Development and Validation of a Novel Resonant Energy Transfer (FRET) Biosensor to Measure Tensile Forces at the LINC Complex in Live Cells

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DISSERTATION: DEVELOPMENT AND VALIDATION OF A NOVEL RESONANT ENERGY TRANSFER (FRET) BIOSENSOR TO MEASURE TENSILE FORCES AT THE LINC COMPLEX IN LIVE CELLS

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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Fluorometer Excitation-Emission Fingerprints of fluorescein (left image) and TAMRA (right image): Single-dye solutions were diluted in PBS(pH7) to 2uM and recorded at room temperature with a fluorometer in 3D mode. Excitation and emission wavelengths were sampled with 5 and 2 nm spacing spanning from 400-600 and 450-750nm respectively. The fluorescent intensity [Au] was color coded in both images with a scale bar shown to the right of the plot. Single-Dye excitation and emission spectra from Figure 10B-C were normalized to unit volume and summed together in this representation. The FRET component was synthesized from the single-dye spectra shown in Figure 11A according to Equation 2.1. The text labels for each component are printed on the excitation-emission peaks. In contrast to emission spectra at a fixed excitation frequency, the FRET component can be clearly delineated from the acceptor peak in the excitation-emission space.

Fitting Process for the deconvolution of 3D spectra. A) The average normalized excitation and emission spectrums for fluorescein (donor) and TAMRA (acceptor) were computed from data shown in Figure 10. B) Entire excitation-emission spectrum of dye components concatenated end-to-end into a 1-dimensional vector. Each Dye component is separately color-coded and the 1uM mixtures of fluorescein and TAMRA is shown in purple. C) Non-negative least squares fitting of the dye components (dotted green line) plotted in B overlaid with the measured mixed spectra of fluorescein and TAMRA (solid Blue line).
Verification of the Invariance of fluorescein and TAMRAs Excitation and Emission Spectra Shape. Unit area normalized excitation and emission cross-sections of single dye excitation-emission matrices from Figure 10A. Several spectra around the peaks of the excitation and emission bands (see Figure 10) showed little shape variance. Each excitation or emission spectra are plotted on top of each other with corresponding labels in the legend on the right portion of the graph. These spectra confirm the assumptions necessary to generate the FRET spectra from the averaged components in Figure 11A-B. Details on the independence of excitation and emission spectra are well established in the literature with few exceptions [55].

Measured and Modeled Excitation-Emission Spectrum of a Mixture of fluorescein and TAMRA. A) Unmixing components detailed in Figure 11 were used to fit a 2uM mixture of fluorescein and TAMRA (B) with a linear least-squares model. The percentage weightings for each component (fluorescein=62%, TAMRA=38%, FRET=0%) are printed on the peaks of image. B) The measured mixture of 2uM fluorescein and TAMRA.

Excitation Pairing Matrices. Excitation-emission recordings shown in Figure 10 were used to generate pairs of emission spectra at two different excitation frequencies: \( F_1(\lambda_{ex_1}, \lambda_{em}) \), \( F_2(\lambda_{ex_2}, \lambda_{em}) \), where \( F_1 \) is the spectra at excitation frequency 1, \( \lambda_{ex_1} \) the fixed excitation frequency 1, and \( \lambda_{em} \) the entire emission spectrum. Each pair of spectra were used as inputs into the spectral unmixing algorithms (LuxFRET-A, sRET-B, and SensorFRET-C). Every possible paired input spectra were used to output an estimated FRET efficiency that is color coded in each matrix. Ideally, all pixels would register 0% FRET (green), however certain excitation pairs have poor signal to noise ratios. Each algorithm showed different sensitivity to the various signal to noise ratios and all failed at the red-edge of the spectrum.
SensorFRET Unmixing of 2uM Dye Mixture using 1-Photon Spectral Imaging: fluorescein and TAMRA were mixed together at 1uM concentrations and imaged on glass slides using spectral mode. Pixels were averaged to form a single spectra per image. Alpha-Fit) Raw spectra (solid or dotted red) at two excitation frequencies ($F_{DA}^1$ and $F_{DA}^2$) were fit to the fluorescein component shape (solid or dotted blue). Beta-Fit) The magnitude of the direct excitation of TAMRA was estimated with the fitting of the TAMRA component shape (dotted red) with solid blue line defined in the legend. C) Using the Beta Fit, the direct excitation was subtracted from the raw spectra (red) to generate the corrected spectra (teal). D) Corrected spectra (teal) were unmixed from the fluorescein (solid blue) and Tamra (solid yellow) component shapes. The approximately zero magnitude acceptor component (solid yellow) yielded a FRET efficiency estimate of -0.02%.

Estimation of the Fluorescent Lifetime of fluorescein: Lifetimes were captured on an inverted microscope using time-correlated single photon counting (TCSCP). Stock fluorescein was diluted in sodium borate buffer (pH 9) to a final concentration of 50uM and imaged as a droplet on glass slides. A) global (256x256) or B) local (10x10) binning of lifetime images were used to aggregate photon counts into two separate decay traces (Photon Counts: $N_A=5.8E6$ and $N_B=4.4E5$). Non-linear least squares fitting a single exponential decay model (red) yielded a decay constant of A) 4.19ns or B) 4.13ns. The measured instrumental response function (IRF) is shown in black and the goodness of fit ($\chi^2$ statistic) measured A) 13.28 and B) 0.98 respectively. Fit residuals are plotted below each decay trace in blue.

fluorescein and TRITC Fluorescent Lifetime and FRET Efficiency Distributions at 1-2uM Concentration: Lifetimes were captured on an inverted microscope using time-correlated single photon counting (TCSCP). A) Fluorescent lifetime distributions for fluorescein (FL-Blue), fluorescein and TRITC at 1uM (FL-TRITC-1uM-Green) and fluorescein and TRITC at 2uM (FL-TRITC-2uM-Red). Means of the lifetime distributions are shown as vertical dotted lines corresponding to the histogram color with averages printed in the legend. B) Inferred FRET efficiency for 1 and 2uM mixtures based on the lifetime distributions in A that were calculated according to equation 2.26.
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24 Comparing FLIM and SensorFRET Pixels: Images were captured at 20X magnification using TCSPC and two-photon spectral imaging modes on the same field of view with approximately 50 to 100 cells measured for each construct. Five protein FRET standards (C5A, CTV, C32V, C17V and C5V) were imaged using live samples on a heated stage. SensorFRET and FLIM-FRET estimates are shown in red and blue boxplots respectively with box-widths displaying the interquartile range and center marking for the median. A) FLIM-FRET efficiency estimates used the published C5A lifetime as the donor-only reference [53].B) FLIM-FRET efficiency estimates used the measured mean C5A lifetime.
SensorFRET and FLIM using Comparative Images: Images were captured at 20X magnification using TCSPC and two-photon spectral imaging modes on the same field of view of live 3T3 fibroblasts. Pixels were converted to FRET Efficiency according to the SensorFRET unmixing algorithm or according to Equation 2.26. SensorFRET pixels were down-sampled using linear interpolation to match the image resolution of the FLIM-image and were registered using cross-correlation. Top Row) Cells expressing CTV protein processed from spectral images using SensorFRET (Top-left) or FLIM traces using SPCI software (Top-right). Bottom Row) Cells expressing C17V protein processed from spectral images using SensorFRET (Bottom-left) or FLIM traces using SPCI software (Bottom-right).

SensorFRET and FLIM using Comparative Pixels: Images were captured at 20X magnification using TCSPC and two-photon spectral imaging modes on the same field of view of live 3T3 fibroblasts. FRET Efficiency pixels from registered SensorFRET (y-axis) and FLIM (x-axis) images (as shown in Figure 25) represent each point in the scatterplot. Each axis is reported in units of FRET Efficiency and point colors encodes the protein FRET constructs.

Comparing FLIM and SensorFRET Pixels: Images were captured at 20X magnification using TCSPC and two-photon spectral imaging modes on the same field of view with approximately 50 to 100 cells measured for each construct. Five protein FRET standards (C5A, CTV, C32V, C17V and C5V) were imaged using live samples on a heated stage. SensorFRET and FLIM-FRET estimates are shown in blue and green boxplots respectively with errorbars displaying the median absolute deviation and bar heights representing the median FRET Efficiency. A) FLIM-FRET efficiency estimates used the measured C5A mean lifetime as the donor-only reference. B) FLIM-FRET efficiency estimates used the published mean C5A lifetime [53].

Series of simulated FRET standard deviation vs Normalized Fit Residual curves for .1 FRET efficiency steps. At each simulated FRET efficiency, 1000 pixels were used to compute the standard deviation and the average normalized residual of the non-negative least squares fit. To generate each curve, Equation 2.1 was used and poisson noise was added based on the noise model shown in Figure 7.
SensorFRET analysis of MDCK cells expressing TV40 unimolecular FRET constructs incorporated into E-cadherin transmembrane proteins (denoted TS). A-E show analysis for the raw data while F-J show analysis results for the same data after a 5 pixel Gaussian blur. Blurring of the image has minimal effect on the mean FRET efficiency or load but significantly reduces the error associated with any given pixel. This allows differences to be discerned between regions within a single image.

Donor (Cy3-green) and acceptor (Cy5-red) TIRF fluorescent intensity line traces through cyclic loading with force-time plot below. During peak forces, the donor signal reaches a maximum and the acceptor a minimum. Figure from [47].

A) FRET-force calibration curves for TSmod and various linker lengths. Linker lengths are specified by the number of amino-acids per linker (green-yellow-25, purple-red-40, and pink-blue-50). C) Measured compliances as a function of linker lengths. Figure from [47].

Nesprin tension biosensor (A) Schematic of Nesprin-2G tension sensor and headless control. (B) The tension sensor had significantly reduced FRET at the nucleus as compared to the force-insensitive headless control, t-test, \( p < 0.01 \). Bar graphs represent FRET pixels from discrete intensity ranges collected across a minimum of 20 cells per condition. Similar results were obtained for three independent experiments. (C) Nesprin-2G tension sensor and headless control localized to the nuclear membrane in NIH3T3 fibroblasts. The FRET at the nuclear envelope was reduced for the tension sensor as compared to the headless control.

(A) Nesprin-2G localization in normal (non-diseased) and HGPS fibroblasts. Normal and HGPS fibroblasts were grown on fibronectin-coated glass coverslips, fixed, and stained for endogenous Nesprin 2G. Nuclei were stained with Hoechst. (B) When examined across multiple cells the intensity of Nesprin-2G at the nuclear membrane in HGPS cells was reduced relative to normal cells.

Live cell images from NIH3T3 cells overexpressing Nesprin-2 tension sensor or headless control. The tension sensor, but not the headless control, organized into linear filament-like structures in the cytoplasm.
35 Immunocytochemistry staining of paraformaldehyde fixed NIH3T3 cells showed that actin-nuclear interactions are maintained in Nesprin-2 tension sensor expressing cells but not in cells expressing the headless control. Transfected cells are indicated with white arrows.

36 Immunocytochemistry staining of paraformaldehyde fixed NIH3T3 cells expressing Nesprin-2 tension sensor. Immunocytochemistry staining of methanol fixed NIH3T3 cells showed that intermediate filaments as shown by vimentin were similar between tension sensor expressing (green expressing) and untransfected cells.

37 Immunocytochemistry staining of paraformaldehyde fixed NIH3T3 cells expressing Nesprin-2 tension sensor. Immunocytochemistry staining of methanol fixed NIH3T3 cells showed that microtubules as determined by alpha-tubulin staining were similar between tension sensor expressing (green cell) and untransfected cells.

38 A) Nesprin-2G tension sensor rescues rearward nuclear movement in Nesprin-2G depleted cells subjected to scratch wounding and lysophosphatidic acid (LPA) treatment. (B) The tension sensor relative FRET index per cell at various cross-sections of the nucleus (apical, equatorial, and basal). Significantly reduced FRET was observed for apical and equatorial planes compared to basal (ANOVA, Newman-Keuls post-hoc test, $p < 0.01$). (C) No significant differences were observed for the headless control at various cross-sections of the nucleus (apical, equatorial, and basal).

39 (A) NIH3T3 cells expressing the Nesprin tension sensor were treated with either 1nM calyculin A or both 10 uM Y-27632 and 10 uM ML7 and were compared to unstimulated cells (B) Statistical analysis of each condition showed that calyculin A treatment significantly reduced FRET whereas Y27632 and ML7 treatment increased FRET, ANOVA Newman-Keuls post-hoc test, $p < 0.01$. Bar graphs represent FRET pixels from discrete intensity ranges collected across a minimum of 20 cells per condition. Similar results were obtained for two independent experiments.
40 Images of Nesprin-2G depleted NIH3T3 cells subjected to scratch wounding and lysophosphatidic acid (LPA) treatment. Cells expressing Nesprin-2G tension sensor, but not GFP or headless control, exhibited rearward nuclear movement.

41 A) Quantification of nuclear and centrosome movement after wounding. B) Western blot to confirm knockdown of endogenous Nesprin-2G.

42 A) Images showing Nesprin-2G tension sensor incorporation into TAN lines, in contrast to the headless control. TAN lines were induced by scratch wounding and LPA treatment. B) Quantification of scored cells that are positive for TAN lines.

43 (A) NIH3T3 cells were treated with Rho Activator I or II per manufacturer instructions (Cytoskeleton, Inc.). (B) Both activators of Rho resulted in significantly decreased FRET, ANOVA, NewmanKeuls post-hoc test, \( p < 0.01 \). Bar graphs represent FRET pixels from a discrete intensity range collected across a minimum of 15 cells per condition. Similar results were obtained for two independent experiments.

44 Stacked histogram of FRET images from Nesprin-TS population. Each colored histogram represents a single image of a Nesprin-ts expressing nuclei. Data extracted from images shown in Figure 32.

