pN (Fig. 6A). As the measurements described here are ensemble recordings, the underlying force distributions within the population of sensor molecules are not readily accessible (Fig. 6B). Calculating an average force per molecule may be misleading because it suggests that all tension sensor molecules experience the same amount of tension. This is unlikely to be the case in most subcellular structures that are often characterized by rather dynamic molecular processes (Wehrle-Haller & Imhof, 2002). An alternative assumption is that some tension sensor molecules are entirely stretched, while others remain in a closed conformation. For experiments using FL-based TSM, which virtually exists in two states (stretched above 5 pN and closed below

Table 4 Troubleshooting Guide for FLIM Experiments

Problem	Possible cause	Solution
IRF displays additional peak	Misalignment in microscope setup	Contact microscope support
Signal is too low	Insufficient construct expression	See Table 3 for troubleshooting of transient transfection
	Suboptimal image acquisition	Increase acquisition time (e.g., by increased number of image repeats)
		Increase laser intensity (ensure all photons can still be detected)
No-force control shows less FRET than tension sensor	Inappropriate donor-only lifetime	Use donor-only control with nonfluorescent acceptor (point mutation)
		Compare donor-only lifetime of constructs matching tension sensor and no-force control
		Test different no-force control
	No-force control experiences different environment	Determine intermolecular FRET to test whether increased intermolecular FRET leads to elevated tension sensor FRET
High spread in FRET data	High spread between experimental days	Optimize and follow standard procedure for sample preparation to minimize biological variability
		Standardize image acquisition (speed, acquisition time, laser intensity) to minimize variability
	High spread in individual days	Check IRF and adjust setup if necessary; secondary peaks in the IRF strongly influence tail fitting
		Test different start and endpoints for tail fitting
		Increase photon counts to increase fit accuracy
		Limit data extraction to even more specific regions of interest
Higher data spread in specific samples	Higher variance in tension sensor data	Expected because tension sensor has an additional source of variation (amount of tension) compared to no-force control
	Higher variance in no-force control data	Compare localization of no-force control and tension sensor
		Adjust signal masking to isolate only specific signal

FLIM, fluorescence lifetime imaging microscopy; FRET, Förster resonance energy transfer; IRF, instrument response function.

3 pN), it seems reasonable to neglect intermediate states. In this case, FRET efficiencies reflect the amount of molecules being exposed to mechanical tension and do not inform on the force magnitude per molecule. The ratio of stretched and non-stretched molecules can be determined using bi-exponential fitting (see Alternate Protocol).

The parallel use of several TSMs with distinct force sensitivities can help to estimate molecular forces. Note that the precise molecular force value might not be of ultimate interest. Instead, the relative changes in molecular tension are often sufficient to investigate molecular processes underlying force transduction in cells.

