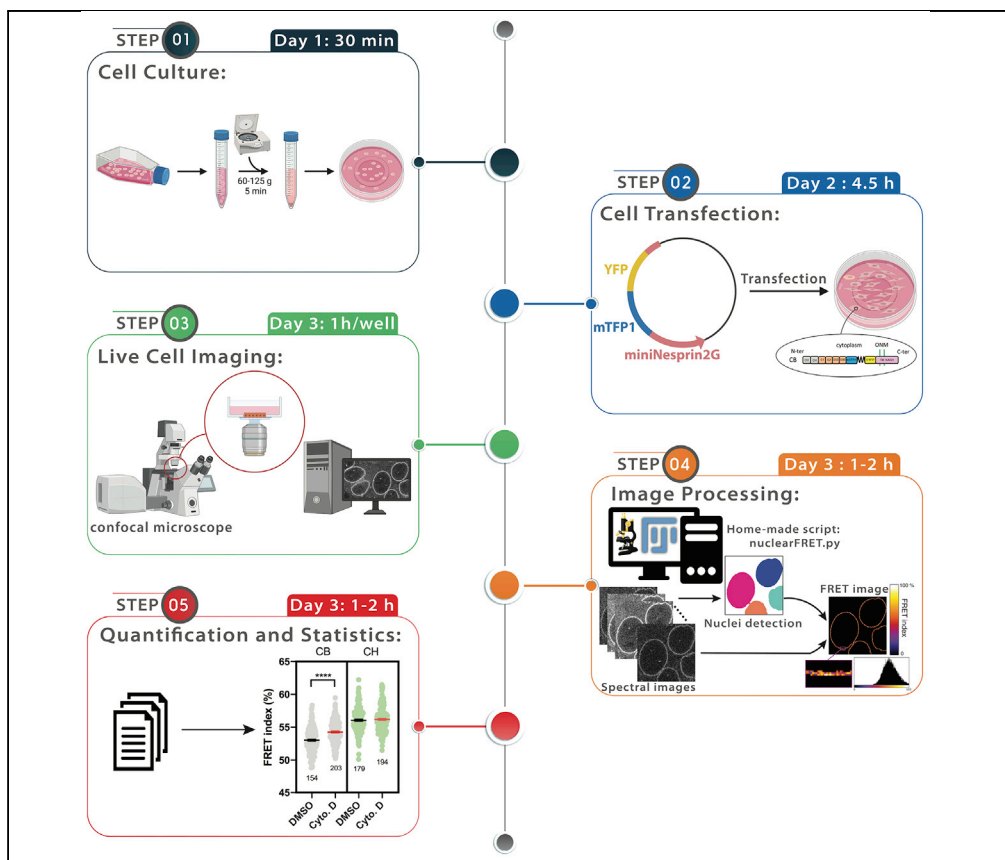


Protocol

Molecular tension microscopy of the LINC complex in live cells



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Highlights

Genetically encoded
molecular tension
biosensors

Spectral imaging on a
scanning confocal
microscope

Microscope
calibration for FRET
imaging

Custom script for
semi-automated
image analysis of
FRET and nucleus
segmentation

We present a protocol to measure the effect of pharmacological treatments on the mechanical tension experienced by nesprins at the cytoplasmic surface of the nuclear envelope of mammalian cells in culture. We apply this protocol to MDCK epithelial cells exposed to the actin depolymerization agent cytochalasin D. To do so, we perform confocal spectral imaging of transiently expressed molecular tension sensors of mini-nesprin 2G and analyze the FRET signal from the sensors with a custom-made Fiji script.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

Molecular tension microscopy of the LINC complex in live cells

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SUMMARY

We present a protocol to measure the effect of pharmacological treatments on the mechanical tension experienced by nesprins at the cytoplasmic surface of the nuclear envelope of mammalian cells in culture. We apply this protocol to MDCK epithelial cells exposed to the actin depolymerization agent cytochalasin D. To do so, we perform confocal spectral imaging of transiently expressed molecular tension sensors of mini-nesprin 2G and analyze the FRET signal from the sensors with a custom-made Fiji script.

For complete details on the use and execution of this protocol, please refer to Déjardin et al. (2020).

BEFORE YOU BEGIN

⌚ Timing: 1 week

1. Cell culture. Start a culture of Madin–Darby canine kidney (MDCK) type II G cells in DMEM supplemented with 10% (vol/vol) FBS with low glucose 1 g/L, 1% Pen/Strep, 2 mM L-Glutamine (cell culture medium). Incubate the cells at 37°C in 5% CO₂ and passage them every time they reach 80%–90% confluence.

Note: MDCK are epithelial cells and grow as tight colonies.

Note: This normal stable cell line allows for many rounds of expansion and splitting. Nevertheless, it is preferable to keep passage number low, never exceed confluence, and thaw a new batch at the first sign of phenotypic drift.

Note: We usually split the cells every week in a ratio of 1:10 and we replace the culture medium every other day to replenish nutrients and eliminate dead cells and debris.

Note: Depending on your application, you can use another cell line and follow the same guidelines before starting the experiment. We have also used this protocol for NIH 3T3 cells.

2. Collagen coating of chambered coverslips. In the tissue culture hood, cover the coverslips with collagen solution (see [materials and equipment](#)). Incubate for 1 h at 18°C–25°C. Then, remove the collagen solution, let dry for 1 h and switch ON the UV lamp for 15 min to sterilize the surface.