45 (A) FRET ratio images of micropatterned 3T3 fibroblasts expressing Nesprin2G-TS on 20 micron wide fibronectin rectangular patterns or unpatterned fibronectin coated glass. (B) Statistical analysis of each condition showed that cells grown on lines had reduced average FRET compared to cells grown on the un-patterned surface. Bar graphs represent FRET pixels from a discrete high-intensity range of pixels collected across a minimum of 15 cells per condition, \( t \)-test \( p \)-value < .05. Similar results were obtained for three independent experiments. (C) Nesprin-2G FRET of NIH3T3 cells grown on micropatterned lines (width of 5, 20, or 40 m) or unpatterned FN. All conditions are significantly different from each other (ANOVA, NewmanKeuls post-hoc test, \( p < 0.01 \)).
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(A) The Nesprin tension sensor was expressed in normal and HGPS primary fibroblasts. (B) There was a significant increase in FRET for HGPS cells as compared to normal cells, t-test, p < 0.01. Bar graphs represent FRET pixels from discrete intensity ranges collected across a minimum of 20 cells per condition. Similar results were obtained for two independent experiments. (C) Histogram analysis of fluorescent intensity of the Nesprin sensor for normal and HGPS cells. .................. 119

Integrated fluorescent intensity images of confluent stable expressing E-cadherin-TS MDCK cells on a (A) pre-tensioned pdms membrane and (B) 60% uni-axial strained pdms membrane. Binary thresholded intensity images (C) and (C) using (A) and (B) as inputs for morphological analysis. Histogram analysis of (E) cell circularity and cell pixel area (F) of a confluent layer of MDCK cells before and after strain application. ........................................ 120

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Fluorescent intensity, thresholded binary image, and FRET ratio images \( \frac{\text{acceptor}}{\text{donor}} \) of patterned MDCK cells expressing E-Cadherin-TS under (High), (Medium) and (Low) uni-axial strain. Each image contains the same cells on an equivalent intensity and ratio scale. .......................... 122

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Mean FRET ratio \(\frac{\text{acceptor}}{\text{donor}}\) of patterned MDCK E-Cadherin-TS expressing cells shown in Figure 51. Errorbars represent the 99% bootstrapped confidence interval about the mean FRET ratio.

NesprinTS MDCK pattern: Patterned MCDK cells that stably express Nesprin-TS A) before strain and B) after strain. Each cell in image A) corresponds to the same cell in image B) where their relative positions are identical. A Gaussian blur (\(\sigma = 1\)) was applied to the unmixed images before computing the ratio. Pixels are color-coded to the smoothed FRET ratio defined as \(\frac{\text{acceptor}}{\text{donor}}\). Both images are color-coded on an identical ratio scale.

Patterned MDCK expressing NesprinTS: The normalized FRET ratio histograms of patterned MCDK cells stably expressing NesprinTS. Patterned (blue or red) and unpatterned (black or green) cells were measured before and after strain application. The membrane was strained roughly 30% however nuclei strain varied per cell. The histogram frequency was bootstrapped by the mean value at each FRET ratio shown as a 95% confidence interval band about the mean line.

FRET Ratio simulation High FRET: A) Simulated Donor and Acceptor emission spectra using Gaussian distributions with means centered at \(\lambda_{em}(490)\) and \(\lambda_{em}(550)\) and equivalent standard deviations. B) Intensity distributions for Donor and Acceptor signals with means centered at 80 and 20 respectively with standard deviations equivalent to the square root of the amplitude. C) Noiseless donor and acceptor spectra sampled from the intensity distributions shown in B. D) Simulated FRET signal with thermal and poisson noise added (purple), non-negative least squares (nnls) fitting (black), and the component fittings of the nnls fit (blue and green dotted lines).

FRET Ratio Error Simulation: A and B) Histogram of ratios of \(\frac{\text{acceptor}}{\text{donor}}\) computed from the nnls fitting of simulated pixels from Figure 57 or Figure 55, with the true(solid-red), median(dotted-black), and mean ratio (dotted-green) shown as vertical vertical lines. C) The absolute \% error of the computed FRET ratio \(\frac{\text{true ratio} - \text{computed ratio}}{\text{true ratio}}\) for each simulated level of FRET efficiency.
FRET Ratio simulation Low FRET: A) Simulated Donor and Acceptor emission spectra using Gaussian distributions with means centered at $\lambda_{em}(490)$ and $\lambda_{em}(550)$ and equivalent standard deviations. B) Intensity distributions for Donor and Acceptor signals with means centered at 95 and 5 respectively with standard deviations equivalent to the square root of the amplitude. C) Noiseless donor and acceptor spectra sampled from the intensity distributions shown in B. D) Simulated FRET signal with thermal and poisson noise added (purple), non-negative least squares (nnls) fitting (black), and the component fittings of the nnls fit (blue and green dotted lines).

3T3 NesprinTS FRET Efficiency: (Inset Image) 20uM micro-patterned 3T3 fibroblasts with color-coded FRET Efficiency. FRET Efficiency pixels were thresholded by excluding normalized residuals errors greater than 6%. (Outer Image) Histogram of error thresholded FRET Efficiency pixels with the median value (solid red line).

SensorFRET unmixing of Nesprin-TS: Patterned 3T3 fibroblast on glass expressing Nesprin-TS shown in Figure 58. (Alpha Fit) teal fitted raw spectra. (Beta Fit) Fitted Venus Direct Excitation. (Subtraction of Direct Excitation) Removal of direct excitation of Venus. (Unmixed Components) Fitting of the corrected Nesprin-TS spectra.

(A-C) 3D renderings of uni-axial, 3D-printed cell stretcher controlled by a single (A) or double (B,C) independent worm gear mechanism. (D) Biaxial cell stretcher: CAD rendering of biaxial cell stretcher designed for a cruciform PDMS membrane. The PDMS membrane attaches to four cylindrical rollers fixed to four worm gears. The worm gears are meshed together by a pulley above the rollers producing equivalent strain in the x-y plane at the center of the membrane. The base of the stretcher fits into stage plate of a Carl Zeiss inverted microscope.

(A) Stress strain analysis of clear .005” PDMS membrane with fitted modulus at low (red-line) or high (green-line) strains. (B) Precision and slippage of the uni-axial cell stretcher as shown by the axial membrane strain as a function of manual worm gear rotation. (C) Image analysis of membrane strain through each manual gear rotation.
Second Trial: (A) boxplot distribution of the mean junction FRET ratio for tracked cell junctions at zero and maxstrain. (B) Change in the FRET ratio of junctions analyzed from data shown in (A). Third Trial: (C) Boxplot of mean FRET ratios for zero and maxstrains for tracked cell junctions and (D) change in FRET ratios per junction analyzed. Fourth Trial: E) Boxplot of mean FRET ratios for zero, mid, and max strain distributions and F) Change in the mean FRET ratio per condition of strain and grouped by image.

Loading curve: (A) Distribution of FRET efficiencies of unloaded (0deg-blue), 30% (90deg-green) and 60% (135deg-red) bi-axially strained MDCK cells expressing Nesprin-TS determined by SensorFRET. Un-loading curve: (B) Distribution of FRET efficiencies of (135deg-blue) 60%, (90deg-green) 30%, and 0% (0deg-red).

Recovery: (A) FRET efficiency histograms of MDCKs expressing Nesprin-TS after 30% biaxial strain. (B) Change in FRET efficiency histograms of MDCK cells expressing Nesprin-TS from 0% to 90% biaxial strain. FRET efficiency histograms were determined using SensorFRET.

Model of Nesprin-2 orientation in response to applied force. (A) Nesprin-2 is oriented radially for non-polarized cells. (B) In elongated cells, in which actin stress fibers are oriented parallel to the longitudinal axis, there is more force applied to the LINC complex, as well as additional Nesprin-2 molecules recruited to the nuclear membrane. Nesprin-2 forces are spatially homogeneous around the periphery of the nucleus, suggesting that Nesprin-2 orients in the longitudinal direction.

Bone cell marker assays on different surface topographies: (A) alkaline phosphatase activity on tissue culture plastic (TCPS), smooth, and rough titanium surfaces for untransfected, GFP, and DN-KASH expressing (MSCs) Mesenchymal Stem Cells. (B) osteocalcin transcription activity on tissue culture plastic (TCPS), smooth, and rough titanium surfaces for untransfected, GFP, and DN-KASH expressing (MSCs) Mesenchymal Stem Cells.
Bone cell marker assays on different surface stiffnesses: (A) Integrin expression on tissue culture plastic (TCP), or PDMS gels of varying stiffness (0.5kPa, 8kPa, and 32kPa) for control GFP or (B) DN-KASH infected patient derived MSC cells. Osteogenic marker expression on tissue culture plastic (TCP), or PDMS gels of varying stiffness (0.5kPa, 8kPa, and 32kPa) for control GFP or DN-KASH infected patient derived MSC cells.

Traction Force Bead Trajectories: Tracked fluorescent bead trajectories shown as colored vectors with cell outlines shown in white for A) DN-KASH and B) GFP adeno-virus treated cells.

Traction Displacements for DN-KASH expressing MSCs: (A) Interpolated traction displacement map for MSC cells infected with DN-KASH or (B) cytosolic GFP. (C) Cell area adjusted traction stresses for GFP (green) or DN-KASH (red) expressing cells on 8kPa PDMS gels.

fluorescein Tamra fluorometer 50uM.
Abstract

DISSERTATION: DEVELOPMENT AND VALIDATION OF A NOVEL RESONANT ENERGY TRANSFER (FRET) BIOSENSOR TO MEASURE TENSILE FORCES AT THE LINC COMPLEX IN LIVE CELLS

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Virginia Commonwealth University, 2017.

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There is a large body of evidence supporting the theory that cell physiology largely depends on the mechanical properties of its surroundings or micro-environment. More recently studies have shown that changes to intra-cellular mechanical properties can also have a meaningful impact on cell function and in some cases lead to the progression of ailments or disease. For example, small changes to the protein sequence of a structural nuclear envelope protein called lamin-A is known to cause a variety of neurological and musculoskeletal diseases referred to as laminopathies. Currently, there is little incite into how these mutations lead to disease progression due in part to an inability to measure protein-specific mechanical changes and how these alterations may relate to disruptions in intra-cellular signaling or function.

To improve upon the ability to measure mechanical properties inside living cells, a previously validated, genetically-encoded resonant energy transfer (FRET)-force
biosensor was modified to localize to the nuclear envelope. This biosensor integrated into the nuclear envelope protein Nesprin-2G and senses small deformations that are resolved by indirect measurements of spectroscopic fluctuations in the fluorescent emission of the sensor. To accurately measure these changes, a new spectral-imaging technique named SensorFRET was developed which can resolve small changes in the FRET sensor under varying levels of fluorescent intensity and with known absolute precision. Using SensorFRET, the Nesprin-2G biosensor (Nesprin-TS) reported changes in actomyosin contractility, nuclear shape, and nuclear deformation. Using Nesprin-TS, fibroblasts derived from patients with Hutchinson-Gilford progeria syndrome (HGPS) reported less force on Nesprin-2G molecules relative to healthy fibroblasts on average.

To demonstrate how intra-cellular forces on the nucleus may impact normal cell physiology, bone-marrow derived mesenchymal stem cells (MSCs) were genetically modified such that the cytoskeleton was decoupled from the nucleus by saturating KASH binding proteins with a non-functional truncated protein called DN-KASH. MSCs treated with DN-KASH preferentially differentiated into osteocytes (bone cells) at a higher rate than MSCs exposed to osteogenic growth factors. This osteogenic preference after DN-KASH treatment was independent of the cell substrate topology and did not significantly alter integrin expression. However, this tendency to differentiate into osteocytes was dependent on substrate stiffness. Overall, the data imply that an intra-cellular force-dependent mechanism connected to the cell nucleus strongly influences MSC differentiation.
1.1 An Overview of Mechanotransduction

In 2006, a ground breaking paper by Engler et. al. demonstrated that Mesenchymal Stem Cell (MSC) differentiation can be driven by the mechanical properties of the extracellular matrix in vitro [1]. Increasing the stiffness of the cellular substrate increases the propensity of Osteocyte differentiation. Conversely, decreasing substrate stiffness increases Adipocyte differentiation given the same growth factors (Figure 1). These observations clearly reveal that cells sense their mechanical environment and this sensing can have a profound impact on the behavior of cells. While it well known that cells respond to changes in the mechanical environment, how they translate their mechanical senses into biochemical intracellular signals remains unclear.

The field of mechanobiology is largely dedicated to deciphering how mechanical cues from the extracellular environment are transduced into biochemical signals. To understand this form of mechanotransduction, knowledge of cell architecture and its corresponding mechanical properties is necessary. Furthermore, an ability to measure cell forces with protein-level resolution is key to understanding how tension, compression and shear forces mechanistically lead to changes in protein confirmations and ultimately gene expression.

1.2 An Overview of the Cellular Cytoskeleton

The mechanical integrity of cells and cellular movement is governed by the cytoskeleton. Mechanical cues from the environment or from within the cell must prop-
agate through load bearing elements and the cytoskeleton is generally regarded as the major load bearing system within cells. It is composed of a series of different types of assembled protein filaments. The three major types of protein filaments are actin, intermediate filaments and microtubules. While all three components contribute to the mechanical properties of eukaryotic cells, the actin cytoskeleton is probably the most characterized.

The actin cytoskeleton is generally considered a tensile polymer network. G-actin monomers are assembled end-to-end into larger F-actin fibers or filaments than span large distances across the cell. The filaments can organize into bundles or networks. The former is a tightly wound collection of parallel filaments that are used to form cellular projections, like microvilli or filiapodia. Networks on the other hand are organized as a less dense orthogonal mesh that give structural integrity to the cell [2].

The actin cytoskeleton is a dynamic structure in the cell. It rearranges as the cell adapts to its environment (such as substrate stiffness or geometry). Furthermore, the tension on actin can be tuned by activating myosin motor proteins. Activation of myosin motor proteins changes the contractility state of intracellular actin much like it does in muscle tissue. At any given time, the mechanical properties of the actin cytoskeleton will at least depend on the equilibrium between the polymerization/depolymerization of F-actin and the activation/deactivation of myosin motor proteins.

A large proportion of cellular actin localizes to the cell periphery. It is sometimes referred to as cortical actin. Cortical actin is believed to play a large role in maintaining cellular shape and assisting in cell movement. Another region of concentrated actin are focal adhesions. Cells adhere to their substrates through the binding of transmembrane proteins (integrins) to extracellular matrix proteins (ECM). ECM proteins are either coated or secreted by cells before they bind substrate surfaces.
On the inside of the cell, these integrin proteins are linked to actin through a series of binding proteins which are collectively referred to as focal adhesions. Large actin bundles span from focal adhesions across large distances in the cell [2]. In some cells, such endothelial cells, large actin filaments terminate at adherens junctions which contribute to cell-to-cell adhesion [3].