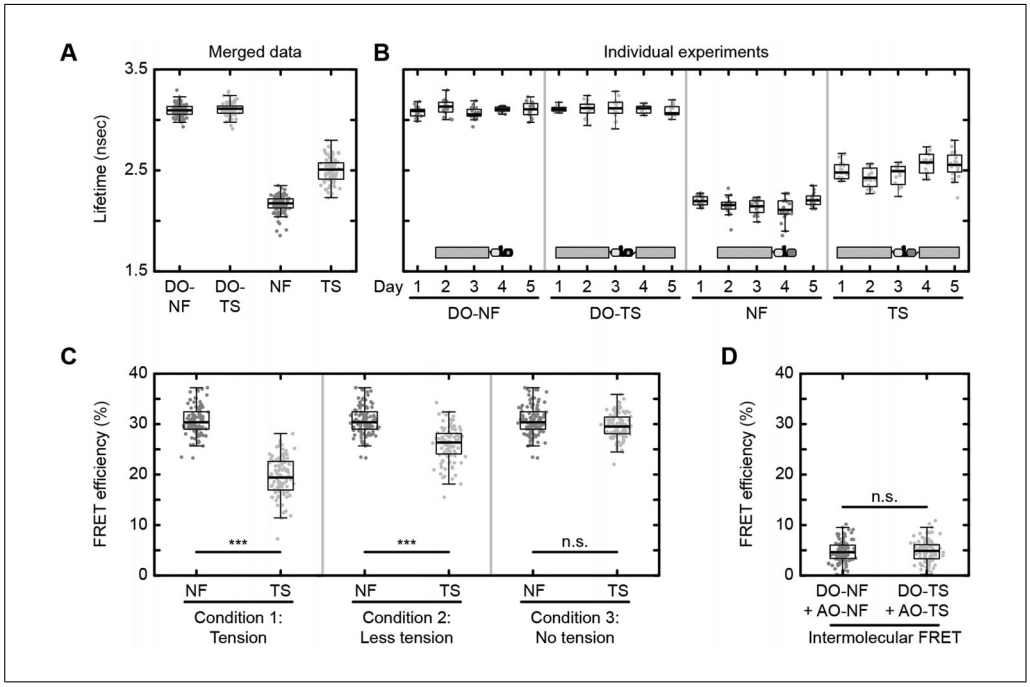

Figure 5 Anticipated results for fluorescence lifetimes and Förster resonance energy transfer (FRET) efficiencies. (**A**) Lifetimes for donor-only controls (DO) matching the no-force control (DO-NF) and tension sensor (DO-TS) are usually comparable. FRET shortens the fluorescence lifetime of the donor; thus the no-force control (NF) has a shorter lifetime. Mechanical forces across TS molecules lead to an increase in fluorescence lifetime as compared to NF. (**B**) Fluorescence lifetimes determined from individual experimental days should show similar values. Variability is usually largest for TS samples as they are subject to biological regulation. (**C**) The FRET efficiency of the TS decreases in response to mechanical tension. Different degrees of tension lead to different FRET efficiencies. The NF should stay rather constant throughout the experiments. If sources of molecular tension are disrupted (biological no-force control, condition 3), the FRET efficiency of the TS sample is expected to increase to the level of NF. (**D**) Intermolecular FRET may contribute to the observed FRET efficiency. FRET efficiencies of intermolecular FRET are usually small compared to the overall FRET. Intermolecular FRET of NF and TS constructs should be similar. (A-D) Simulated data based on experimental values observed for a tension sensor with YPet/mCherry FRET pair and FL linker peptide derived from 20 images on 5 experimental days resulting in 100 images per condition. Two-sided Kolmogorov-Smirnov test; ***$p < 0.001$ and n.s. (not significant) $p > 0.05$.

Time Considerations

Tension sensor development

Developing a new tension sensor is a time-consuming task. Generating, applying, and evaluating new tension sensor constructs may take years because several integration sites may need to be tested. In addition, the protein functionality after TSM integration has to be carefully validated before any meaningful measurements can be performed. Moreover, data analysis tools may need to be developed. Thus, using established tension sensor constructs considerably reduces time requirements.

Tension sensor experiment

For an individual experiment, transient transfection takes about 30 min experimental time 1 or 2 days prior to microscopy measurement. Preparing cells for live-cell imaging depends on the cell type used but typically requires not more than 1 hr. The most time-consuming part of the experiment is the microscopy because sufficient data should be collected for each condition and construct (ideally about 20 cells per condition and measurement day). Depending on the transfection efficiency and microscopy setup, measurement may take up to 6 hr per day. Thus, a full tension sensor experiment with five independent experimental repeats can be performed within a week.

Data analysis

Setting up a data analysis pipeline can be cumbersome. Once established, data analysis can be completed within a few hours.