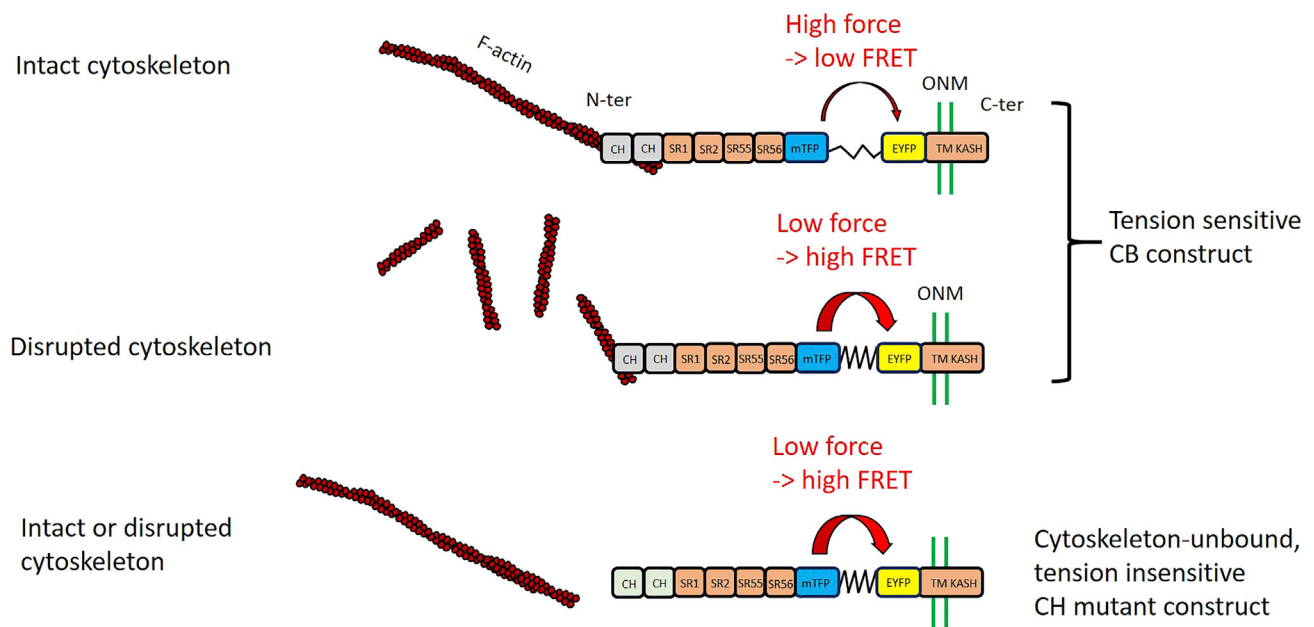


Figure 1. Tension sensor constructs and conditions

The nesprin tension sensor construct CB binds to the cytoskeleton through the Calponin Homology domains (CH), contains spectrin repeats SR1, 2, 55, 56 of the endogenous nesprin2G, two fluorophores mTFP1 and EYFP separated by a polypeptidic spring that serve as a tension sensor, and the transmembrane (TM) and KASH domains of the endogenous Nesprin 2G. The higher the tension, the lower the FRET between the fluorophores. Upon cytoskeleton disruption, tension decreases and FRET increases. The CH mutant construct contains mutations in the CH domains that prevent proper cytoskeleton binding, making it insensitive to changes in cytoskeletal tension. See (Déjardin et al., 2020) for construction details and characterization.

Note: Use the UV light of the cell culture hood. Multiple coverslips may be prepared in advance and stored at 18°C–25°C indefinitely in a sterile packaging. The following protocol will require at least three 2-well coverslips. MDCK do not adhere well on bare glass, such collagen coating allows full cell spreading within a couple of hours. We have noticed no substantial difference with type I collagen from rat tail but MDCK cells tend to adhere more slowly on fibronectin. We have noticed no detrimental effect of UV exposure.

3. Amplify, purify and quantify the plasmids encoding nesprin tension sensors and FRET standards.

Note: We generated the nesprin tension sensor (CB: cytoskeleton-binding) construct by inserting a tension sensor module (TSMoD, (Grashoff et al., 2010)) between the transmembrane domain and the neighboring spectrin repeat (SR) of a short variant of nesprin2G (mN2G, (Luxton et al., 2010)). The TSMoD consists in a pair of fluorescent proteins (mTFP/mYFP) flanking a polypeptidic spring and the version used originates from (Borghi et al., 2012). Details of the nesprin tension sensor construction can be found in (Déjardin et al., 2020) (Figure 1). As a tension-less control, we mutated the CB construct in the Calponin Homology (CH) domain to impair cytoskeleton binding (Déjardin et al., 2020) (Figure 1). FRET standards consist in FRET pairs separated by fixed-length linkers of known FRET efficiencies (Day et al., 2008).

Note: The quality of the plasmid preparation affects the efficiency of transfection. It is better to use a midi-prep or maxi-prep kit rather than a mini-prep kit and to elute DNA with ultrapure sterile water. DNA concentration is estimated by measuring A260 absorbance using a spectrophotometer. To evaluate DNA purity, we measure A260/A280 absorbance ratio. DNA preps with an A260/A280 ratio of 1.8–1.9 are suitable for reliable transfection results.

4. Install software and custom codes for analysis.

Download and install (Fiji Is Just) ImageJ (FIJI) (Rueden et al., 2017). Install the libraries LOCI (<https://imagej.net/orgs/loci>) and MorphoLibJ (<https://imagej.net/plugins/morpholibj>) (Legland et al., 2016). Download the Fiji script 'nuclearFRET.py' from the GitHub webpage <https://github.com/phigirard/nuclearFRET>. This script was tested in Fiji 2.1.0/1.53f.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
DMEM, low glucose, pyruvate	Thermo Fisher Scientific	Cat#31885049
Penicillin-Streptomycin (10,000 U/mL)	Thermo Fisher Scientific	Cat#15140122
Fetal Bovine Serum, qualified, heat inactivated, Brazil	Thermo Fisher Scientific	Cat#10270106
L-Glutamine (200 mM)	Thermo Fisher Scientific	Cat#25030024
FluoroBrite™ DMEM	Thermo Fisher Scientific	Cat#A1896701
Collagen from human placenta, Collagen Type 4	Sigma-Aldrich	Cat#C7521, CAS Number 9007-34-5
HEPES (1 M)	Thermo Fisher Scientific	Cat#15630080
DPBS, no calcium, no magnesium	Thermo Fisher Scientific	Cat#14190144
Acetic acid glacial, ReagentPlus, ≥ 99%	Sigma-Aldrich	Cat#A6283, CAS Number 64-19-7
Trypsin-EDTA (0.5%), no phenol red	Thermo Fisher Scientific	Cat# 15400054
Cytochalasin D	Sigma-Aldrich	Cat#C8273, CAS Number 22144-77-0
Mineral Oil	Sigma-Aldrich	Cat# M5904, CAS Number 8042-47-5
TurboFect Transfection Reagent	Thermo Fisher Scientific	Cat#R0531
Deposited data		
Raw image series	https://github.com/phigirard/nuclearFRET	https://doi.org/10.5281/zenodo.6642854
Experimental models: Cell lines		
Madin–Darby canine kidney (MDCK) type II G	ECACC	MDCK II (ECACC 00062107)
Recombinant DNA		
mN2G-TSMod (CB)	(Déjardin et al., 2020)	https://doi.org/10.1083/jcb.201908036
mN2G-TSMod (CH)	(Déjardin et al., 2020)	https://doi.org/10.1083/jcb.201908036
mTFP1-GPGGA-Venus (5AA)	(Day et al., 2008)	https://doi.org/10.1117/1.2939094
mTFP1-TRAF-Venus (TRAF)	(Day et al., 2008)	https://doi.org/10.1117/1.2939094
Software and algorithms		
FIJI version 2.1.0/1.53 with libraries: LOCI MorphoLibJ release v1.4.3	(Rueden et al., 2017) (Wisconsin-Madison University) (Legland et al., 2016)	RRID: SCR_002285 https://imagej.net/orgs/loci https://imagej.net/plugins/morpholibj
nuclearFRET.py	https://github.com/phigirard/nuclearFRET	https://doi.org/10.5281/zenodo.6642854
Zen 2011 (Black edition)	Carl Zeiss	RRID: SCR_018163
Prism VIII software	GraphPad	RRID: SCR_002798
Other		
2-well Chambered Coverglass w/ non-removable wells	Thermo Fisher Scientific	Cat#155380PK
Commercial laser scanning confocal inverted microscope equipped with an incubator	Carl Zeiss	NA
Microscope objective: 63×/1.4NA Plan-Apochromat oil immersion.	Carl Zeiss	NA

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Microscope detection: Spectral fluorescence GaAsP array detector.	Carl Zeiss	NA
Argon laser -the 458 nm line is optimal for mTFP1 excitation- or equivalent, 25 mW	Carl Zeiss	NA
Top stage incubator for live cell time lapse imaging (with temperature, humidity, CO ₂ control)	Carl Zeiss	NA
Main beam splitter 458	Carl Zeiss	NA
Epifluorescence Illuminator HXP 120 V + filter set 38 GFP (EX 470/40).	Carl Zeiss	NA
Table-top centrifuge and rotor for 15 mL Falcon tubes	Generic	NA

MATERIALS AND EQUIPMENT

Recipe: Collagen solution for collagen coating of chambered coverslips.