Newer research suggests that stress fibers may directly link focal adhesions to the nuclear envelope through the LINC complex (Linker of Nucleoskeleton to Cytoskeleton). While direct physical evidence that stress fibers contiguously connect focal adhesions to nucleus is lacking, it is becoming clear that forces from focal adhesions are transmitted onto the nucleus through the actomyosin cytoskeleton [4]. It was shown that pulling on integrins at the cell membrane simultaneously leads to deformation of the mitochondria and the nucleus [5]. Furthermore, 3D reconstruction of phalloidin stained F-actin appears to show that stress fibers originating at focal adhesions also terminate at the apical region of the nucleus [6, 7]. Finally, laser ablation of stress fibers on the apical nuclear surface in smooth muscle cells leads to a large, instantaneous deformation of the nucleus and retraction of the stress fibers (Figure 2).

While it is generally believed that the actin cytoskeleton exerts forces on the nucleus by tensioning actin filaments through the activation myosin, it is unclear how microtubules and intermediate filaments distribute forces on the nucleus and whether these forces play an direct role in nuclear mechanotransduction.

Microtubules are larger in diameter (25nm) than actin filaments and are hollow in the center. They are made up of polymerized tubulin protein. Generally, microtubules are regarded as compression bearing components that are stiff and resist bending [2, 8]. In mammalian cells microtubules emanate from the microtubule organizing center (MTOC), located near the centroid of the cell. Similar to actin, microtubules grow
and decay according to the rates of tubulin assembly/disassembly, a process controlled by GTP hydrolysis. Microtubules also have their own motor proteins, dynein and kinesin. These motor proteins walk along microtubules much like the head of myosin as it pulls along actin fibers. Dynein and kinesin differ in the directions that they walk on microtubules.

During cell migration or mitosis, microtubules undergo physical changes that mechanically aid the processes of cell polarization and cell division respectively. During cell division, the mitotic spindle forms and daughter chromosomes are pulled apart by microtubules after the centrosomes duplicate. Opposing microtubules (polar microtubules) overlap at their ends pushing the spindle apart [2]. The process of cell division exerts both compressive forces on polar microtubules and tensile forces on astral microtubules. The tensile forces are believed to be driven by dynein motors that are cortically anchored and walk along the microtubules [9]. During migration, microtubules have been shown to exert both tensile and compressive forces. For example, during the migration of 3T3 fibroblasts, microtubules mimic the movements of actin as it retracts toward the cell center [10]. However, in migrating cells microtubules in the lamellapodia project outward toward the leading edge, suggesting compressive forces dominate as the membrane is pushed outward [11]. A similar phenomenon occurs in neuronal axons, where axonal projections are believed to be pushed outward by dynein motor proteins that exert forces on microtubules [12]. Thus, microtubules are clearly drivers of force mediated cellular behavior, but it is unclear how much force, if any, is transmitted onto the nucleus through LINC connected microtubules.

The final and third structural component to describe are intermediate filaments. They are called intermediate filaments because they are larger than actin, but smaller than microtubules in diameter (typical diameter is 7nm). While actin and microtubule cytoskeleton are formed by the polymerization of actin and tubulin respectively, inter-
mediate filaments have at least 50 different protein subunits. These protein subunits are further sub-classified into six major groups (Table 1). Most of the intermediate filament subunit proteins are expressed in only a subset of cell types [13, 14]. However, the nuclear lamina of all cell types is composed of intermediate filaments called lamins. For the most part intermediate filaments are more stable than either actin or microtubules. While actin and microtubules are continuously remodeled by assembly/disassembly, intermediate filaments are relatively static. A notable exception is the disassembly of lamins in the nucleus during cell division. This disassembly is driven by kinases, such as Cdc2, that phosphorylate the binding ends of lamins. Much like actin and adherens junctions, intermediate filaments bind to junction proteins referred to as desmosomes. Desmosomes are structural junction complexes that are expressed in epithelial tissue and cardiac muscle tissue. Mutations in desmosomes are known to play a role in certain cardiomyopathies and skin blistering diseases [2].

While actin and microtubules have clear roles in mechanically driven processes, such as cell contraction or division, much less is known about the types of forces experienced by intermediate filaments and their binding partners. However, it is known that intermediate filaments bind to the nucleus through the LINC complex, much like actin and microtubules. Thus, LINC complexes connect all three types of structural protein filaments to the nucleus (Figure 3). The wide variety of structural proteins that connect to the nucleus suggest that it experiences a range of forces. Many have suggested that the nuclear cytoskeleton is a hotbed for mechanotransduction, since it is physically associated with DNA [15, 16, 17, 7, 18, 19, 20, 21, 22]. An attractive hypothesis is that LINC associated linkages transmit force directly onto DNA, causing changes in the chromatin state that in turn influence gene expression.
1.3 The LINC complex and nuclear mechanotransduction

If one were to search for putative load bearing proteins involved in nuclear mechanotransduction, the LINC complex is probably the best place to start. Structural protein filaments that connect the cytoskeleton to the nucleus all terminate on a subset of LINC proteins called Nesprins. A variety of Nesprin isoforms are expressed in mammalian cells. Nesprins 1-2 connect to actin on the cytoplasmic end. Nesprins 1-2 also bind to kinesin motor proteins that are attached to microtubules in the cytoplasm. Nesprin 3 associates with plectin which is attached to cytoplasmic intermediate filaments. All Nesprins isoforms enter the outer nuclear membrane and bind to Sun proteins in the perinuclear space. Sun proteins have two isoforms: Sun-1 and Sun-2. Any forces that propagate from the cytoskeleton are likely to transmit through Sun-1 or Sun-2. In the nucleoplasm, sun proteins physically attach to the nuclear lamina (Figure 4).

1.4 Why study nuclear mechanotransduction?

Research purists often believe the study of basic cell biology is in and of itself a worthy cause. Conversely, the pragmatist may argue this type of research should be grounded in an application or be translatable to the field of medicine. The study of nuclear mechanotransduction could easily fall into the categories of either basic research or practical research in medicine. This is because after the sequencing of the human genome, mutations in nuclear lamina genes were found to be the cause of a variety of diseases, known as laminopathies. These laminopathies include the accelerated aging disease progeria, werner syndrome, and a host of muscular dystrophy diseases [22]. Many of these laminopathies share a common feature, the loss or disruption of mesenchymal tissue (such as fat, bone, muscle and neuronal) [23, 24, 25].
Recent studies have found that disruption of either lamins or sun proteins impairs the differentiation of mesenchymal stem cells \textit{in vitro} [26, 27]. Together these studies suggest a connection between LINC proteins and mesenchymal stem cell function. How these two quantities are related is unknown. A more detailed understanding of nuclear mechanotransduction may lead to a better understanding of laminopathies and possibly elucidate the mechanisms of force-driven stem cell differentiation.

![Image](image.png)

Fig. 1. The effect of substrate stiffness on MSC differentiation. Image from ([28]).
Fig. 2. The effect of laser ablation on stress fiber and nuclear displacement in smooth muscle cells. Raw images taken from [29] and processed in ImageJ.

Table 1. Overview of physical attachments to the LINC complex. Table from [15]

<table>
<thead>
<tr>
<th>Type</th>
<th>Protein</th>
<th>Size (kd)</th>
<th>Site of expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Acidic keratins (&lt;15 proteins)</td>
<td>40–60</td>
<td>Epithelial cells</td>
</tr>
<tr>
<td>II</td>
<td>Neutral or basic keratins (&lt;15 proteins)</td>
<td>50–70</td>
<td>Epithelial cells</td>
</tr>
<tr>
<td>III</td>
<td>Vimentin</td>
<td>54</td>
<td>Fibroblasts, white blood cells, and other cell types</td>
</tr>
<tr>
<td></td>
<td>Desmin</td>
<td>53</td>
<td>Muscle cells</td>
</tr>
<tr>
<td></td>
<td>Glial fibrillary acidic protein</td>
<td>51</td>
<td>Glial cells</td>
</tr>
<tr>
<td></td>
<td>Peripherin</td>
<td>57</td>
<td>Peripheral neurons</td>
</tr>
<tr>
<td>IV</td>
<td>Neurofilament proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NF-L</td>
<td>67</td>
<td>Neurons</td>
</tr>
<tr>
<td></td>
<td>NF-M</td>
<td>150</td>
<td>Neurons</td>
</tr>
<tr>
<td></td>
<td>NF-H</td>
<td>200</td>
<td>Neurons</td>
</tr>
<tr>
<td></td>
<td>α-Internexin</td>
<td>66</td>
<td>Neurons</td>
</tr>
<tr>
<td>V</td>
<td>Nuclear laminas</td>
<td>60–75</td>
<td>Nuclear lamina of all cell types</td>
</tr>
<tr>
<td>VI</td>
<td>Nestin</td>
<td>200</td>
<td>Stem cells of central nervous system</td>
</tr>
</tbody>
</table>
Fig. 3. Overview of physical attachments to the LINC complex. Figure taken from [15]
Fig. 4. Cytoskeletal attachments to the LINC complex. Image from [30]
CHAPTER 2

DEVELOPMENT OF METHODS TO QUANTIFY LIVE CELL FRET MEASUREMENTS USING SPECTRAL IMAGING

2.1 Abstract

In this chapter, a novel approach to determining the FRET efficiency and spectral bleed-through of unimolecular fluorescent biosensors was developed that does not require the use of calibration samples (Acceptor-only and Donor-only samples). Measurements can be carried out on any microscope with spectral resolution and at least two excitation wavelengths. The analysis approach only requires two registered images acquired at different excitation wavelengths but with no constraints on the power and gain settings of each. This method was validated through simulation with ideal and noisy spectra, as well as experiment using Cerulean-Venus FRET standards of known efficiency. The normalized fit residual of the emission spectra was used to estimate the expected standard deviation of the FRET efficiency, allowing the measurement uncertainty to be mapped on a pixel by pixel basis. We applied this method to Teal-Venus fluorescent protein force sensitive biosensors that integrate into E-cadherin junction proteins to map cell-cell adhesion forces in epithelial cells. By knowing the measurement uncertainty of each pixel, we determined that differences in tensile forces on E-cadherin could not be spatially resolved with statistical power unless a pixel binning with a radius of 5 pixels was applied.
2.2 Introduction

Forster Resonant Energy Transfer (FRET) is an invaluable tool for the nanoscale examination of a variety of interactions in live-cells. FRET arises from the non-radiative transfer of energy between two chromophores termed donor and acceptor molecules [31]. The engineering of a variety of fluorescent proteins has led to the increased use of genetically-encoded FRET-based biosensors in which the donor and acceptor molecules are held together by peptide linkers [32, 33]. These sensors are capable of measuring various intra-cellular processes that occur on a scale between 1-10 nanometers, well below the diffraction-limit of optical microscopes. A large number of FRET biosensors have been engineered to study protein cleavage, protein conformation changes, local redox and pH sensing, and determining the mechanical load on force bearing proteins [34].

Genetically-encoded, unimolecular FRET biosensors are particularly useful because they are generally less-toxic than cellular dyes and they can be directed to specific regions or organelles in the cell [35]. Measurements of FRET in unimolecular biosensors are simplified by the fact that donor and acceptor fluorophores are expressed in the same molecule [36]. A simple ratio image of the resolved emissions of the donor and acceptor yields a non-linear FRET index that is correlated, but not equal to the FRET transfer efficiency [37]. This form of FRET imaging monitors the sensitized emission of the acceptor and quenched emission of the donor, a direct measure of the transfer of energy between the donor and acceptor molecules [38].

Measuring sensitized-emission FRET (SeFRET) is a common technique to monitor unimolecular sensors due to the ease of capturing ratio images and the speed at which they can be acquired [39]. For studies that require increased temporal resolution, measurements of SeFRET are preferred since photo toxicity and image
acquisition time is minimal when compared to photo-bleaching or fluorescent lifetime imaging (FLIM) respectively [40]. However, a major drawback to SeFRET imaging is the difficulty in accurately removing spectral bleed-through (also known as cross-talk) to determine transfer efficiency [41]. Using current methods, spectral bleed-through from the direct excitation of the acceptor fluorophore cannot be removed from SeFRET images without calibration measurements requiring donor-only or acceptor-only control samples and the implementation of correction algorithms after image capture [42, 43, 44, 41]. While uncorrected ratio-images can be used to monitor relative changes in FRET, they cannot be used to quantitatively measure the transfer efficiency of FRET. The transfer efficiency is generally the most useful parameter in FRET experiments because it is independent of the measuring equipment and can be used to estimate the distance between fluorophores in the sensor [45]. Knowing the true FRET efficiency is particularly pertinent for FRET-force probes since the force calibrations for these sensors are reported in units of FRET transfer efficiency [46, 47, 48, 49, 50].

A rapid and simple method to measure spectral bleed-through in experimental samples that does not require control samples or complicated corrections would make quantitative seFRET more attractive to researchers that need fast and quantitative measurements of FRET efficiency. In this article, we present a novel method to quantitatively measure FRET efficiency using SeFRET that does not require a lengthy calibration and the uncertainty of the FRET efficiency can be determined on a per-pixel basis. Since this method relies on a curve-fitting approach, the uncertainty of the spectral bleed-through correction and FRET efficiency can be estimated by computing normalized residuals on each image pixel. Using the normalized residual error as an error metric, SensorFRET images can be thresholded by the estimated uncertainty in each pixel depending on the precision requirements of the experiment.
This method can be implemented on any type of microscope equipped with at least two excitation wavelengths and a detector with spectral resolution.

The acquisition routine of this method is substantially easier to implement than other established spectral imaging methods. For SensorFRET as well as other published spectral methods (luxFRET, sRET, and pFRET) [44, 43, 41], images of the same region must be acquired using two different excitation wavelengths. SensorFRET is unique from other spectral methods since no additional calibration images are required and the laser power and gain settings for each of these excitation wavelengths may be adjusted independently in order to achieve the best imaging conditions. This is in stark contrast to the calibration requirements needed for the other methods, which require one or more single fluorophore standards to be imaged prior to every experiment and the laser power/gain settings must be maintained between the calibration and experiment. LuxFRET requires imaging of two cell cultures expressing only donor or acceptor fluorophores, sRET requires imaging of two solutions with known concentrations of the donor or acceptor fluorophore, and pFRET requires imaging of a single cell culture expressing only the acceptor fluorophore, but has additional restrictions on which excitation wavelengths can be used [44, 43, 41]. SensorFRET, which does not require specialized equipment (as required by FLIM) or the additional complication, time, and expense associated with calibration sample preparation, provides an easy and accessible method for accurately determining the FRET efficiency, enabling FRET analysis to be utilized by a broader range of the research community.