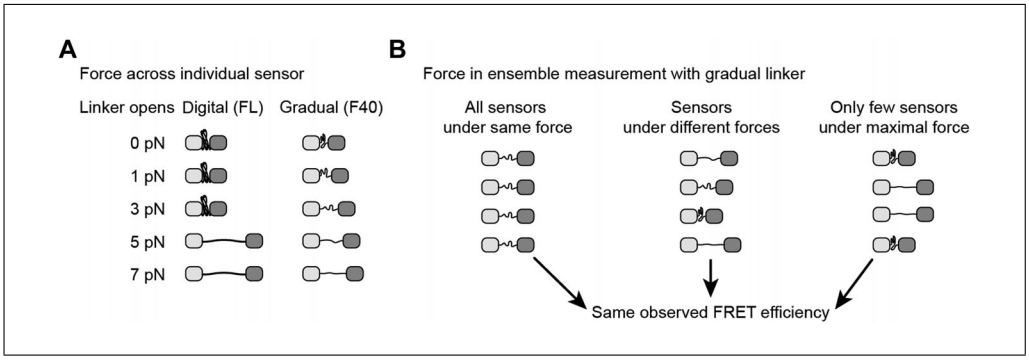

Figure 6 Interpretation of results. (**A**) For data interpretation, it is important to consider the unfolding properties of the employed linker peptide. For example, the FL peptide is characterized by an almost digital transition and therefore will exist in either the non-stretched (low forces, high FRET) or stretched (high forces, no FRET) conformation. By contrast, the F40 peptide opens gradually in response to force and will display a range of FRET efficiencies. (**B**) In ensemble measurements, where the signal of many molecules is averaged, observed FRET efficiencies could be explained by different underlying tension sensor states. If gradually opening sensors are used, a given FRET efficiency could result from all molecules being stretched to the same extent, molecules being exposed to different forces, or molecules being either stretched or non-stretched. Applying additional TSMs with distinct force response characteristics may help to determine which scenario is reflective of the physiological situation.

Acknowledgements

We thank Verena Kanoldt for sharing cloning and transfection protocols and Christoph Klingner for commenting on the manuscript. C.G. was supported by the Max Planck Förderstiftung, the Human Frontier Science Program (RGP0024), and the German Research Council through the SFB863, SPP1782, and the DFG-ANR program.

Conflicts of Interest

The authors declare no conflicts of interest.

Literature Cited

Alsteens, D., Martinez, N., Jamin, M., & Jacob-Dubuisson, F. (2013). Sequential unfolding of beta helical protein by single-molecule atomic force microscopy. *PLoS One*, *8*, e73572. doi: 10.1371/journal.pone.0073572.

Austen, K., Ringer, P., Mehlich, A., Chrostek-Grashoff, A., Kluger, C., Klingner, C., ... Grashoff, C. (2015). Extracellular rigidity sensing by talin isoform-specific mechanical linkages. *Nature Cell Biology*, *17*, 1597–1606. doi: 10.1038/ncb3268.

Bajar, B., Wang, E., Zhang, S., Lin, M. Z., & Chu, J. (2016). A guide to fluorescent protein FRET pairs. *Sensors*, *16*, E1488. doi: 10.3390/s16091488.

Borghi, N., Sorokina, M., Shcherbakova, O. G., Weis, W. I., Pruitt, B. L., Nelson, W. J., & Dunn, A. R. (2012). E-cadherin is under constitutive actomyosin-generated tension that is increased at cell–cell contacts upon externally applied stretch. *Proceedings of the National Academy of Sciences of the United States of America*, *109*, 12568–12573. doi: 10.1073/pnas.1204390109.

Brenner, M. D., Zhou, R., Conway, D. E., Lanzano, L., Gratton, E., Schwartz, M. A., & Ha, T. (2016). Spider silk peptide is a compact, linear nanospring ideal for intracellular tension sensing. *Nano Letters*, *16*, 2096–2102. doi: 10.1021/acs.nanolett.6b00305.

Cost, A.-L., Ringer, P., Chrostek-Grashoff, A., & Grashoff, C. (2015). How to measure molecular forces in cells: A guide to evaluating genetically-encoded FRET-based tension sensors. *Cellular and Molecular Bioengineering*, *8*, 96–105. doi: 10.1007/s12195-014-0368-1.

Day, R. N., & Davidson, M. W. (2012). Fluorescent proteins for FRET microscopy: Monitoring protein interactions in living cells. *Bioessays*, *34*, 341–350. doi: 10.1002/bies.201100098.

del Rio, A., Perez-Jimenez, R., Liu, R., Roca-Cusachs, P., Fernandez, J. M., & Sheetz, M. P. (2009). Stretching single talin rod molecules activates vinculin binding. *Science*, *323*, 638–641. doi: 10.1126/science.1162912.