- Dilute Acetic Acid (stock 17.4 M) in MilliQ water (final concentration 0.5 M) by adding 2.87 mL Acetic Acid in 100 mL ddH₂O.
- Dilute collagen in Acetic Acid on ice (to obtain a 20× collagen stock solution) by adding 5 mg collagen in 5 mL Acetic Acid 0.5 M.
- Prepare a working solution (50 µg/mL) by diluting 1 mL 20× collagen stock solution in 20 mL Acetic Acid 0.5 M.

Note: never add water to acid.

Recipe: Imaging medium (storage at 4°C maximum 1 month).

- Pour 43 mL of FluoroBrite DMEM medium in an opaque 50 mL Falcon tube.
- Add L-Glutamine, Pen/Strep, FBS and HEPES according to the table below to reach the final volume of 50 mL.

Reagent	Final concentration	Amount
L-Glutamine (200 mM)	2 mM	0.5 mL
Penicillin-Streptomycin (10,000 U/mL)	100 U/mL	0.5 mL
HEPES (1 M) (optional)	20 mM	1 mL
FBS	10%	5 mL
FluoroBrite™ DMEM	NA	43 mL
Total	NA	50 mL

Note: We recommend using FluoroBrite for live cell imaging for its very low fluorescence compared to other standard phenol red-free DMEM.

△ CRITICAL: Use an opaque Falcon tube to protect the imaging medium from light and prevent the production of fluorescent compounds. Alternatively, wrap the tube in an aluminum foil. You can also cover the top of your chambered coverslips with aluminum until microscope imaging.

Alternatives: You can perform microscopy imaging without 5% CO₂-controlled atmosphere for up to 10 h if you buffer the medium at pH7.4 with 20 mM HEPES.

Material:

- Commercial laser-scanning confocal inverted microscope equipped with:
 - An incubator (37°C and 5% CO₂ in a humidified atmosphere).
 - High numerical aperture immersion objective.
 - Excitation: Argon laser -the 458 nm line is optimal for mTFP1 excitation- or equivalent, and a beam splitter that transmits above the excitation wavelength.
 - Spectral acquisition: microscopes that use a grating in the emission path and an array of detectors allow for simultaneous acquisition of the donor and acceptor emission spectra with a spectral resolution below 10 nm.

Note: The type of immersion medium does not matter but we have noticed that some oils can contribute to background fluorescence and should be avoided. Water, in turn, is less suitable for long term time lapses due to evaporation.

STEP-BY-STEP METHOD DETAILS

Cell seeding on imaging coverslips (Day 1)

⌚ **Timing:** 30 min

1. Detach cells from a culture flask (T25 flask).
 - a. Prepare a new T25 flask containing 4.5 mL of fresh DMEM and place it into the incubator.
 - b. Aspirate old medium with a Pasteur pipette.
 - c. Wash once with 1 mL of PBS (Ca²⁺ and Mg²⁺ free) and gently swirl to dilute remaining DMEM and cell debris.
 - d. Aspirate the supernatant.
 - e. Add 1 mL of Trypsin-EDTA solution to the cell culture flask and swirl to cover the cells.
 - f. Incubate about 10 min for cells to detach from the surface and each other.
 - g. Gently shake the flask to facilitate cell detachment.
 - h. Visually assess cell detachment using an inverted phase-contrast microscope.
2. Collect the detached cells:
 - a. Once cells are detached, add 4 mL of cell culture medium to stop trypsin enzymatic reaction, gently pipette up and down to help cell detachment and collect all the cell suspension.
 - b. Transfer 1:10 of the volume (0.5 mL) into the new flask and put back in the incubator for future experiments.
 - c. Transfer the remaining volume (4.5 mL) of the old flask to a 15 mL Falcon tube;
 - d. Pellet the cells by centrifugation at 60–125 g for 5 min.
 - e. Discard the supernatant and gently resuspend the pellet in 3 mL of imaging medium (stock cell suspension).
3. Seed cells on coverslips in imaging medium.
 - a. Count cells with a hemacytometer or equivalent device.
 - b. Dilute stock cell suspension in imaging medium at 5×10^4 cell/mL.

Critical: Rinse collagen-coated coverslips with PBS and then rinse again with imaging medium to remove any acid residues.

- c. Transfer 1.5 mL of the 5×10^4 cell/mL suspension in each well of the 2-well chambered coverslip.
- d. Incubate the cells at least 12 h to allow for cell attachment.

△ CRITICAL: When seeding cells, pipette up and down twice in the well to facilitate homogeneous seeding and avoid cell aggregation at the center.

Note: Plating cells on coverslips with imaging medium before rather than after transfection allows for minimal perturbation just before imaging as cells are ready to be imaged when transfection is over.

Note: Cells can be transfected just after cell attachment (at least 1 h after seeding) but transfection efficiency may not be optimal.

Note: When using other types of imaging dishes, seeding volume of the diluted stock cell suspension must be adapted such that the cell surface density is around 18.000 cells/cm².

Cell transfection (Day 2)

⌚ Timing: 30 min (preparation) + 4 h incubation

Each coverslip well can be used for a different transfection. You can use two wells of a coverslip for the nesprin tension sensor (CB), two wells of another for its control (CH mutant) and two wells of a last coverslip for the FRET standards (5AA and TRAF) (see [key resources table](#)).

4. Preparation for DNA-lipid particles formation (Quantities for 1 well, scale accordingly for more).
 - a. Prepare a 1.5 mL tube with 100 μ L FluoroBrite medium (without serum).
 - b. Pipette 1 μ g DNA into this tube and vortex during 10 s.
 - c. Briefly vortex the transfection reagent and add 2 μ L to the diluted DNA. Mix immediately by vortexing for 10 s.
 - d. Incubate 15 min at 18°C–25°C.
 - e. Meanwhile, prepare another 1.5 mL tube with 900 μ L of imaging medium.
 - f. Add 100 μ L of the DNA/lipid particles mixture (prepared in step 4c) into the second tube. Flick the tube to homogeneously distribute DNA/lipid particles.
5. Cell transfection.
 - a. Gently aspirate and discard the imaging medium from the well with a micropipette and filter tip.
 - b. Add 1 mL of the tube prepared in step 4f to the well.
 - c. Incubate the cells at 37°C (5% CO₂) for 4 h.
6. Medium change.
 - a. Use a micropipette and a filter tip to aspirate and discard the medium from the transfected well.
 - b. Add 1 mL of imaging medium to the well.
 - c. Incubate the cells at 37°C (5% CO₂) for at least 24 h (up to 48 h).

Note: The volumes indicated above refer to cells seeded in one well of a 2-well plate. For other types of dishes, scale accordingly.