2.3 Methods

Simulations: Simulations of FRET spectra were created using the ipython notebook. Idealized FRET spectra were simulated using parameters available in the
The FRET spectra, $F_{DA}$, is a function of the emission wavelength, $\lambda_{em}$, excitation wavelength, $\lambda_{ex}$, and FRET efficiency, $E$, was calculated according to:

$$
F_{DA}(\lambda_{em}, \lambda_{ex}, E) = I(\lambda_{ex}) \ast [DA] \ast \left[ s_D \ast \hat{\epsilon}_D(\lambda_{ex}) \ast (1 - E) \ast Q_D \ast \hat{\epsilon}_D(\lambda_{em}) + E \ast Q_A \ast \hat{\epsilon}_A(\lambda_{em}) + s_A \ast \hat{\epsilon}_A(\lambda_{ex}) \ast Q_A \ast \hat{\epsilon}_A(\lambda_{em}) \right]
$$

(2.1)

$I(\lambda_{ex})$ = intensity at the sample

$[DA]$ = concentration of FRET construct

$\hat{\epsilon}_D(\lambda_{ex})$ and $\hat{\epsilon}_A(\lambda_{ex})$ = normalized donor and acceptor excitation spectra

$\hat{\epsilon}_D(\lambda_{em})$ and $\hat{\epsilon}_A(\lambda_{em})$ = normalized donor and acceptor emission spectra

$s_D$ and $s_A$ = scaling factors for the donor and acceptor excitation spectra

$Q_D$ and $Q_A$ = quantum efficiencies of the donor and acceptor

The normalized emission spectra, excitation spectra and quantum efficiencies were all readily available in the literature for both 1 photon [51] and 2 photon [44] excitation. In order to have comparable spectral resolution to the experimental results, the literature emission spectra were re-sampled at 27 wavelengths between 465 and 718 nm. The $s_D$ and $s_A$ scaling factors are unknown, however the calculation of $E$ is independent of these parameters. $s_D = 1$ and $s_A = 5$ were chosen such that the shape at a given FRET efficiency is close to what is observed experimentally. $I(\lambda_{ex})$ and $[DA]$ were adjusted such that the spectra peak has a maximum amplitude of 65535, corresponding to the ideal signal amplitude for a 16 bit acquisition system.

For the luxFRET, sRET, and pFRET methods it was also necessary to simulate single fluorophore spectra for the calibration processes required by each of these
analysis approaches using the following equation.

\[
F_X(\lambda_{em}, \lambda_{ex}) = I(\lambda_{ex})*[X]*s_X*\dot{e}_X(\lambda_{ex})*Q_X*\dot{e}_X(\lambda_{em})
\]  

(2.2)

where X denotes either D or A for the donor or acceptor single fluorophore respectively. The \( I(\lambda_{ex}) \) term was maintained between the FRET construct spectra and calibration spectra and the \([X]\) terms were maintained at both excitation frequencies, as required by the luxFRET, sRET, and pFRET analysis approaches. Excitation wavelengths of 405 and 458 were used in the simulation of SensorFRET, luxFRET, and sRET, while excitation wavelengths of 458 and 515 were simulated for pFRET. The simulated noiseless spectra are shown in Figure 5.

Cell culture: NIH-3T3 mouse fibroblasts were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal calf serum and xMol of Penicillin-Strepomycin antibiotics. Primary human fibroblasts were cultured in DMEM with 10% fetal bovine serum. Cells were maintained in an atmospherically controlled incubator at 37 degrees celsius and 5% CO2 atmosphere. Media were changed every other day. Madin-Darby canine kidney cells (MDCK) were a gift from Rob Tombes (VCU Biology) and were maintained in high glucose DMEM (ThermoFisher) to which was added 10% fetal bovine serum (ThermoFisher) and 1% penicillin/streptomycin (ThermoFisher) under standard cell culture conditions.

To generate stable cell lines expressing canine full-length E-cad TSmod or TL (EcadΔcyto), cells were transfected with lipofectamine 2000 and selected using 500 ug/ml G418 (ThermoFisher). Full-length canine E-cad TSmod and EcadΔcyto were gift of Alex Dunn [52].

DNA Preparation: Soluble Cerulean-Venus FRET standards were acquired from the Addgene.org plasmid repository. Plasmids were received as E-coli vectors ex-
pressing C5V, C32V, CTV, CTA, and Venus plasmids. E-coli cultures were amplified in LB-broth overnight and plasmid DNA was isolated by affinity column purification using the NucleoBond Xtra Midi kit distributed by Macherey-Nagel.

**Plasmid Transfection:** DNA plasmids were transfected into cells using Lipofectamine 2000 or 3000 (Life Technologies) per manufacturer instructions. In all experiments cells were allowed to adhere to fibronectin coated glass bottom dishes or coverslips overnight before imaging.

**Single-Photon Imaging:** Images were acquired from cells grown on glass bottom dishes on an inverted Zeiss LSM 710 confocal using a either 405nm or 458nm excitation wavelengths from an argon laser source. A plan-apochromat 20x objective lens (NA=0.8) was used for all images involving the FRET standards or single fluorophore reference samples. Live cells expressing either soluble Cerulean-Traf-Amber or Venus were imaged in spectral mode using a 32-channel spectral META detector to record spectral fingerprints of Cerulean and Venus fluorophores respectively. For the FRET standards, images were captured in spectral mode with the emission frequencies spanning 460-720nm with 10nm spacing per channel. Images were captured in 16 bit mode, scanned bi-directionally, and averaged 4 times.

**Two-Photon Imaging:** Images were acquired from cells grown on glass bottom dishes on an upright Zeiss LSM 510 META NLO multi-photon laser scanning microscope with water immersion objectives. Images were captured with an internal de-scanned meta detector with emission frequencies spanning 367-699nm at 11nm spacing between channels. Excitation frequencies (850nm and 920nm) were tuned using a Spectra-Physics Mia-Tai broadband tunable Ti:sapphire laser.

**Fluorometer Measurements** Fluorescein and carboxy-tetramethylrhodamine (TAMRA or TRITC) were purchased from Sigma in their Reference Dye Sampler Kit (R14782). Stock 1mM solutions were diluted in phosphate buffered saline (PBS, pH 7.4) to final
concentrations (50μM and 1μM). Working solutions were transferred to a 3.5mL 4-sided Quartz cuvette with a path length of 10mm and an optical working range of 334 to 2500nm (Starna 3-G-10). All absorption and emission measurements were recorded by a Varian Cary Eclipse Fluorometer (SN:EL00043440) in 3D mode. Absorption and Emission wavelengths were captured at 5 and 2nm increments respectively. The absorption and emission bands spanned from (400-700nm) and (475-750nm) respectively. All measurements were averaged for .1s and the pmt voltage was adjusted such that the peak emission intensity approached approximately 80 % of the saturation intensity of the detector. All data analysis was performed in the ipython notebook where the mixed spectra were deconvolved using the non-negative least squares (nnls) scientific python package.

**FLIM Imaging:** Fluorescent lifetime imaging was performed on a two-photon Zeiss 780 NLO microscope equipped with a 32-channel descan spectral GaAsP (Gallium Arsenide Phosphide) detector. The Zeiss 780 was coupled with a Ti:Sapphire laser (Chameleon Vision-II, ultrafast) tunable from 680nm to 1080nm. The excitation frequency was tuned 860nm. To capture photons from fluorescein-only an HQ510-50m dichroic filter was used. A Becker and Hickl FLIM hybrid detector (HPM-100-40) coupled to the 780 NDD port was used for time-correlated single photon counting (TCSPC). To ensure instrument calibration, the standard dye fluorescein was used at a previously published concentration of 50μM [53]. Specifically, stock 1mM fluorescein pre-dissolved in DMSO was diluted in sodium borate buffer (pH 9) to a final concentration of 50μM. The 50μM diluted fluorescein was pipetted onto a glass slide and the fluorescent droplet was immediately imaged at 22°C. Photons were captured for total duration of 30 seconds at rate of approximately 200,000 events per second. Lifetime images were imported into Becker and Hickl software SPCI and fit using a single exponential model. The fitting shift had to be manually fixed to value that
minimized the Chi-square statistic. The offset was manually fixed to 0.

2.4 Results

2.4.1 An Overview of the SensorFRET method:

SensorFRET enables the calculation of FRET efficiency on a per pixel basis using spectral images acquired at two different excitation wavelengths. This analysis assumes the FRET sensor is unimolecular, such that all donor molecules are paired with acceptor molecules. For the purposes of our analysis, the fluorescent output of a FRET construct, $F_{DA}(\lambda_{em}, \lambda_{ex})$, is a function of emission wavelength, $\lambda_{em}$, and excitation wavelength, $\lambda_{ex}$, and can be thought of as the linear combination of three components:

$$F_{DA}(\lambda_{em}, \lambda_{ex}) = \text{DonorFRET} + \text{AcceptorFRET} + \text{Acceptor Direct Excitation}$$ (2.3)

The main challenge in accurately determining the FRET efficiency is separating the acceptor emission due to FRET from the acceptor emission due to direct excitation. Since both components have the same emission spectra, linear unmixing of a single spectra cannot separate the two contributions. SensorFRET takes advantage of the fact that the acceptor emission due to FRET has a different dependence on excitation wavelength than the acceptor emission due to direct excitation. This allows the calculation and removal of the acceptor direct excitation term using images of the same region of interest at two different excitation wavelengths, 1 and 2.

A full derivation of the SensorFRET approach is provided in section 2.4.2. The results of this analysis show that there are only three key parameters needed to calculate and remove the acceptor fluorophore direct excitation component. The first
of these, \( \alpha \), is defined as:

\[
\alpha \equiv \frac{F_{DA}(\text{donor}, \lambda_{ex2})}{F_{DA}(\text{donor}, \lambda_{ex1})} \tag{2.4}
\]

where \( F_{DA}(\text{donor}, \lambda_{exi}) \) is the magnitude of the donor component of the FRET spectra at excitation wavelength \( i \), determined through linear unmixing (Figure 6A). The second parameter needed is \( \beta \), which is defined as:

\[
\beta \times \hat{e}_A \equiv F_{DA}(\lambda_{em}, \lambda_{ex2}) - \alpha \times F_{DA}(\lambda_{em}, \lambda_{ex1}) \tag{2.5}
\]

where \( F_{DA}(\lambda_{em}, \lambda_{exi}) \) is the raw FRET spectra at excitation wavelength \( i \), and \( \beta \) is determined by least squares fitting the right hand side of equation 2.5 to the normalized acceptor emission shape, \( \hat{e}_A \) (Figure 6B). The final parameter required is \( \gamma \), which is defined as:

\[
\gamma \equiv \frac{\varepsilon_{D2}}{\varepsilon_{D1}} \times \frac{\varepsilon_{A1}}{\varepsilon_{A2}} = \frac{\hat{\varepsilon}_{D2}}{\varepsilon_{D1}} \times \frac{\hat{\varepsilon}_{A1}}{\varepsilon_{A2}} \text{(from literature)} \tag{2.6}
\]

or

\[
= \frac{F_D(\lambda_{em}, \lambda_{ex2})}{F_D(\lambda_{em}, \lambda_{ex1})} \times \frac{F_A(\lambda_{em}, \lambda_{ex1})}{F_A(\lambda_{em}, \lambda_{ex2})} \text{(from single fluorophore cell cultures)}
\]

where \( \varepsilon_X \) is the molar extinction coefficient of fluorophore \( X \) at excitation wavelength \( i \), \( \hat{\varepsilon}_X \) is the value of the excitation spectra of fluorophore \( X \) at excitation wavelength \( i \), and \( F_X(\lambda_{em}, \lambda_{exi}) \) is the raw spectra at excitation wavelength \( i \) from a cell expressing a single fluorophore \( X \) (Figure 6C). This parameter can be determined from normalized excitation spectra which are readily available in the literature for commonly used fluorescent proteins [54] allowing the FRET efficiency to be determined without any of the standards needed by other spectral intensity approaches [42, 43, 44, 41]. However, it should be cautioned that literature values of the excitation spectra are generally
reported as normalized spectrums, not absolute extinction coefficient spectra. At high concentrations the fluorophore absorption spectrums can undergo significant shape changes [55]. If concentration artifacts are a concern or if the absorption spectra are not available then this parameter can also be determined experimentally from two cell cultures, one expressing only the donor fluorophore and the other expressing only the acceptor fluorophore. For the experimental determination of $\gamma$, it is important that calibration images taken at both excitation wavelengths are in register for a given cell culture and that the laser power and gain settings are maintained while imaging donor-only and acceptor-only cell cultures when using a given excitation pair of wavelengths (see section 2.4.2 for justification of this requirement). If $\gamma$ is measured properly, it is independent of the instrumentation parameters and depends solely on the fluorophores in the sensor and the two excitation wavelengths used.

With these three parameters, the acceptor direct excitation can be computed at excitation wavelengths 1 and 2, as shown in Figure 6 C, according to the following equations:

$$F_{DA_{corr}}(\lambda_{em}, \lambda_{ex1}) = F_{DA}(\lambda_{em}, \lambda_{ex1}) - \frac{\beta}{\alpha(\gamma^{-1} - 1)} \times \hat{e}_A$$  \hspace{1cm} (2.7)

$$F_{DA_{corr}}(\lambda_{em}, \lambda_{ex2}) = F_{DA}(\lambda_{em}, \lambda_{ex1}) - \frac{\beta}{1 - \gamma} \times \hat{e}_A$$  \hspace{1cm} (2.8)

Once the acceptor direct excitation component is removed, the corrected spectra can be unmixed into donor FRET and acceptor FRET components using linear unmixing (Figure 6 D) and the FRET efficiency at excitation wavelength $i$, $E(\lambda_{exi})$, is determined according to:

$$E(\lambda_{exi}) = \left( \frac{F_{DA_{corr}}(donor, \lambda_{exi}) \times Q_A}{F_{DA_{corr}}(acceptor, \lambda_{exi}) \times Q_D + 1} \right)^{-1}$$  \hspace{1cm} (2.9)

where $F_{DA_{corr}}(donor, \lambda_{exi})$ is the magnitude of the donor component of the corrected spectra at excitation wavelength $i$, $F_{DA_{corr}}(acceptor, \lambda_{exi})$ is the magnitude of the
acceptor component of the corrected spectra at excitation wavelength $i$, $Q_A$ is the quantum efficiency of the acceptor fluorophore, and $Q_D$ is the quantum efficiency of the donor fluorophore.