Demeautis, C., Sipieter, F., Roul, J., Chapuis, C., Padilla-Parra, S., Riquet, F. B., & Tramier, M. (2017). Multiplexing PKA and ERK1&2 kinases FRET biosensors in living cells using single excitation wavelength dual colour FLIM. *Scientific Reports*, *7*, 41026. doi: 10.1038/srep41026.

Dietz, H., & Rief, M. (2004). Exploring the energy landscape of GFP by single-molecule mechanical experiments. *Proceedings of the National Academy of Sciences of the United States of America*, *101*, 16192–16197. doi: 10.1073/pnas.0404549101.

Finer, J. T., Simmons, R. M., & Spudich, J. A. (1994). Single myosin molecule mechanics: Piconewton forces and nanometre steps. *Nature*, *368*, 113–119. doi: 10.1038/368113a0.

Freikamp, A., Cost, A.-L., & Grashoff, C. (2016). The piconewton force awakens: Quantifying mechanics in cells. *Trends in Cell Biology*, *26*, 838–847. doi: 10.1016/j.tcb.2016.07.005.

Freikamp, A., Mehlich, A., Klingner, C., & Grashoff, C. (2017). Investigating piconewton forces in cells by FRET-based molecular force microscopy. *Journal of Structural Biology*, *197*, 37–42. doi: 10.1016/j.jsb.2016.03.011.

Gayrard, C., & Borghi, N. (2016). FRET-based molecular tension microscopy. *Methods*, *94*, 33–42. doi: 10.1016/j.ymeth.2015.07.010.

Gennerich, A., Carter, A. P., Reck-Peterson, S. L., & Vale, R. D. (2007). Force-induced bidirectional stepping of cytoplasmic dynein. *Cell*, *131*, 952–965. doi: 10.1016/j.cell.2007.10.016.

Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., Hutchison III, C. A., & Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*, *6*, 343–345. doi: 10.1038/nmeth.1318.

Grashoff, C., Hoffman, B. D., Brenner, M. D., Zhou, R., Parsons, M., Yang, M. T., ... Schwartz, M. A. (2010). Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. *Nature*, *466*, 263–266. doi: 10.1038/nature09198.

Hendrix, J., Flors, C., Dedecker, P., Hofkens, J., & Engelborghs, Y. (2008). Dark states in monomeric red fluorescent proteins studied by fluorescence correlation and single molecule spectroscopy. *Biophysical Journal*, *94*, 4103–4113. doi: 10.1529/biophysj.107.123596.

Jurchenko, C., & Salaita, K. S. (2015). Lighting up the force: Investigating mechanisms of mechanotransduction using fluorescent tension probes. *Journal of Molecular Cell Biology*, *35*, 2570–2582. doi: 10.1128/MCB.00195-15.

Kong, F., García, A. J., Mould, A. P., Humphries, M. J., & Zhu, C. (2009). Demonstration of catch bonds between an integrin and its ligand. *The Journal of Cell Biology*, *185*, 1275–1284. doi: 10.1083/jcb.200810002.

Kumar, A., Ouyang, M., Van den Dries, K., McGhee, E. J., Tanaka, K., Anderson, M. D., ... Schwartz, M. A. (2016). Talin tension sensor reveals novel features of focal adhesion force transmission and mechanosensitivity. *The Journal of Cell Biology*, *213*, 371–383. doi: 10.1083/jcb.201510012.

LaCroix, A. S., Lynch, A. D., Berginski, M. E., & Hoffman, B. D. (2018). Tunable molecular tension sensors reveal extension-based control of vinculin loading. *eLife*, *7*, e33927. doi: 10.7554/eLife.33927.

Lagendijk, A. K., Gomez, G. A., Baek, S., Hesselson, D., Hughes, W. E., Paterson, S., ... Hogan, B. M. (2017). Live imaging molecular changes in junctional tension upon VE-cadherin in zebrafish. *Nature Communications*, *8*, 1402. doi: 10.1038/s41467-017-01325-6.