△ CRITICAL: For best results, the transfection reagent to DNA ratio must be 2:1. It is very important to follow this recommendation, otherwise the transfection efficiency may drastically decrease. Never transfect more than 1 μ g DNA/mL of final volume. For example, for 2 mL (typically, one well of a 6-well plate), we use no more than 2 μ g DNA.

Note: Changing medium (step 6) is not mandatory following the manufacturer guidelines but we recommend to do it to boost the growth of transfected cells.

Live cell imaging (Day 3)

⌚ Timing: 1 h (depending on the number of wells)

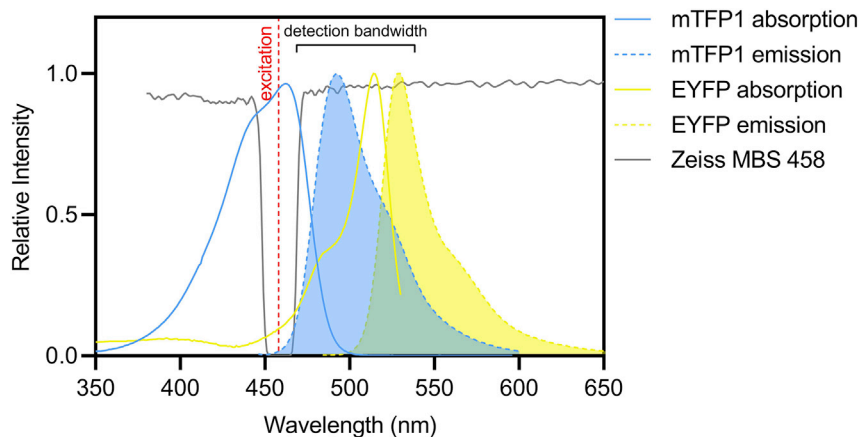


Figure 2. Absorption and emission spectra of the different fluorescent proteins, with microscope characteristics
Modified from FPbase (Lambert, 2019).

This protocol is valid for imaging of any FRET construct with a blue/yellow pair and particularly suitable for mTFP1/YFP (or Venus) as in the nesprin biosensor (and control construct) and FRET standards 5AA and TRAF (Figure 2). Below is its application on a Zeiss 780 laser scanning confocal microscope controlled by Zen software (see [key resources table](#)).

7. Prepare the microscope (Figure 3).
 - a. Turn on the microscope.
 - b. About 1 h before imaging (minimum 30 min), set the incubation parameters to 37°C and 5% CO₂.
 - c. Select the 63×/1.4NA Plan-Apochromat Oil objective and appropriate immersion oil, or equivalent.
 - d. Select an appropriate Dichroic mirror in the excitation path, such as to excite a GFP protein (for instance FSet38 wf).
8. Prepare the cells (volumes below are for 2-well coverslips of 1 mL volume each, adapt accordingly for other dishes).
 - a. For each well, prepare 250 μL of pre-warmed imaging medium with or without drug (depending on your application) at 4× final concentration.
 - b. From each well, gently remove 250 μL using a micropipette with filter tips.
 - c. Add 250 μL of pre-warmed imaging medium with and/or without drug.
 - d. Allow cells to rest for 15 min in the cell culture incubator.
9. Prepare the acquisition (Locate tab, Figure 3).
10. Set up the imaging coverslip with cells on the microscope stage.
 - a. Add mineral oil on top of the imaging medium to prevent evaporation.
 - b. Visualize fluorescent cells using epifluorescence.
 - i. Slowly bring the cells into focus, being careful not to smash the objective into the glass bottom dish.
 - ii. Verify cells are transfected and if the construct is well expressed at the expected location (at the nuclear envelope for nesprin constructs, in the cytoplasm and nucleus for 5AA, in the cytoplasm only for TRAF).
 - iii. Choose cells with a good enough signal-to-noise ratio, avoid cells expressing too much construct.
11. Acquire images (Acquisition tab, Figure 4).
 - a. Select the lambda mode and set the acquisition range from 471 nm to 562 nm with a spectral resolution of 9.0 nm on the spectral channel (ChS).
 - b. Select the excitation beam splitter MBS458.

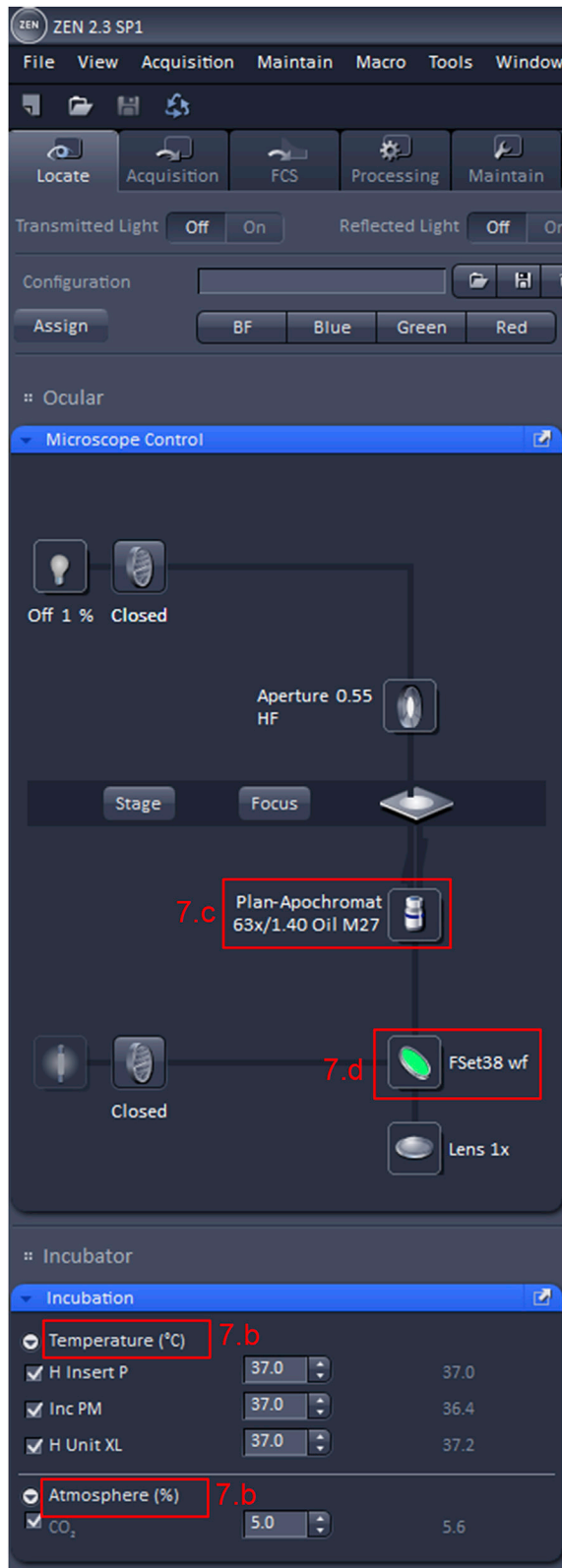


Figure 3. Acquisition panel to visualize fluorescent cells
See text for step-by-step protocol.