### 2.4.2 Complete Derivation of the SensorFRET method

The magnitude and shape of a single donor fluorophore’s spectra can be described as [56]:

$$ F_D(\lambda_{em}, \lambda_{ex}) = I(\lambda_{ex}) *[D]*s_D*\hat{\epsilon}_D(\lambda_{ex})*Q_D*\hat{\epsilon}_D(\lambda_{em}) \quad (2.10) $$

$F_D(\lambda_{em}, \lambda_{ex}) = \text{complete fluorescent spectra}$

- $\lambda_{em} = \text{emission wavelengths}$
- $\lambda_{ex} = \text{excitation wavelengths}$
- $I(\lambda_{ex}) = \text{laser excitation power}$
- $[D] = \text{concentration of donor molecules}$
- $s_D = \text{donor absorption coefficient scalar}$
- $\hat{\epsilon}_D(\lambda_{ex}) = \text{normalized excitation spectrum}$
- $Q_D = \text{quantum efficiency}$
- $\hat{\epsilon}_D(\lambda_{em}) = \text{normalized emission spectrum}$

The magnitude and shape of a single FRET sensor’s spectra can be described as:
\[ F_{DA}(\lambda_{em}, \lambda_{ex}) = I(\lambda_{ex}) \cdot [DA] \left[ s_D \cdot \hat{\epsilon}_D(\lambda_{ex}) \cdot (1 - E) \cdot Q_D \cdot \hat{\epsilon}_D(\lambda_{em}) + E \cdot Q_A \cdot \hat{\epsilon}_A(\lambda_{em}) + s_A \cdot \hat{\epsilon}_A(\lambda_{ex}) \cdot Q_A \cdot \hat{\epsilon}_A(\lambda_{em}) \right] \]

\[(2.11)\]

This equation is the sum of three components, the Donor FRET signal, the Acceptor FRET signal, and the Acceptor Direct Excitation, defined here as:

\[ F_{DA}(\lambda_{em}, \lambda_{ex}) = \text{complete fluorescent FRET spectra} \]

\[ E = \text{FRET efficiency} \]

\[ \hat{\epsilon}_A(\lambda_{ex}) = \text{normalized acceptor excitation spectrum} \]

\[ \hat{\epsilon}_A(\lambda_{em}) = \text{normalized acceptor emission spectrum} \]

\[ s_A = \text{acceptor absorption scalar} \]

\[ Q_A = \text{quantum efficiency of the acceptor} \]

\[ [DA] = \text{concentration of FRET molecules} \]

Donor FRET = \[ I(\lambda_{ex}) \cdot [DA] \cdot s_D \cdot \hat{\epsilon}_D(\lambda_{ex}) \cdot (1 - E) \cdot Q_D \cdot \hat{\epsilon}_D(\lambda_{em}) \]

Acceptor FRET = \[ I(\lambda_{ex}) \cdot [DA] \cdot s_D \cdot \hat{\epsilon}_D(\lambda_{ex}) \cdot E \cdot Q_A \cdot \hat{\epsilon}_A(\lambda_{em}) \]

Acceptor Direct Excitation = \[ I(\lambda_{ex}) \cdot [DA] \cdot s_A \cdot \hat{\epsilon}_A(\lambda_{ex}) \cdot Q_A \cdot \hat{\epsilon}_A(\lambda_{em}) \]

Linear unmixing using the normalized emission shapes of the donor and acceptor
fluorophores ($\hat{e}_D$ and $\hat{e}_A$) yields the magnitude of the donor and acceptor components:

\[
F_{DA}(\text{donor}, \lambda_{exi}) = I(\lambda_{exi}) \cdot [DA] \cdot s_D \cdot \hat{e}_D(\lambda_{exi}) \cdot (1 - E) \cdot Q_D
\]

\[
F_{DA}(\text{acceptor}, \lambda_{exi}) = I(\lambda_{exi}) \cdot [DA] \cdot s_D \cdot \hat{e}_D(\lambda_{exi}) \cdot E \cdot Q_A + I(\lambda_{exi}) \cdot [DA] \cdot s_A \cdot \hat{e}_A(\lambda_{exi}) \cdot E \cdot Q_A
\]

Taking the ratio of the donor component magnitudes at two different excitation wavelengths leaves a quantity that is proportional to the ratio of the donor absorption coefficients scaled by the excitation power coefficients at excitation wavelengths 1 and 2 as shown below:

\[
\frac{F_{DA}(\text{donor}, \lambda_{ex2})}{F_{DA}(\text{donor}, \lambda_{ex1})} = \frac{I(\lambda_{ex2}) \cdot [DA] \cdot s_D \cdot \hat{e}_D(\lambda_{ex2}) \cdot (1 - E) \cdot Q_D}{I(\lambda_{ex1}) \cdot [DA] \cdot s_D \cdot \hat{e}_D(\lambda_{ex1}) \cdot (1 - E) \cdot Q_D} = \frac{I(\lambda_{ex2}) \cdot \hat{e}_D(\lambda_{ex2})}{I(\lambda_{ex1}) \cdot \hat{e}_D(\lambda_{ex1})} \equiv \alpha
\]

This quantity is called $\alpha$, which is a scalar quantity that is constant for a given FRET sensor at a fixed pair of excitation frequencies and power ratio. The $\alpha$ term can then be used to scale $F_{DA}(\lambda_{ex1}, \lambda_{em})$ such that the Donor FRET and Acceptor FRET terms in 2.12 are equivalent at both excitation wavelengths as shown below (the equivalent Donor FRET and Acceptor FRET terms are shown in large brackets):

\[
\alpha \cdot F_{DA}(\lambda_{em}, \lambda_{ex1}) = \left[ I(\lambda_{ex2}) \cdot [DA] \cdot s_D \cdot \hat{e}_D(\lambda_{ex2}) \cdot (1 - E) \cdot Q_D \cdot \hat{e}_D(\lambda_{em}) + E \cdot Q_A \cdot \hat{e}_A(\lambda_{em}) \right] + I(\lambda_{ex2}) \cdot [DA] \cdot \frac{\hat{e}_D(\lambda_{ex2})}{\hat{e}_D(\lambda_{ex1})} \cdot s_A \cdot \hat{e}_A(\lambda_{ex1}) \cdot Q_A \cdot \hat{e}_A(\lambda_{em})
\]
\[
F_{DA}(\lambda_{em}, \lambda_{ex2}) = \left[ I(\lambda_{ex2}) \ast [DA] \ast s_D \ast \hat{e}_D(\lambda_{ex2}) \left( (1 - E) \ast Q_D \ast \hat{e}_D(\lambda_{em}) +
E \ast Q_A \ast \hat{e}_A(\lambda_{em}) \right) \right] + \\
I(\lambda_{ex2}) \ast [DA] \ast s_A \ast \hat{e}_A(\lambda_{ex2}) \ast Q_A \ast \hat{e}_A(\lambda_{em})
\]

(2.16)

Taking the difference between \(F_{DA}(\lambda_{em}, \lambda_{ex2})\) and \(\alpha \ast F_{DA}(\lambda_{em}, \lambda_{ex1})\) produces a term related to the magnitude of the acceptor direct excitation, defined as \(\beta\):

\[
\beta \ast \hat{e}_A(\lambda_{em}) = F_{DA}(\lambda_{em}, \lambda_{ex2}) - \alpha \ast F_{DA}(\lambda_{em}, \lambda_{ex1})
\]

\[
= I(\lambda_{ex2}) \ast [DA] s_A \ast \hat{e}_A(\lambda_{ex2}) \ast Q_A \ast \hat{e}_A(\lambda_{em}) - I(\lambda_{ex2}) \ast [DA] * \\
\frac{\hat{e}_{D2}}{\hat{e}_{D1}} \ast s_A \ast \hat{e}_A(\lambda_{ex1}) \ast Q_A \ast \hat{e}_A(\lambda_{em})
\]

\[
= I(\lambda_{ex2}) \ast [DA] s_A \ast \hat{e}_A(\lambda_{ex2}) \ast Q_A \ast \hat{e}_A(\lambda_{em}) \left( 1 - \frac{\hat{e}_{A1}}{\hat{e}_{A2}} \ast \frac{\hat{e}_{D2}}{\hat{e}_{D1}} \right)
\]

(2.17)

or

\[
\beta = F_{DA}(\text{acceptor}, \lambda_{ex2}) - \alpha \ast F_{DA}(\text{acceptor}, \lambda_{ex1})
\]

Eq. 2.17 shows that the scalar quantity \(\beta\) can be computed by least squares fitting of \(\hat{e}_A\). Since \(F_{DA}(\lambda_{em}, \lambda_{ex2}) - \alpha \ast F_{DA}(\lambda_{em}, \lambda_{ex1})\) is ideally the same shape as the acceptor emission, this approach provides a useful visual check that the direct excitation can be characterized above any noise in the signal. Alternatively, \(\beta\) can be calculated directly from the acceptor component magnitudes \(F_{DA}(\text{acceptor}, \lambda_{ex2}) - \alpha \ast F_{DA}(\text{acceptor}, \lambda_{ex1})\), which is computationally less intensive than a second fitting operation.

The final parameter required to determine the acceptor direct excitation term is
\( \gamma \), defined as:

\[
\gamma \equiv \frac{\hat{\epsilon}_D(\lambda_{ex2}) * \hat{\epsilon}_A(\lambda_{ex1})}{\hat{\epsilon}_D(\lambda_{ex1}) * \hat{\epsilon}_A(\lambda_{ex2})}
\]  
(2.18)

Since the excitation spectra of most fluorophores have been previously characterized in the literature, each of the \( \hat{\epsilon}_D(\lambda_{ex2}) \), \( \hat{\epsilon}_D(\lambda_{ex1}) \), \( \hat{\epsilon}_A(\lambda_{ex1}) \), and \( \hat{\epsilon}_A(\lambda_{ex2}) \) terms can be determined directly from those. If the fluorophores have not been previously characterized or if the fluorescence is expected to be significantly different due to the local environment within a particular cell type, this parameter can be determined experimentally using two cell cultures expressing donor only and acceptor only fluorophores. Four spectra are required (donor only cells at \( \lambda_{ex2} \), donor only cells at \( \lambda_{ex1} \), acceptor only cells at \( \lambda_{ex1} \), and acceptor only cells at \( \lambda_{ex2} \)).

\[
\gamma = \frac{F_D(\lambda_{em}, \lambda_{ex2}) * F_A(\lambda_{em}, \lambda_{ex1})}{F_D(\lambda_{em}, \lambda_{ex1}) * F_A(\lambda_{em}, \lambda_{ex2})}
\]  
\[
= \frac{I(\lambda_{ex2}) * [D] * s_D * \hat{\epsilon}_D(\lambda_{ex2}) * Q_D * \hat{\epsilon}_D(\lambda_{em}) * I(\lambda_{ex1}) * [A] * s_A * \hat{\epsilon}_A(\lambda_{ex1}) * Q_A * \hat{\epsilon}_A(\lambda_{em})}{I(\lambda_{ex1}) * [D] * s_D * \hat{\epsilon}_D(\lambda_{ex1}) * Q_D * \hat{\epsilon}_D(\lambda_{em}) * I(\lambda_{ex2}) * [A] * s_A * \hat{\epsilon}_A(\lambda_{ex2}) * Q_A * \hat{\epsilon}_A(\lambda_{em})}
\]
\[
= \frac{\hat{\epsilon}_D(\lambda_{ex2}) * \hat{\epsilon}_A(\lambda_{ex1})}{\hat{\epsilon}_D(\lambda_{ex1}) * \hat{\epsilon}_A(\lambda_{ex2})}
\]  
(2.19)

In order for the intensity and concentration terms to cancel out as shown above, it is important to maintain some of the experimental parameters between certain images for this calibration. The laser power and gain settings must be maintained so that \( I(\lambda_{exi}) \) for the donor is the same as \( I(\lambda_{exi}) \) for the acceptor. Additionally, it is important that the images are in registration so that the \([D] \) or \([A] \) term is constant at both excitation wavelengths for any given pixel. For the Cerulean Venus FRET construct, the \( \gamma \) term determined from literature [44] was 0.045 while the \( \gamma \) term from the above calibration in 3T3 cells was 0.069. This yields a change in the measured C32V FRET efficiency of 0.6% FRET, which is much smaller than the experimental
variation due to noise even with significant blurring.