Lakowicz, J. R. (2006). *Principles of fluorescence spectroscopy*, *Vol*. 3. New York: Springer.

Lemke, S. B., Weidemann, T., Cost, A.-L., Grashoff, C., & Schnorrer, F. (2018). A small proportion of talin molecules transmit forces to achieve muscle attachment in vivo. Preprint. *https://www.biorxiv.org/content/10.1101/446336v1*.

Liu, Y., Galior, K., Ma, V. P.-Y., & Salaita, K. (2017). Molecular tension probes for imaging forces at the cell surface. *Accounts of Chemical Research*, *50*, 2915–2924. doi: 10.1021/acs.accounts.7b00305.

Maki, K., Han, S.-W., Hirano, Y., Yonemura, S., Hakoshima, T., & Adachi, T. (2016). Mechano-adaptive sensory mechanism of α-catenin under tension. *Scientific Reports*, *6*, 24878. doi: 10.1038/srep24878.

Meng, F., & Sachs, F. (2011). Visualizing dynamic cytoplasmic forces with a compliance-matched FRET sensor. *Journal of Cell Science*, *124*, 261–269. doi: 10.1242/jcs.071928.

Meng, F., Suchyna, T. M., Lazakovitch, E., Gronostajski, R. M., & Sachs, F. (2011). Real time FRET based detection of mechanical stress in cytoskeletal and extracellular matrix proteins. *Cellular and Molecular Bioengineering*, *4*, 148–159. doi: 10.1007/s12195-010-0140-0.

Meng, F., Suchyna, T. M., & Sachs, F. (2008). A fluorescence energy transfer-based mechanical stress sensor for specific proteins in situ. *The FEBS Journal*, *275*, 3072–3087. doi: 10.1111/j.1742-4658.2008.06461.x.

Morimatsu, M., Mekhdjian, A. H., Adhikari, A. S., & Dunn, A. R. (2013). Molecular tension sensors report forces generated by single integrin molecules in living cells. *Nano Letters*, *13*, 3985–3989. doi: 10.1021/nl4005145.

Oberhauser, A. F., Badilla-Fernandez, C., Carrion-Vazquez, M., & Fernandez, J. M. (2002). The mechanical hierarchies of fibronectin observed with single-molecule AFM. *Journal of Molecular Biology*, *319*, 433–447. doi: 10.1016/S0022-2836(02)00306-6.

Padilla-Parra, S., & Tramier, M. (2012). FRET microscopy in the living cell: Different approaches, strengths and weaknesses. *Bioessays*, *34*, 369–376. doi: 10.1002/bies.201100086.

Piston, D. W., & Kremers, G.-J. (2007). Fluorescent protein FRET: The good, the bad and the ugly. *Trends in Biochemical Sciences*, *32*, 407–414. doi: 10.1016/j.tibs.2007.08.003.

Polacheck, W. J., & Chen, C. S. (2016). Measuring cell-generated forces: A guide to the available tools. *Nature Methods*, *13*, 415–423. doi: 10.1038/nmeth.3834.

Price, A. J., Cost, A.-L., Ungewiß, H., Waschke, J., Dunn, A. R., & Grashoff, C. (2018). Mechanical loading of desmosomes depends on the magnitude and orientation of external stress. *Nature Communications*, *9*, 5284. doi: 10.1038/s41467-018-07523-0.

Rief, M., Gautel, M., Oesterhelt, F., Fernandez, J. M., & Gaub, H. E. (1997). Reversible unfolding of individual titin immunoglobulin domains by AFM. *Science*, *276*, 1109–1112. doi: 10.1126/science.276.5315.1109.

Ringer, P., Weißl, A., Cost, A.-L., Freikamp, A., Sabass, B., Mehlich, A., ... Grashoff, C. (2017). Multiplexing molecular tension sensors reveals piconewton force gradient across talin-1. *Nature Methods*, *14*, 1090–1096. doi: 10.1038/nmeth.4431.