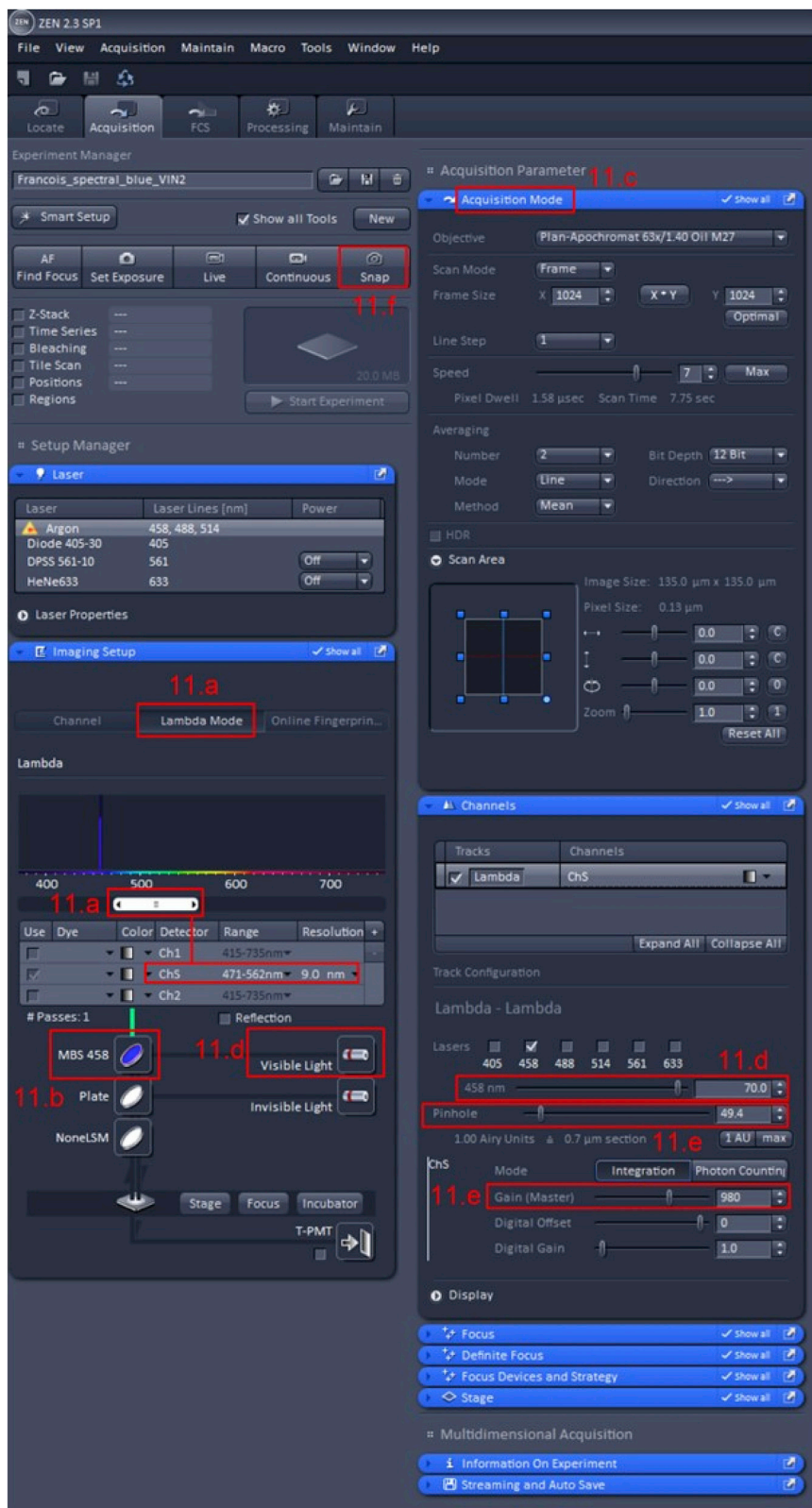


Figure 4. Acquisition panel with Imaging Settings
See text for step-by-step protocol.

- c. Set the scan parameters as you would for regular scanning confocal imaging. Typically, we use unidirectional, 2× line averaging at speed 7 for a 1024 × 1024 pixels² frame corresponding to a real size of 135 × 135 μm² with pixel size 0.13 × 0.13 μm².
 - d. Check the 458 nm excitation laser and set the power to about 70%.
 - e. Set pinhole and detector parameters as you would for regular scanning confocal imaging. We typically use 49.4 μm (1 Airy Unit).
 - f. Start the acquisition.
12. Save your acquisition and repeat on another field of view as you wish. Eventually, you will obtain a collection of .lsm or .czi files that contain your multichannel images.

Note: Choose the objective with highest numerical aperture, typically 1.4 for standard objectives, to maximize resolution and signal intensity. This should also come with apochromatic correction.

△ CRITICAL: Beware of photo-bleaching before and during acquisition as it will affect FRET measurement. To do so keep epifluorescence light source and laser low enough and do not let cells exposed to excitation light for too long before acquisition.

Note: In continuous mode to preview your sample, you can select HiLo LUT to visualize saturated pixels and background and adjust imaging parameters (laser power, scan speed, pinhole) to optimize the signal-to-noise ratio. Avoid saturation as it will bias FRET measurement.

Note: In the present protocol, we measure FRET in end point. You can also perform manual or automated time-lapses to follow FRET, and tension changes through time. Be cautious to not photo-bleach your sample with too short time intervals and/or too long experiments.

Note: Optionally, click on the “Positions” box and indicate the number of fields you want to image.

Image processing and FRET analysis

⌚ **Timing:** varies depending on the number of images acquired

We use a custom-made Python script ‘nuclearFRET.py’ in Fiji version 2.1.0/1.53 (Rueden et al., 2017) with the libraries LOCI and MorpholibJ v1.4.3 (Legland et al., 2016). The script with the user guide for image processing is available from the GitHub webpage <https://github.com/phigirard/nuclearFRET>. The source code is freely available under GNU General Public License v3.0. The script can generate a FRET index image of semi-automatically extracted nuclear envelopes and a list of mean FRET index per nucleus (‘Nuclei’ workflow) when applied on images of cells expressing the CB or CH constructs. Alternatively, the script generates the FRET index image of FRET standard constructs 5AA and TRAF expressed in the cytoplasm (‘Whole cell’ workflow). The script is organized in 4 tasks that follow one another. For step-by step visual guide of the protocol please refer to the [Figure 5](#).

TASK 1.