Using the definitions for $\alpha$, $\beta$, and $\gamma$, the magnitude of the acceptor direct excitation can be written in terms of these parameters and correct the acceptor component magnitude such that only the FRET component is included:

$$\text{Acceptor Direct Excitation}(\lambda_{ex2}) = \frac{\beta}{(1 - \gamma)}$$

and

$$\text{Acceptor Direct Excitation}(\lambda_{ex1}) = \frac{\beta}{\alpha(\gamma^{-1} - 1)}$$

$$F_{DAcorr}(\lambda_{em}, \lambda_{exi}) = F_D(\lambda_{em}, \lambda_{exi}) - \text{Acceptor Direct Excitation}(\lambda_{exi})$$

$$= I(\lambda_{exi}) \times [DA] \times s_D \times \hat{e}_D(\lambda_{exi}) \times (1 - E) \times Q_D \times \hat{e}_D(\lambda_{em}) +$$

$$E \times Q_A \times \hat{e}_A(\lambda_{em})$$

The corrected FRET spectra at either frequency can then be unmixed and rearranged to solve for the FRET efficiency, $E$:

$$F_{DAcorr}(\text{donor}, \lambda_{exi}) = I(\lambda_{exi}) \times [DA] \times s_D \times \hat{e}_D(\lambda_{exi}) \times (1 - E) \times Q_D$$

$$F_{DAcorr}(\text{acceptor}, \lambda_{exi}) = I(\lambda_{exi}) \times [DA] \times s_D \times \hat{e}_D(\lambda_{exi}) \times E \times Q_A$$

$$E(\lambda_{exi}) = \left( \frac{F_{DAcorr}(\text{donor}, \lambda_{exi}) \times Q_A}{F_{DAcorr}(\text{acceptor}, \lambda_{exi}) \times Q_D + 1} \right)^{-1}$$

### 2.4.3 Validation of SensorFRET using simulated spectra

In order to validate this method and compare it to the sRET [44], luxFRET [43], and pFRET [41] spectral imaging methods, FRET spectra with a pre-determined FRET efficiency (35%) were generated from excitation and emission spectra for Cerulean and Venus fluorophores available in the literature [51]. The spectra were
Table 2. Comparison of SensorFRET measurements (using both single and two photon excitation) to sRET and FLIM-FRET measurements for established FRET standards. Values are reported as mean ± std (n = # of cells measured). Values denoted with a were obtained from Thaler et al. [44]. Values denoted with b were obtained from Koushick et al. [53].

<table>
<thead>
<tr>
<th>Method/FRET Standard</th>
<th>CTV</th>
<th>C32V</th>
<th>C5V</th>
</tr>
</thead>
<tbody>
<tr>
<td>SensorFRET(405/458nm)</td>
<td>2.1±5.6 (n=14)</td>
<td>36.2±2.9 (n=23)</td>
<td>43.8±6.5 (n=24)</td>
</tr>
<tr>
<td>SensorFRET(850/920nm)</td>
<td>0.8±5.3 (n=16)</td>
<td>30.7±5.4 (n=10)</td>
<td>47.5±3.8 (n=12)</td>
</tr>
<tr>
<td>sRET</td>
<td>1.7±7.0 (n=12)a</td>
<td>29.5±8.0 (n=60)b</td>
<td>41.5±8.7 (n=60)b</td>
</tr>
<tr>
<td>FLIM-FRET</td>
<td>6.3±2.6 (n=30)a</td>
<td>33.3±4.5 (n=10)b</td>
<td>44.3±1.7 (n=10)b</td>
</tr>
</tbody>
</table>

generated according to equation 2.11 as detailed in section 2.3 and shown in Figure 5. Wavelengths 1 and 2 were chosen to be 405 and 458 nm, corresponding to the laser lines used in the subsequent experimental section 2.4.6. The α and β terms were determined using the fits shown in Figure 6A and B, respectively, while the γ term was calculated from the literature excitation spectra to have a value of 0.045. sRET, luxFRET, and pFRET algorithms were implemented according to their original references [44, 43, 41]. Under these ideal and noiseless conditions, all 4 approaches yielded a FRET efficiency of exactly 35%, corresponding to the pre-determined simulated value within floating point precision.

2.4.4 Determining the Noise Tolerance of SensorFRET using Simulated Noisy Pixels

Besides confirming the mathematical approach of SensorFRET, another goal of these simulations was to characterize the noise dependence of this method and compare it to the other spectral intensity based methods. In order to add realistic noise to simulated pixels of a known FRET efficiency, the experimental noise was
characterized by determining the standard deviation of the noise as a function of spectra amplitude, shown in Figure 7A. While the observed noise appears to vary as a function of emission frequency (Figure 7A), that dependence results from amplitude fluctuation of the spectra across the recorded emission wavelengths. The amplitude of the noise is linearly dependent on the square root of the signal amplitude, as shown in Figure 7B. This type of noise dependence is known as shot noise and is characteristic of the photomultiplier tube (PMT) detector [57]. Using the linear fit parameters from Figure 7B, the noise added to simulated spectra are nearly indistinguishable from experimentally observed pixels, as shown in Figure 7C.

By simulating pixels covering a range of signal to noise ratios, the expected standard deviation in the measured FRET efficiency as a function of the normalized spectra fit residual was determined (Figure 8A). The normalized residual is used as a metric for the amount of noise in the spectra because it can be calculated on a per-pixel basis and is intensity independent. While the poisson noise in the measured spectra is dependent on the intensity, the normalized residual returns the error of the fit independent of the spectra amplitude. The same simulated input spectra used to form Figure 8A was analyzed using the sRET, luxFRET and pFRET methods (while using perfect, noiseless calibration spectra required for each analysis approach) to obtain analogous estimations of the mean and standard deviation of the FRET as a function of the residual. These are plotted in Figure 8B and 8C, which shows no appreciable difference in the noise tolerance between any of the methods.

2.4.5 Validation of SensorFRET using standard fluorescent dyes

While simulations show that SensorFRET and other spectral imaging methods are mathematically correct, they do not measure the accuracy of the algorithm under conditions where noise and instrumental errors can bias the measurement. Further-
more, it is possible that the fluorescent spectra model outlined in section 2.4.2 is either incomplete or invalid and fails under realistic experimental conditions. To test the validity of the fluorescent spectra model (section 2.4.2) and the noise tolerance of SensorFRET, an experiment using a FRET-compatible dye pair with an unknown FRET efficiency was conducted.

While fluorescent protein FRET standards have already been developed by the Vogel lab [53] and used by other laboratories to validate FRET methodologies, our extensive experimentation with these standards revealed occasionally inconsistent FRET efficiency estimates that could not be explained by instrumentation error (see section 2.4.7). A series of explanations could explain why these fluorescent protein standards have varying FRET efficiencies. One explanation is that the cell microenvironment or fluorescent concentration changes markedly from batch to batch or across different cell types. There are also published discrepancies between FRET efficiencies determined by lifetime measurements when compared to sensitized emission measurements. This is particularly the case for the CTV construct which was reported to have a FRET efficiency of anywhere from 0 to 10 % from well respected laboratories [44, 41, 58, 59, 60]. While various estimates of the same construct have been published, to date there have been no attempts to understand the source of these discrepancies.

In an effort to generate a FRET standard that has a stationary FRET efficiency value regardless of the methodology, a FRET compatible standard fluorescent dye pair consisting of fluorocein and TAMRA were developed. There are many advantages of using fluorescent dyes for validation measurements. First, they have well characterized excitation and emission spectra. Second, their concentration can be manipulated. Third, accurate fluorescent lifetime data for standard dyes can be used to independently validate the instrumentation and cross-check steady-state sensitized
emission FRET measurements. Fourth, unlike fluorescent proteins the fluorescent lifetime of these dyes follows a simple single exponential decay model. Fifth, these dyes can be easily loaded into a spectrofluorometer that measures the entire absorption/emission spectrum. Finally, the average radius between donor and acceptor pairs can be varied by changing the concentration of dye-pair in solution.

\[
\frac{1}{p} \pm \frac{1}{3} = \left( \frac{1}{p_0} \pm \frac{1}{3} \right) \left( 1 + \frac{4\pi N_c R_0^6}{15(2a)^3} \times 10^{-3} \right)
\] (2.23)

It was shown by G. Weber that depolarization of dyes \( \frac{1}{p} \) in solution due to FRET could be modeled as a linear function of concentration \( c \) (Equation 2.23)[61]. It is important to note this model strongly depends on the assumed Forster radius \( R \) and the effective molecular radius \( 2a \) of the dye(s). Without knowing the exact molecular or Forster radius, this model can be used as a first approximation for concentration-based FRET studies. The model predicts that the concentration at which a randomly distributed, isotropically oriented single dye (such as fluorocein) begins to FRET in solution at concentrations above 1µM (Figure 9A). At concentrations around 1µM or less, a 0-1% FRET efficiency would be expected (Figure 9A). At concentrations of 10µM or more, a measurable amount of FRET is predicted to occur (7-8%) (Figure 9A). It should be noted that Weber’s model was based on a single type of dye in solution rather than two different dyes. Any charged interactions between the dyes that could change the effective molecular radius will produce errors in the expected concentrations that FRET might occur.

One approach to validating the SensorFRET algorithm is to use a pair of dyes that are capable of energy transfer but at a concentration where 0 percent FRET occurs (where the average distance between dye molecules is > twice the Forster radius). This approach validates that the unmixing algorithm and calibration measurement
are both accurate enough to remove non-FRET acceptor emission in the presence of measurement noise and imperfect instrumentation. This could be thought of as a “zero FRET control” or negative control.

Fluoresein and TAMRA dyes were diluted in phosphate buffered saline (pH 7) to a concentration of 2µM. These dyes were chosen for two reasons. First, they are well characterized standard reference dyes that can be purchased together from Sigma’s standards reference dye kit. Second, they have the spectral properties necessary for FRET to occur, specifically an overlap between the donor emission and acceptor absorption wavelengths (Figure 9B). Using a spectrofluorometer, the entire absorption and emission spectrum of each single dye solution was acquired (Figure 10B-C). The single dye absorption-emission matrix forms the basis of a three dimensional fluorescent “fingerprint” that can be used to deconvolve a mixed sample.

One problem with deconvolving these 3D signals is that the sensitized emission FRET component is not directly measurable like the single-dye fingerprints. This is due to the fact that sensitized-emission FRET can only be observed in a sample containing both the donor and acceptor fluorophores. However, all of the information of the sensitized emission is embedded in the Donor-only and Acceptor-only spectra. As outlined in section 2.4.2, the sensitized emission shape is a product of the normalized donor excitation spectrum and the normalized acceptor emission spectrum (see Equation 2.12). Since this information is captured in the spectra fingerprints (Figure 10B-C), the FRET component shape can be modeled to produce three components for the unmixing algorithm: 1) the donor fingerprint 2) the acceptor fingerprint and 3) the FRET fingerprint. Each of these fingerprints represents a unique 3-dimensional peak in the absorption-emission space (Figure 10).

The sensitized emission FRET component was modeled by taking single wavelength cross-sections of the donor excitation spectrum and acceptor emission spec-
trum. This is equivalent to taking a vertical slice from the fluorocein fingerprint (Figure 10B) and a horizontal slice from the TAMRA fingerprint (Figure 10C). It is arbitrary which wavelength is chosen so long as the signal to noise is favorable. In this approach, the average excitation and emission spectrums were used to model the FRET component fingerprint (Figure 11A). The synthesis of this FRET component assumes that the shape of the donor excitation and acceptor emission spectrums are invariant in the excitation-emission space. Fortunately, this assumption can be verified by examining the shape of these normalized spectra at multiple excitation and emission wavelengths. The normalized excitation and emission spectrums from Fluorocein and TAMRA respectively showed trivial shape variance at many different wavelengths (Figure 12).

After determining the shapes of the unmixing components, the mixed dye sample was measured with a spectrofluorometer. Fluorocein and TAMRA were prepared at 1μM concentration and mixed together, bringing the total dye concentration to 2μM. To implement the unmixing algorithm, the excitation-emission matrices were converted to 1-dimensional vectors such that each emission spectrum was appended end-to-end (Figure 11B). The excitation-emission matrices were transformed this way to take advantage of the non-negative-least squares (nnls) fitting algorithm integrated in the scientific python package. The nnls function was used previously in Section 2.4.3 for unmixing emission spectra and provides a very computationally efficient way to fit spectra. This package is a wrapper for the FORTRAN non-negative least squares solver which is designed to minimize the residual of the following linear system:

$$\min \|Ax - \tilde{b}\|$$  \hspace{1cm} (2.24)

For this particular application, the unmixing components form matrix $A$, the
vector \( \vec{x} \) contains the coefficients to weight each column of matrix \( A \) and vector \( \vec{b} \) represents the measured spectra vector. Since \( A \) and \( \vec{b} \) are known in our application, the algorithm solves for the coefficients in \( \vec{x} \).

To visualize the accuracy of the nnls fitting, the modeled spectra is plotted with the measured spectra of Fluorocein and TAMRA (Figure 11C). The close agreement between these two curves demonstrates that the modeled fit is nearly identical to the measured spectra. To quantify the accuracy of the fit, a reduced-chi-square statistic was used. This is the same statistic used in fluorescent lifetime measurements, except that in FLIM measurements the independent variable is time whereas in this application the independent variable is excitation frequency. [62]. The reduced chi-square statistic is defined by the FLIM instrument manufacturer Becker and Hickl [63] as:

\[
\chi_r^2 = \frac{1}{N-p} \sum_{i=1}^{N} \frac{(d_i - f_i)^2}{d_i}
\]

(2.25)

Where \( d_i \) is a measurement at wavelength \( i \), \( f_i \) is the fitted model at wavelength \( i \), \( N \) is the number of sampled wavelengths and \( p \) is the number of components or parameters used in the model. The lower the value of this statistic, the closer fit. In the fitting illustrated in Figure 11C, the \( \chi_r^2 \) value is 0.092. As a reference point, typical FLIM \( \chi_r^2 \) values for individual pixels generally range between 3-.9 (see section 2.4.6.

The coefficients of this fitting show that the modeled dye mixture is composed of Fluorocein and TAMRA only. The modeled coefficient for the FRET component was zero. When viewed in the excitation-emission space, there was no discernible FRET peak (Figure 13B). The modeled fit was transformed back into the excitation space with the percentage weight of the components overlaid on each spectra peak (Figure 13A). Fluorocein and TAMRA were 62 and 38 percent of the component weightings.
respectively. The unequal component weightings is likely due to the different quantum yields (the efficiency of their emission process) of these dyes. Fluorocein and TAMRA have published quantum yields of .92 and .68 respectively [64, 65].

It was shown that no FRET signal could be detected in the excitation-emission space of a 2µM mixture of Fluorocein and TAMRA. This demonstrates that this dye mixture can be used as a zero FRET control at 2µM. To test the validity of the SensorFRET algorithm, all pairings of the mixed dye emission at different excitation frequencies were evaluated. If the algorithm was perfect, the 2µM dye mixture would measure 0% FRET at every pairing. In practice, unfavorable signal to noise erodes the accuracy of the measurement at low intensities.