Sun, Y., Rombola, C., Jyothikumar, V., & Periasamy, A. (2013). Förster resonance energy transfer microscopy and spectroscopy for localizing protein–protein interactions in living cells. *Cytometry Part A*, *83*, 780–793. doi: 10.1002/cyto.a.22321.

Svoboda, K., Schmidt, C. F., Schnapp, B. J., & Block, S. M. (1993). Direct observation of kinesin stepping by optical trapping interferometry. *Nature*, *365*, 721–727. doi: 10.1038/365721a0.

Wahl, M. (2014). *Technical note: Time-correlated single photon counting*. Berlin: PicoQuant. *https://www.picoquant.com/images/uploads/page/files/7253/technote_tcspc.pdf*.

Wehrle-Haller, B., & Imhof, B. A. (2002). The inner lives of focal adhesions. *Trends in Cell Biology*, *12*, 382–389. doi: 10.1016/S0962-8924(02)02321-8.

Yao, M., Goult, B. T., Klapholz, B., Hu, X., Toseland, C. P., Guo, Y., ... Yan, J. (2016). The mechanical response of talin. *Nature Communications*, *7*, 11966. doi: 10.1038/ncomms11966.

Zeug, A., Woehler, A., Neher, E., & Ponimaskin, E. G. (2012). Quantitative intensity-based FRET approaches—a comparative snapshot. *Biophysical Journal*, *103*, 1821–1827. doi: 10.1016/j.bpj.2012.09.031.

Zhang, X., Halvorsen, K., Zhang, C.-Z., Wong, W. P., & Springer, T. A. (2009). Mechanoenzymatic cleavage of the ultralarge vascular protein von Willebrand factor. *Science*, *324*, 1330–1334. doi: 10.1126/science.1170905.

Zhang, Y., Ge, C., Zhu, C., & Salaita, K. (2014). DNA-based digital tension probes reveal integrin forces during early cell adhesion. *Nature Communications*, *5*, 5167. doi: 10.1038/ncomms6167.

Key References

Bajar et al. (2016). See above.

Summarizes different FRET measurement techniques and FRET pairs.

Cost et al. (2015). See above.

Summarizes different approaches to measure mechanical tension across single molecules and how to validate protein and tension sensor functionality.

Freikamp et al. (2016). See above.

Summarizes applications of the tension sensor technique and its limitations.

Freikamp et al. (2017). See above.

Describes how to develop new linker peptides for FRET-based tension sensor modules.

Grashoff et al. (2010). See above.

First single-molecule calibrated tension sensor used to measure mechanical forces across the focal adhesion protein vinculin at cell–matrix contacts.

Lagendijk et al. (2017). See above.

First transgenic zebrafish with a VE-cadherin tension sensor to measure forces at cell–cell contacts in vivo.

Lakowicz (2006). See above.

Explains fluorophore properties, FRET theory, measurement methods, and applications.

Price et al. (2018). See above.

Comparison of different donor-only lifetimes at the example of a desmoplakin II tension sensor.

Ringer et al. (2017). See above.

First description of how to determine the relative amount of stretched and non-stretched sensor molecules.

Wahl (2014). See above.

Technical note describing TCSPC setup and measurement.

Internet Resources

https://international.neb.com/Protocols/0001/01/01/pcr-protocol-m0530

PCR protocol for Phusion ® polymerase.

https://www.addgene.org/protocols/gel-electrophoresis/

Explanation of how to pour and run agarose gels.

https://www.addgene.org/protocols/restriction-digest/

Explanation of how to perform a restriction digest.

https://nebcloner.neb.com/#!/redigest

Tool to select appropriate buffers for New England Biolabs restriction enzymes.

http://nebiocalculator.neb.com/#!/ligation

Calculator for pmol amounts of DNA.

https://www.thermofisher.com/de/de/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator.html

Calculator for primer melting temperatures.

https://www.tcspc.com/doku.php/howto:how_to_measure_the_instrument_response_function_irf

Explanation and protocol for the determination of the instrument response function.