13. Use File > Open... to open the script ‘nuclearFRET.py’ in Fiji and click ‘Run’ on the bottom of the script editor window (you can also go to : Run > Run in the Script Fiji menu). Then, in the dialog box that appears:
 - a. select the multichannel ZEISS .lsm or .czi file you wish to analyze,
 - b. select one of the two image analysis workflows: ‘Nuclei’ option to analyze the signal from nuclear envelope or ‘Whole cell’ option to analyze the signal from the cytoplasm,

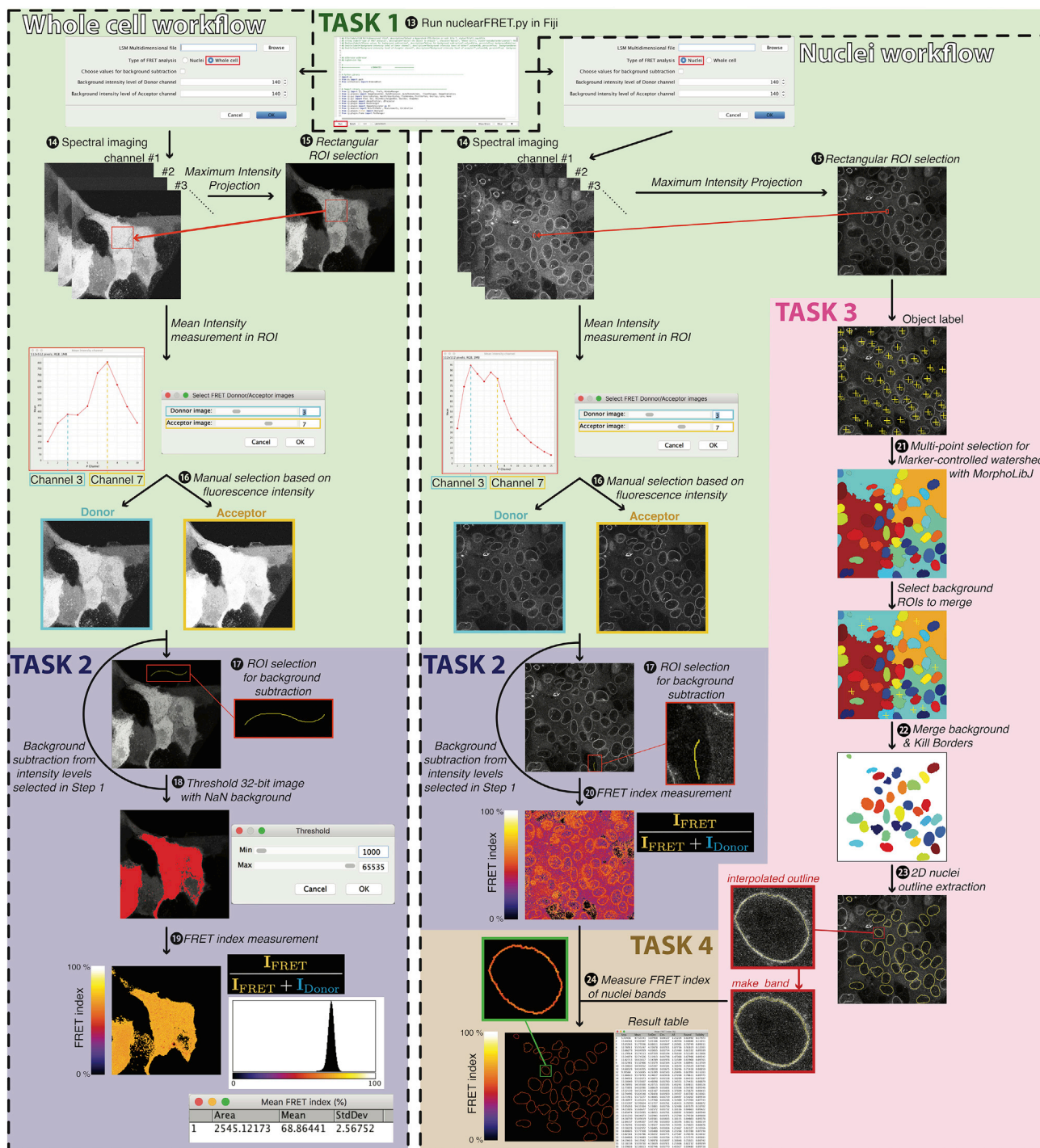


Figure 5. Analysis workflow with custom script
See text for step-by-step protocol.

- c. select [Choose values for background subtraction] if you want to subtract the background intensity levels by inserting manually the background values in the corresponding Donor/Acceptor channel numeric fields. This option will bypass the background measurement from a ROI (Region of Interest) in the acquired images (step 17).

14. The script opens the file and checks its dimensions. If the number of Z-slices or frames is >1 (Z or T >1) or if there is only one channel (C=1), the script will abort and warn the user. If the file contains multiple positions, select with the slider in the popup window the series you want to analyze. The script creates a folder with the name '[filename(without extension)]+_S+[selected series number]' to store all the analyzed data files (images and result tables).
15. The script displays the Maximum Intensity Projection (MIP) generated from the multichannel stack. In this image, use the rectangular tool to manually select a ROI that contains the fluorescent protein signal. The script then displays the mean intensity profile of this ROI through the multichannel image, which reveals the fluorescence emission spectrum of the construct.
16. In the popup, select the channels that correspond to the donor and acceptor intensity maxima based on the emission spectrum. The script applies a Gaussian filter with a radius of 2 pixels to both selected channels in order to reduce noise and assigns Not A Number (NaN) value to saturated and 0-value pixels.

TASK 2.

17. In the image window, manually select a line ROI with the freehand line tool in the darkest area of the image. For each channel, the script measures the mean intensity value within the ROI and subtracts it from the whole image. Each background-subtracted channel is saved in the 'analyzed data' folder created in task 1 ('c1': donor, 'c2': acceptor image).

Note: the script bypasses background measurement if you have checked [Choose values for background subtraction] in the dialog of step 13. While background is usually low or even negligible in confocal images of cells expressing reasonable levels of fluorescent protein, background subtraction is critical for unbiased FRET calculation in low expressing conditions. We thus recommend to subtract the background anyway.

Note: from here, the script will either follow steps 18 and 19 of the 'Whole cell' workflow or steps 20–24 of the 'Nuclei' workflow.

'Whole cell' workflow:

18. In the popup window, select the min and max intensity values of the pixels for which you want to display the FRET index with the threshold sliders. Press 'OK' to generate thresholded donor and acceptor images in which the selected pixels retain their original values, while the other pixels are set to NaN.
19. The script generates a FRET index image (in %) from the background-corrected and thresholded donor and acceptor images by applying the following formula to each non-NaN pixel:

$$\text{FRET index (in \%)} = 100 \times \frac{I_{\text{FRET}}}{I_{\text{FRET}} + I_{\text{Donor}}},$$

where I_{FRET} and I_{Donor} are the intensity values in the acceptor and donor channels, respectively. The script will then display:

- a. an image where every selected pixel displays a FRET index value in % with a 'Fire' color map. The resulting image is saved as 'FRET index (%).tif',
- b. a single row Result Table called 'Mean FRET index (%)' with the area (in the unit of the original image) of the analyzed region, the mean FRET index and its standard deviation. This table is not saved.

This is the end of the 'Whole cell' workflow, you can now repeat the Image processing and FRET analysis from TASK 1 on through the 'Nuclei' workflow and/or go to the [expected outcomes](#) section.

'Nuclei' workflow:

20. The script generates a FRET index image (in %) from the background-corrected donor and acceptor images obtained in step 17 by applying the same formula as in step 19 of 'Whole cell' workflow. The resulting image is saved as 'FRET index (%).tif'.

TASK 3.