A contour plot of all different excitation pairs shows the color coded estimated FRET efficiency using the SensorFRET algorithm (Figure 14B). Each pixel represents a FRET estimate where the input spectra excitation frequencies correspond to the coordinate location in the image. A noiseless perfect measurement would show all pixels with a FRET efficiency of 0%. In this particular representation all pixels would appear uniformly green (Figure 14B). Instead, SensorFRET estimates approximately 0% FRET in specific regions of the excitation space (Figure 14B-green color). These regions correspond to areas of the excitation space where there is an acceptable SNR for the donor. The largest errors of SensorFRET tend to be with excitation pairs that are in the red spectrum of the excitation space (Figure 14B-bottom right corner).

Using the same approach SensorFRET was compared against two different spectral unmixing algorithms, sRET and luxFRET. Both of these algorithms are similar to SensorFRET in that they require two emission spectras at different excitation wavelengths. However, both of these algorithms have different calibration routines and imaging constraints relative to SensorFRET. Regardless of these differences, all three methods can be compared in the same excitation space shown in (Figure 14B).
LuxFRET appears to correctly estimate the FRET efficiency if the donor excitation or acceptor emission have a favorable SNR for one of the excitation frequencies (Figure 14A). In contrast to SensorFRET, the estimated FRET efficiency from luxFRET is not symmetric with respect to the excitation axes (compare Figure 14A with Figure 14B). Both sRET and SensorFRET have symmetric FRET efficiency estimates in the excitation space however sRET appears to outperform both luxFRET and SensorFRET in terms of the number of accurate excitation pairings (Figure 14C). It is important to note that sRET has the most laborious calibration routine that requires known concentrations of the donor and acceptor fluorophores. In most applications, the absolute concentration of fluorophore is not known. Unlike SensorFRET, both luxFRET and sRET require that the acquisition settings of the measuring equipment during the calibration match the settings of the sample measurements.

![Spectra for sensorFRET, luxFRET, sRET, and pFRET analysis approaches](Fig. 5. Noiseless spectra required for SensorFRET, luxFRET, sRET, and pFRET analysis approaches simulated using normalized excitation spectra, emission spectra, and quantum efficiencies from literature [51])
2.4.6 Cross-validation of sensitized emission measurements with fluorescent lifetime imaging (FLIM) using standard fluorescent dyes

To independently verify the FRET efficiency estimates of the fluorescein-TAMRA dye mixture, fluorescent lifetime imaging was performed. The lifetime of fluorescein-only was measured at two different pH levels (9.7 pH) and two concentrations (50.2 uM). Basic (pH 9) and high concentration (50uM) measurements were recorded because previously published lifetime measurements for fluorescein were recorded in these conditions [53]. Low concentration (2uM) and neutral (pH 7) measurements were recorded 1) to ensure the effective molecular radius between fluorescein and TAMRA molecules was large enough such that there was no transfer of energy (see Figure 9A for a graphical justification of this requirement) and 2) to maximize the emission signal from both fluorophores. According to the literature [53], the lifetime of fluorescein at high pH is in the range of (4.0-4.2 ns). In sodium borate buffer (pH 9) at 50uM concentration, the average lifetime measured 4.19±0.037ns using a global binning of pixels and 4.13±.037ns using a 10-pixel binning (Figure 16B, Figure 16, Figure 19). This falls within the confidence interval of the expected lifetime (4.0-4.2ns), confirming that 1) the instrument was properly calibrated and 2) the exponential decay fitting model was accurate [66]. With a pH of 7 at 50uM in PBS, the lifetime measured 4.14±0.039ns (Figure 19). As expected, the lifetime decreased as the pH dropped as previously published [67] (Figure 19).

To determine if energy transfer occurs between fluorescein and TAMRA, an accurate lifetime of the donor fluorophore was required. It was previously reported that the lifetime of fluorescein was largely independent of its concentration [67]. Rather than assume the lifetime of fluorescein is independent of concentration, both high
(50uM) and low (2uM) concentrations were measured (Figure 19). The lifetime of fluorescein substantially decreased as the concentration decreased from 50uM to 2uM at neutral pH (Figure 19). Changes in concentration led to larger shifts in the fluorescent lifetime than pH. This was in contrast to observations made by Hammer et. al. [67], where the measured lifetime was independent of the concentration and strongly dependent on the pH. The process of photon re-absorption where emitted photons are re-absorbed by the donor fluorophore can occur at high fluorophore concentrations. It is possible that photon re-absorption could explain the increase in fluorescent lifetime of fluorescein from 2uM to 50uM [68].

\[ E = 1 - \frac{\tau_{DA}}{\tau_D} \]  

(2.26)

To measure the transfer of energy between fluorescein and TAMRA, dye lifetimes were measured in mixed samples. The FRET efficiency was calculated based on the change in lifetime of fluorescein in these mixtures. Specifically, the FRET efficiency was calculated according to Equation 2.26, where \( \tau_D \) was defined as the average lifetime of the fluorescein-only samples, \( \tau_{DA} \) the lifetime of fluorescein in the mixed samples including TAMRA, and \( E \) referring to the FRET transfer efficiency. A single exponential decay model was used for all least-squares fittings to estimate the lifetime decay constants. Without knowing whether the concentration of fluorophore solvent (DMSO) or the donor fluorophore concentration should be held constant in the mixed samples relative to the donor-only sample, both were measured. Since concentration and solvent effects were potential independent factors, four mixed concentrations were measured where each fluorophore’s final concentration was either 50uM, 25uM, 2uM or 1uM. Holding the concentration or solvent constant yielded nearly the same result in terms of the inferred FRET efficiency (Figures 18B and 17B). The results
showed fluorescein’s average lifetime was approximately 250 picoseconds faster in the presence of TAMRA (Figure 17A). This reduction in lifetime represented a FRET efficiency of 1% at the low concentration mixtures (1uM or 2uM) (Figure 17B). This was very similar to independent measurements using a fluorometer (Section 2.4.5) and spectrally resolved confocal measurements (Figure 15). At high concentration mixtures, the change in lifetime implied a 6% FRET in solution (Figures 18A-B).

In conclusion, the 1uM mixture of fluorescein and TRITC can be used as a 0% FRET standard. Spectral confocal microscopy, fluorescent lifetime-imaging, and excitation-emission fluorometer readings all independently confirmed the 2uM mixture of fluorescein and TRITC has approximately zero energy transfer. These dyes could be useful for any researcher that needs to validate their FRET imaging methodology or develop new FRET imaging techniques. In addition, the dye pair could be particularly useful for verifying the unmixing of cross-talk or direct excitation in spectral imaging. This represents an improvement from the CTV protein FRET standard which in theory should measure 0% FRET efficiency based on the predicted distance of the TRAF domain linker. However, in practice the apparent FRET of CTV ranges from 2-15 % FRET efficiency depending on the methodology acquisition. As FRET standards, fluorescein and TAMRA have clear advantages over the protein standards. Assuming these reference fluorophores are chemically pure, measurements shouldn’t vary from batch to batch since the dye solution can be carefully controlled in contrast to the cell microenvironment. The measured standard deviation of the FRET efficiency in the dye mixture is substantially smaller than the FRET efficiency determined in cells expressing protein FRET standards using FLIM. The large variance in the FRET efficiency of the protein FRET standards is likely due to a confluence of factors related to the complex microenvironment inside cells. The cell environment is dynamic, potentially subject to changes in the refractive index, concentration, local
pH, and/or quenchers. Measuring all of these variables is not feasible for cellular FRET measurements. Thus, any changes to one of these confounding variables will increase the overall variance in the measured FRET efficiency. Without knowing how to parse these individual components that collectively contribute to the overall measurement variance, the instrumentation or methodological error is nearly impossible to determine without additional experiments. However, with the fluorescein-TAMRA dye mixture the largest source of error in the FRET efficiency estimation will likely be the instrumentation. This could allow researchers to estimate the maximum level of precision in their FRET efficiency measurements.

Validation using fluorescent protein FRET Standards

In addition to the simulations carried out above, 3T3 cell cultures were transfected with plasmid DNA encoded to produce soluble Cerulean-Venus FRET constructs with a known FRET efficiency. Analysis of the C32V FRET standard is shown in detail in this section, while results for other FRET standards (C5V, and CTV) are provided in Table 2. Spectral images of the same cells were acquired using both 405 and 458 nm wavelength laser lines. Two photon excitation at 850 and 920 nm was also used to characterize all three FRET standards provided in Table 2. In addition to the raw images, images using a Gaussian blur filter with $\sigma = 1$ and 3 pixels was used to generate images with reduced spectra noise, as shown in Figure 20A. Using each of the registered images and the $\gamma$ term determined from the literature ($\gamma = 0.045$), the FRET efficiency is calculated on a per-pixel basis as shown in Figure 20B. Finally, the normalized fit residual calculated for each pixel is used to determine the expected error in the FRET efficiency on a per pixel basis (Figure 20C), based on the simulated standard deviation vs residual curve shown in Figure 8C.

In their characterization of these same C32V standards, Koushik et al [53] re-
ported a FRET efficiency range of $29.5\pm8.0$ using sRET, and $33.3\pm4.5$ using FLIM-FRET (mean±std of 60 and 10 cells respectively). The measured FRET efficiency of the pixels in Figure 4B were $33.3\pm14.2$, $34.1\pm2.7$, and $34.2\pm1.6$ (mean±std of individual pixels) for the raw, $\sigma = 1$, and $\sigma = 3$ images, respectively, which fall within the range of reported FRET efficiency.

Since the C32V FRET construct should have a spatially uniform efficiency, we are able to aggregate pixels to determine the standard deviation as a function of the normalized fit residual as shown in Figure 21A. There is strong agreement with the simulated standard deviation where there are a large number of pixels at that residual level to estimate the experimental standard deviation (Figure 21B). The curves diverge at the tails of the pixel histograms where there are much fewer pixels to calculate the standard deviation.

2.4.7 Comparing steady-state sensitized emission FRET using Sensor-FRET with fluorescent-lifetime Imaging (FLIM) using protein FRET standards

In principle, the FRET efficiency estimated by steady-state sensitized emission measurements should be identical to the FRET measured by lifetime imaging. This was approximately the case from the experimental results outlined in section 2.4.2 and summarized in Table 2. While fluorescent energy transfer measured by FLIM is considered the most accurate and simple way to quantify FRET (compare Equation 2.26 with section 2.4.2), there are no publications that quantitatively compare these methods side-by-side using the same instrument and same samples. More specifically, there is 1) a lack of literature quantifying whether sensitized emission and FLIM are equivalent measures of FRET and 2) how precise these methodologies are relative to each other.
To directly compare the two methodologies, both the fluorescent lifetime and spectral emission of the same live 3T3 cells expressing various protein FRET standards were measured on a two-photon microscope with a spectral detector and FLIM detector equipped for time-correlated single photon counting (TCSCP). In these experiments, it was expected that the FRET efficiency would be similar to those previously published [53] and approximately match those determined in Section 2.4.2.

To calculate the FRET as measured by FLIM (FLIM-FRET), the donor lifetime was determined in two different ways. First, the average lifetime of the donor was fixed to the lifetime determined by the literature value for these standards that was published by the Vogel lab [53]. In the second approach, the lifetime of the donor was fixed to the measured average lifetime of the C5A (Cerulean-5aminoacids-Amber) construct. This construct is identical to the C5V (Cerulean-5-Venus) standard except that Venus was mutated to Amber (Tyrosine 67 to Cysteine) such that it longer emits photons. In the literature it was reported that the difference between the fluorescent lifetime of C5A and Cerulean-alone was small (200 picoseconds) but significant [53]. Compared to Cerulean-alone, the C5A is structurally more similar to the protein standards in terms of size and composition. It was also reported that all FRET standards with the amber mutation (C5A,C17A and C32A) had statistically indistinguishable lifetimes. For this reason, the measured donor lifetime and emission were determined from C5A only.

Using the literature donor lifetime ($\tau = 2.75\,\text{ns}$), the average FRET efficiency for C5A as measured by FLIM was approximately 25% (Figure 24A). On the same sample with identical cells in view, SensorFRET measured 0% FRET efficiency on average (Figure 24A,25). This was expected because the donor emission shaped was determined from the C5A sample, so by definition this sample should have a mean value of 0% FRET. Spectral images of the same sample were aggregated into a single
mean spectra and analyzed by SensorFRET (Figure 23). The SensorFRET Beta Fit, a direct measure of the magnitude of the acceptor, had near zero amplitude (Figure 23-Beta Fit). There was essentially no detectable Venus emission in the C5A sample. The absence of venus emission confirmed: 1) the presence of the dark mutation (amber construct) and 2) that no energy transfer was measurable by steady-state emission (see Figure 23-Unmixed Components-Acceptor Fit). The variability of C5A FLIM-FRET was roughly 50 times greater than the FRET efficiency variance measured by SensorFRET (see Figure 26-red points) according to the median absolute deviation. The median absolute deviation was used instead of the standard deviation due the presence of a few extremely large outliers which were excluded from Figures 24A,24B because they did not fit into the plotting range. If the donor lifetime was computed as the mean lifetime of C5A ($\tau = 2.04\, ns$), then the mean FLIM-FRET measured 0% FRET by definition (Figure 24B). Regardless of how the donor lifetime was computed, the measurement variance of FLIM-FRET was significantly higher in the C5A sample as shown by the interquartile range and the median absolute deviation when compared to steady-state sensitized emission (Figures 24A-B and 27). Therefore, some factor(s) unrelated to FRET must have contributed to the variability of the FLIM measurements.

A large discrepancy between SensorFRET and FLIM-FRET was also observed in the CTV sample using the literature donor lifetime (Figure 25 and 24A). If the donor lifetime was fixed to the mean C5A lifetime, then the FRET efficiency difference between SensorFRET and FLIM-FRET decreased. However, the difference between each construct remained significantly different ($p < .001$) according to ANOVA or Kruskal-Wallis H-tests between all pairings except the C5V standard where the donor lifetime was set to the literature value (Figures 24A,24A,27,27). The differences between SensorFRET and FLIM could not be explained by error in the steady-
state emission measurements. As an example, the average overall spectra from C32V showed donor and acceptor fits that were nearly identical to the observed spectra (Figure 22). The Beta Fit (used to compute acceptor direct excitation) from this analysis showed near perfect registration with the measurement (Figure 22). Similar results were obtained for CTV, C5V, and C17V.

For all constructs examined, as the linker lengths decreased the measured FRET efficiency increased as predicted. In addition, the difference between SensorFRET and FLIM diminished (Figure 26). This was clearly noticeable for C17V and C5V if the literature donor lifetime was used to compute the FRET efficiency (Figure 25, 27).