21. In the MIP image, manually point with the multi-point selection tool all nuclei. The coordinates of these markers are saved as a set of ROIs under the name 'RoiSet_Markers.zip'. The script segments all selected regions with the Marker-controlled Watershed plugin from the MorphoLibJ library.
22. In the segmented image, select background regions to fuse with the multi-point selection tool. The function 'Merge Label and Kill border' of the MorphoLibJ library then merges together the selected background regions and the nucleus regions touching the border of the image. The resulting image with the 'Jet' color map and white background is saved as 'LabelBordersRGB.tif'.
23. The script then interpolates labeled nuclei in closed polygon ROIs with points spaced one pixel apart by applying a 3-point running average. Polygons with less than 10 points are discarded. The set of resulting ROIs are saved as 'RoiSet_NucleiContour.zip'. A 3-pixels-thick band is then generated from each ROI. The set of resulting ROIs is saved as 'RoiSet_NucleiBand.zip'. A Result Table is generated with one row per pixel within the band of each nucleus contour and the following columns: the index of the nucleus ('IObject'), the ROI name of the nucleus given by ROI Manager ('Iname'), the index of the pixel within the band ('IcontourPoints'), the coordinates of this pixel ('PointX' and 'PointY') and the FRET index in % of this pixel ('FRET index (%)'). The table is saved as 'ContourMeasurements.csv'.

TASK 4.

24. The script sets all pixels excluded from the 3-pixels thick nucleus contour to NaN in the FRET index image obtained in step 20 of 'Nuclei' workflow and displays:
 - a. an image where the pixel values in the nucleus contour are the FRET indices in % with a 'Fire' color map. This image is saved as 'FRET index Nuclei.tif'.
 - b. a Result Table called 'Mean FRET index (%)' with, for each nucleus, its area (in the unit of the original image), the mean and standard deviation of the FRET index (in %) and the shape descriptors (circularity, aspect ratio, roundness and solidity) of the nucleus contour. This table is saved as 'MeanFRETMeasurements.csv'.

Note: This script was successfully run on laptop computers (both Windows and Mac) with at least 1.80 GHz Processor and 8 GB of RAM.

Note: The segmentation step will highly depend on the signal-to-noise ratio of the initial image and the transfection homogeneity across the cell population.

Note: the script also works on .tif files provided the dimensionality is compatible ($C>1, Z=1, T=1$).

Note: To assess the script, we provide raw multichannel images of MDCK cells expressing the CB and CH constructs with and without drug treatment, and the 5AA and TRAF constructs, all acquired on a Zeiss LSM780 confocal microscope according to the present protocol.

EXPECTED OUTCOMES

The FRET maps will display FRET index values between 0 and 100 by definition of the FRET index wherever within a detected region of interest (Figure 6). The actual value is instrument-dependent

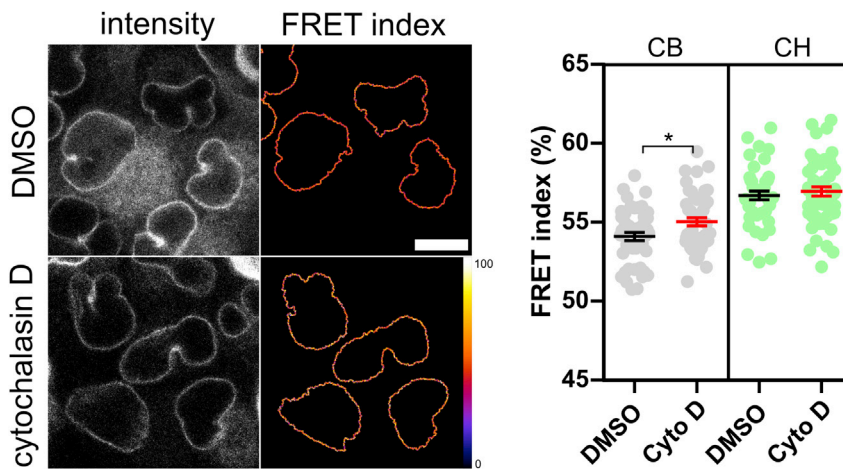


Figure 6. Typical result

Left: MDCK cells expressing the CB construct exposed for 20 min to DMSO or cytochalasin D: raw intensity images and FRET index maps. Bar=10 μ m. Right: Quantification of the FRET indices for populations of cells expressing either the CB or the CH construct exposed to either DMSO or cytochalasin D (cyto D). 1 point=1 nucleus. Mean \pm SEM. Mann-Whitney test (* $<$ 0.05).

and cannot be compared with a measurement on a different microscope, unless a calibration of the microscope is performed (see [FRET index to Force calibration](#) below). Nevertheless, comparisons can be made between experimental conditions and constructs if acquired on the same microscope and if the microscope is stable, which can be assessed with FRET standards 5AA and TRAF.

FRET indices of 5AA and TRAF display intra- and even intercellular variability even though they are standards. Nevertheless, their mean should be high and low, respectively. FRET indices of CB and CH constructs likewise display intra- and intercellular variability ([Figure 6](#)). Their mean should be low and high, respectively, and lower than that of the 5AA construct.

By design, the CH construct is insensitive to cytoskeletal tension, treatments are not expected to change its FRET index on average, unless they involve processes different from cytoskeletal tension. In practice, pharmacological perturbations of the actin, myosin and microtubules ([Déjardin et al., 2020](#)) have not significantly affected the FRET index of the CH construct ([Figure 6](#)).

The CB construct is sensitive to cytoskeletal tension and will display a FRET index that depends on the condition, it may be as low as that of the TRAF standard ([Déjardin et al., 2020](#)). Upon exposure to 0.5 μ M cytochalasin D for 20 min, the anticipated outcome is an increase in FRET ([Figure 6](#)). Mind that other factors than pharmacological perturbations may affect tension: treated and untreated conditions should therefore differ as little as possible apart from the presence of the drug.

These differences and changes between constructs and conditions can be qualitatively assessed directly on the FRET images and mean FRET values output from the Fiji script. However, only a proper statistical analysis can tell whether they are significant (see [quantification and statistical analysis](#) below).

Moreover, and independently of any statistical significance, however big or small differences and changes in FRET index seem to be on their own, they can only be considered so by comparison to a biologically meaningful reference. To address this, a conversion of FRET index to force must be performed (see [quantification and statistical analysis](#) below).

Note: FRET measurements on FRET standards do not need to be performed together with every experiment on nesprin tension sensors as they serve to account for presumably constant

instrument and fluorophore properties. Nevertheless, instrument properties, such as detection efficiency across the spectrum, may change as the hardware ages. We recommend to assess instrument stability regularly every few months and after any maintenance to ensure comparability between experiments performed at distant times.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification of treatment effect on nesprin tension

1. Repeat the protocol from steps 'Cell seeding on imaging coverslips' to 'image processing and FRET analysis' at least 3 times for the nesprin tension sensor CB and its control CH.
2. In Prism, pool mean FRET indices output from the script from all images of the same construct (CB, CH), condition (treated, untreated), and experimental repeat in the same group of a column table. Repeat for every construct, condition and experimental repeat.
3. To assess for inter-experimental repeat variability, run a Kruskal-Wallis test between experimental repeats of the same construct and condition. Pool groups that are not statistically different. Repeat for each construct and condition.
4. To assess for an effect of the treatment, run a Mann-Whitney test between treated and untreated conditions, for each construct.

Note: It is preferable to have similar numbers of measurements for every construct, condition and experimental repeat.

△ CRITICAL: The treatment has an effect on FRET if treated and untreated conditions are statistically different, for any construct (Figure 6). The treatment has an effect on nesprin tension if treatment effects on CB and CH constructs are statistically different. In most cases, the treatment has a significant effect on the CB construct but not on the CH construct and the conclusion is trivial (Figure 6). Otherwise, if the treatment has significant effects on both CB and CH constructs, then they should be statistically compared, that is, differences in FRET upon treatment should be compared. In that case, an experimental scheme with a FRET measurement before and after treatment to obtain a FRET difference per nucleus is better suited. Doing so, tension-dependent and tension-independent effects on FRET of the treatment can be discriminated.

Note: Statistical differences between experimental repeats of the same condition or construct are signs of uncontrolled biases. Nevertheless, it is still possible to assess for differences between conditions and constructs within the same experimental repeat.

FRET index to force calibration

5. Execute the protocol from steps 'Cell seeding on imaging coverslips' to 'image processing and FRET analysis' for the 5AA and TRAF constructs.
6. In Prism, pool measurements from the same construct in the same group of a column table.
7. Extract the mean of each group from the column statistics.
8. Compute $a = (E_H(1 - E_{R,H}) - E_L(1 - E_{R,L}) + E_H E_L (E_{R,H} - E_{R,L})) / c$ and $b = (E_H(1 - E_{R,L}) - E_L(1 - E_{R,H}) + E_{R,L} - E_{R,H}) / c$, with $c = (E_H - E_L)(1 - E_{R,H})(1 - E_{R,L})$, where $E_{R,H}$ and $E_{R,L}$ are the mean FRET indices of 5AA and TRAF obtained in the previous step and E_H and E_L their FRET efficiencies published elsewhere (Day et al., 2008).
9. You can now compute the FRET efficiency $E = (1 - a(1 - E_R)) / (1 - b(1 - E_R))$ for any FRET index E_R of the CB and CH constructs, whether E_R is the raw index in every pixel of an image, an average over each nucleus, or an average over a population of nuclei.
10. Compute the difference in E between the CB and CH constructs for any condition and use the previously published FRET efficiency to force calibration (Grashoff et al., 2010) to convert the difference in E into the force exerted by the cytoskeleton on nesprins in pN.

Note: *a* and *b* are fluorophore- and instrument-dependent coefficients that account for the donor's spectral bleed-through, the acceptor's direct excitation and differences in donor vs acceptor absorption cross-sections and detection efficiencies (Lee et al., 2005). Other FRET standards can be used to retrieve *a* and *b* provided that their fluorophores share the same photophysical properties with that of the biosensors.

LIMITATIONS

This protocol involves transiently transfected cells. It is possible to repeat acquisitions within a 24–72 h window post-transfection. To avoid repeating transfections after this time window, it is possible to make stable cell lines by selection under 200 µg/mL Hygromycin for CB and CH constructs and G418 for FRET standards for a couple weeks before FACS. Note that as passage number increases, some cells in the stable population may display a loss of fluorescence emission of one of the two fluorophores, perhaps as a result of homologous recombination. In that case, spectral imaging is instrumental to identify these cells and discard them from further analysis.

This protocol relies on scanning confocal imaging in spectral mode. While spectral imaging allows simultaneous acquisition of all wavelengths within the specified boundaries, the scanning mode may appear slow for large fields of view compared to other acquisition modes such as widefield or spinning disk confocal. To increase speed without sacrificing signal, it is possible to narrow the field of view. Using other acquisition modes may allow faster acquisition but also come with drawbacks such as the inability to acquire a spectrum and temporal and spatial shifts between donor and acceptor channels.

This protocol involves an end-point acquisition. It is also possible to perform time-lapse imaging but care should be taken to avoid photobleaching.

This protocol does not rely on the measure of acceptor fluorescence upon direct acceptor excitation. This avoids an additional acquisition following every spectrum acquisition upon donor excitation, with another laser line whose power relative to that of donor excitation must be kept constant at all times. Although this approach saves time, computer memory and limit additional sources of variability, it does not allow to verify the expected 1:1 fluorophore stoichiometry within the sensor. We consider this is a valid assumption wherever the signal originates from regions where the functional protein is expected to be.

TROUBLESHOOTING

Problem 1

Seeded cells aggregate at the center of the wells.

Potential solution 1

When seeding, make sure the cell suspension covers the uniformly the coverslip by gently shaking the dish linearly in X and Y, not circularly. We also recommend gently pipetting up and down several times with a micropipette and filter tips.

Problem 2

When previsualizing your cells by epifluorescence, the signal fades away.

Potential solution 2

Photobleaching is occurring. Reduce the excitation power.

Problem 3

Untreated cells look sick during the experiment.

Potential solution 3

Make sure CO₂ and temperature are correctly adjusted and that mineral oil covers the whole surface of the imaging medium to prevent evaporation.

Problem 4

The microscope is not equipped with a 458 nm laser line.

Potential solution 4

A confocal microscope equipped with a 440 nm laser line can be used instead but mTFP1 will not be optimally excited.

Problem 5

Continuous scan of your sample shows saturated pixels.

Potential solution 5

Reduce laser power.

Problem 6

When screening your cells by continuous scan, the signal fades away.

Potential solution 6

Photobleaching is occurring. Reduce laser power and/or increase scan speed.

Problem 7

The fluorescence emission spectrum displays only one maximum.

Potential solution 7

Do not analyze further. The cell has likely not processed the full construct properly. Alternatively, the construct is corrupted, re-sequence the plasmid.

Problem 8

The FRET index distribution on FRET maps displays a peak at 50, well above a roughly Gaussian distribution.

Potential solution 8

Some pixels are saturated. Identify their localization in the donor and acceptor raw intensity images and mask them in the FRET map.

Problem 9

The treatment has no effect or kills the cells.

Potential solution 9

Increase or reduce the treatment concentration, respectively.

Problem 10

Intermolecular FRET between sensors is a valid concern in principle but generally not an issue in practice. Intermolecular FRET can be assessed by measuring the FRET index as a function of biosensor expression level: if intermolecular FRET occurs, the FRET index increases with increasing expression level (Borghi et al., 2012).

Potential solution 10

Working at not too high expression levels in cells expressing the endogenous protein minimizes intermolecular FRET. Comparing conditions at equivalent expression level minimizes biases from intermolecular FRET.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Nicolas Borghi nicolas.borghi@ijm.fr.

Materials availability

5AA TRAF, CB, and CH construct plasmids are available upon request.

Data and code availability

The Fiji script 'nuclearFRET.py' and raw multichannel images of cells expressing the CB, CH, 5AA, and TRAF constructs are available at <https://github.com/phigirard/nuclearFRET>.

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AUTHOR CONTRIBUTIONS

F.S., P.P.G., and N.B. wrote the manuscript. P.P.G. wrote the "nuclearFRET.py" script. L.L. provided experimental data and performed analyses.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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