**Application to Tension Sensors**

Although biosensors based on unimolecular FRET constructs have a wide range of applications, they have been shown to be particularly useful in understanding how inter and intra-cellular forces affect biological processes. In this section, a method to generate force maps from spectral imaging of MDCK cells expressing force sensitive unimolecular Teal-Venus FRET constructs (TV40) is outlined [46].

To determine the FRET efficiency in both the loaded and unloaded conditions, two cell lines were developed with TV40 FRET sensors integrated into the E-cadherin [52]. The first of these, denoted TL for tailless, generates TV40 labeled E-cadherin proteins which cannot attach to the rest of the cytoskeleton, preventing any stress from being applied to the sensor. The average FRET efficiency determined from these cells gives a measure of the FRET efficiency of the sensor in the unloaded condition. The second of these, denoted TS for tension sensor, function similarly to native E-cadherin but transfers load through the FRET construct, allowing any decrease in FRET efficiency relative to the TL sample to be interpreted as increased force on the
E-cadherin proteins. Since the linker protein that separates Teal and Venus in TV40 behaves as an elastic spring[47], decreases in FRET efficiency in the TS sample can be transformed to changes in force on E-cadherin if we assume the change in FRET efficiency is a result of changing distance between Teal and Venus.

As with the Cerulean-Venus FRET standards, spectral images were acquired at 458 and 405 nm excitation wavelengths. These images, in conjunction with the $\gamma$ term determined from literature [69]($\gamma = 0.101$ for the Teal-Venus/405-458nm fluorophore/excitation combination), enables the calculation of FRET efficiency on a pixel by pixel basis as shown in Figure 29B and 29G. Since the FRET efficiency is not spatially uniform (in contrast to the Cerulean Venus FRET standards), in order to determine the expected standard deviation, residual vs standard deviation curves (analogous to Figure 8 C) were simulated over a range of FRET efficiencies, as shown in Figure 28. Interpolation of these curves allows the expected standard deviation to be determined for any given pixel as a function of the measured FRET efficiency and normalized fit residual, as shown in Figure 29C and 29H. It is clear from these plots that the blurring procedure does not affect the measured FRET efficiency on average, but significantly reduces the standard deviation of the individual pixel measurements. One of the main advantages of this approach is that it allows the user to quantify their measurement error and reduce it using blurring (or other filtering methods) until it reaches a level which is acceptable for their particular experimental requirements. In this particular application, we aimed to reduce the FRET pixel standard deviation below 0.02 to spatially resolve forces on E-cadherin with statistical significance. The blurring was increased until this condition was met, finally requiring a 5 pixel gaussian blur as shown in Figure 29F-J.

In order to convert the measured FRET efficiency to force, the mechanical response of the peptide chain linking the two fluorophores must be known. For the
particular peptide linker used in the TV40 FRET construct, the FRET efficiency vs load behavior was characterized using optical tweezers to apply loads to single molecules [46, 47]. The work by Grashoff et al. used Cy3 and Cy5 fluorophores in single molecule experiments due to their higher brightness and improved signal to noise ratio. Because the Cy3 and Cy5 fluorophores have a different effective fluorophore diameter and Forester radius than the Teal and Venus fluorophores, the FRET efficiency vs load response is scaled such that the mean FRET efficiency of the TL expressing cells (E=0.315) corresponds to 0 pN load and as the load approaches \( \infty \) the FRET efficiency asymptotes towards E=0.

This FRET efficiency vs force calibration curve is shown in Figure 29D and 29I, superimposed on the histogram of the measured FRET efficiencies for the whole image. These plots show that by averaging the pixels in the whole image there is a measurable difference in the FRET efficiency between the TS and TL, corresponding to a 1.77 pN (raw) or 1.73 pN (blurred) force applied to the E-cadherin on average. The force is also able to be calculated on a per pixel basis, as shown in Figure 29E and 29J, for the raw and blurred data, respectively. Drawing strong conclusions about differences in force between regions within a single image is challenging when using the raw data set due to the large amount of scatter, shown in the inset boxplots of Figure 29E generated from the magenta and green regions. In the blurred image, however, the scatter in the same regions is much less than the difference in the mean observed (inset boxplot of Figure 29J), allowing statistically significant differences to be measured when comparing different parts of the cell boundaries.

2.5 Conclusion

It was demonstrated that the SensorFRET analysis allows simultaneous measurements of spectral bleed-through and FRET efficiency on a per-pixel basis using
spectral imaging microscopy. SensorFRET is an improvement over existing FRET imaging methods because it does not require single fluorophore references, assuming the normalized excitation and emission spectra of the sensor fluorophores are known and invariant in the measured environment. If the cellular environment (pH, redox, etc.) leads to significant distortions in the fluorescent emission of the donor or acceptor used, a calibration measurement can be used to calculate $\gamma$ (see Section 2.4.1 for definition) experimentally and correct for any differences in the emission spectra. The validity of this approach was verified by simulations and experimental measurements which used: 1) unimolecular protein FRET standards encoding Cerulean-Venus fluorescent proteins and 2) standard reference dyes that were used as 0% FRET standards first the time.

Noiseless FRET simulations demonstrated the mathematical basis of SensorFRET while simulations with Poisson (i.e., shot) and thermal noise showed the accuracy and precision of SensorFRET was indistinguishable from luxFRET, pFRET, and sRET. In theory, any FRET pair and excitation wavelengths could be used with this method, however, in practice the excitation wavelength pair must be chosen such that sufficient donor fluorophore brightness can be achieved at both wavelengths to improve signal to noise (Figure 14). Competing with this requirement, however, is the limitation that as $\gamma$ approaches 1, any error in the $\gamma$ estimate will have a larger effect on the calculation of the acceptor direct excitation and lead to significant errors in the measured FRET efficiency. This was clearly demonstrated in Section 2.4.5, where it was shown that only a subset of the excitation space yields accurate estimates of the FRET efficiency. In addition to SensorFRET, this limitation applies to all spectral FRET algorithms (Figure 14)

Experiments using the protein FRET standards show that SensorFRET can estimate the FRET efficiency in these constructs with similar accuracy to established
methodologies [53]. Single and two-photon measurements yielded estimates of the FRET efficiency that was well within the expected measurement variance (Table 2). However, it is worth noting that in some circumstances and for unknown reasons (Section 2.4.7) the protein FRET standards yielded completely different FRET efficiencies compared to the results shown in Table 2. The inconsistent FRET efficiency estimates from sections 2.4.6 and 2.4.2 were completely unexpected. Furthermore, a large and statistically significant difference between FLIM and sensitized emission FRET were measured for all protein standards examined. This was in contrast to results obtained by Koushik et. al., where steady-state emission FRET and FLIM-FRET were statistically indistinguishable by ANOVA for C5V, C17V and C32V standards [53]. A reasonable explanation for the difference between FLIM and SensorFRET is that errors in the analysis of the steady-state emission spectra led to divergent estimates of the FRET. However, this explanation was ruled out after the same spectra were analyzed with the LuxFRET algorithm. LuxFRET estimates were nearly identical to SensorFRET. The fact that previous experiments using the exact same cell type (3T3 fibroblasts) and FRET standards (CTV, C32V, C5V) yielded different results (see Section 2.4.6) suggests that variability in DNA transfections or protein expression levels may impact FRET efficiency estimates of the protein standards. Alternatively, the physiological state of transfected cells could be radically different from batch to batch. From a practical point of view, a typical researcher measuring FRET does not have the time or money to spend on determining the causes of measurement variance in a biological standard. An ideal standard should have an invariant output measurement such that methodological errors can be ruled out. Using this criteria, the existing protein FRET standards are far from ideal.

Another surprising result from the protein FRET standards was that FLIM-FRET estimates consistently showed higher measurement variance and a different
mean value of FRET efficiency when compared with SensorFRET. Much of the variance in the fluorescent lifetime measurements could not be explained by variations in the FRET efficiency alone. Large fluctuations in the lifetime of the donor-only sample relative to the steady-state emission of the same sample revealed that something other than the average molecular distance was impacting the measurement. While FLIM-FRET and sensorFRET were highly correlated in general, this correlation tended to breakdown dramatically with the CTV construct. CTV was created as a low FRET construct since the known molecular distance across the TRAF domain linker and the fluorophores is a minimum distance of least 80 Angstroms [44]. Assuming a forster radius of 54 angstroms [69] for Cerulean and Venus, the maximum expected FRET efficiency in this construct is 8.6% (using Equation 2.27) which is consistent with FLIM estimates. Alternatively, the maximum predicted distance between Cerulean and Venus could be as high as 100-110 Angstroms with a predicted FRET efficiency of roughly 1.4-2.4%. These efficiencies are consistent with sRET, LuxFRET and SensorFRET estimates of the CTV construct. Without a crystal structure of CTV, the actual average distance between Cerulean and Venus will remain undetermined and it remains unclear which methodology is a more accurate measure of distance.

$$E = \left(1 + \left(\frac{R}{R_o}\right)^6\right)^{-1}$$  \hspace{1cm} (2.27)

After a series of confusing results with the protein FRET standards, it was determined that 1) the variability in replicate experiments and 2) the large differences between FLIM and steady-state emission measurements warranted the development of a fluorescent dye-pair FRET standard. A series of experiments outlined in sections 2.4.5-2.4.6 confirmed that fluorescein and TAMRA are an excellent 0% FRET efficiency standard. Measurements using single photon spectral microscopy, single-photon fluorometry and two-photon fluorescent lifetime imaging all demonstrated
a lack of energy transfer using this dye mixture at 2uM. Two-photon spectral microscopy was attempted as well. Unfortunately, the 2P-excitation spectrum was substantially red-shifted to the point that an acceptable SNR could not be obtained. Higher concentration mixtures (50uM) appeared to show energy transfer (≈8%) according to FLIM and steady-state emission measurements (Figure 70). However, due to substantial changes in the absorption band of the donor (Fluorscein) after mixing the dyes together, a quantitative analysis was not feasible due to large fitting errors. It is possible that higher concentration dye mixtures could be used as a positive FRET standards, however a better understanding of the complex changes to the absorption spectrum would be necessary. Unlike the protein standards, the fluorescent dyes showed little measurement variance in recordings of either lifetime or steady-state emission measurements. In addition, the preparation of the fluorescent dye mixtures were easier and faster than transfecting live-cells with the protein standards. Since the concentration of dyes can be carefully controlled, measurements of the dye mixtures should be more reproducible than the protein standards, as was the case in sections 2.4.5 and 2.4.6.

Finally, it was shown that combining the SensorFRET method with a novel noise model, piconewton scale forces could be measured on the force bearing cell junction molecule E-cadherin in MDCK epithelial cells with known measurement uncertainty on a per pixel basis. The data show that E-cadherin molecules in MDCK cells have a median resting force of 1.5 pN ±4.8 pN. Applying a 5-pixel radius gaussian blur reduced the standard deviation of force to ±0.5 pN, enabling statistically significant differences to be spatially resolved in a single image. By greatly simplifying the experimental requirements for quantitative FRET determination, the SensorFRET approach allows this nano characterization technique to be accessible to a much broader range of the research community.
Fig. 6. Fitting procedures for calculating the FRET efficiency on simulated FRET spectra with 35% transfer efficiency. A) Fitting of the donor contribution to determine \( \alpha \), B) Fitting of the \( \beta \) term, with the calculation shown in the legend C) Subtraction of the acceptor direct excitation (Removal of cross-talk), D) Linear unmixing of the donor and acceptor components in the corrected spectra.
Fig. 7. Determination of an accurate noise model. A) Signal amplitude and signal noise as a function of emission wavelength in Cerulean-only sample. B) signal noise vs the square root of the amplitude. C) comparison of experimental and simulated noisy pixels.
Fig. 8. Comparison of noise tolerance of the SensorFRET method to sFRET, luxFRET, and pFRET. No appreciable difference between the methods is observed. A) FRET efficiency vs normalized fit residual for 60000 simulated pixels (3000 pixels at 20 signal to noise ratios). B) Median estimate for each of the methods as a function of residual. C) Standard deviation for each of the methods as a function of residual.
Fig. 9. Fluorescent Dye FRET Simulation: Using G. Weber’s model for concentration depolarization in solution (see Section 2.4.5, Equation 2.23), the change in fluorescent polarization is plotted against the concentration of fluorophore in logscale Molarity. Vertical lines represent the concentrations examined in this study with the expected FRET at these concentrations printed in the legend. Approximate Overlap Integral $J(\lambda)$ of Fluorocein and TAMRA. Using fluorometry data shown in Figure 10B-C, the overlap integral is highlighted in grey. This area can be used to compute $J(\lambda)$, which must be greater than zero in order for FRET to occur. The emission and absorption curves were normalized to unit area. The average emission and absorptions spectrums were computed from the fingerprints shown in Figure 10B-C.
Fig. 10. Fluorometer Excitation-Emission Fingerprints of fluorescein (left image) and TAMRA (right image): Single-dye solutions were diluted in PBS(pH7) to 2μM and recorded at room temperature with a fluorometer in 3D mode. Excitation and emission wavelengths were sampled with 5 and 2 nm spacing spanning from 400-600 and 450-750nm respectively. The fluorescent intensity [Au] was color coded in both images with a scale bar shown to the right of the plot. Single-Dye excitation and emission spectra from Figure 10B-C were normalized to unit volume and summed together in this representation. The FRET component was synthesized from the single-dye spectra shown in Figure 11A according to Equation 2.1. The text labels for each component are printed on the excitation-emission peaks. In contrast to emission spectra at a fixed excitation frequency, the FRET component can be clearly delineated from the acceptor peak in the excitation-emission space.
Fig. 11. Fitting Process for the deconvolution of 3D spectra. A) The average normalized excitation and emission spectrums for fluorescein (donor) and TAMRA (acceptor) were computed from data shown in Figure 10. B) Entire excitation-emission spectrum of dye components concatenated end-to-end into a 1-dimensional vector. Each Dye component is separately color-coded and the 1uM mixtures of fluorescein and TAMRA is shown in purple. C) Non-negative least squares fitting of the dye components (dotted green line) plotted in B overlaid with the measured mixed spectra of fluorescein and TAMRA (solid Blue line).
Fig. 12. Verification of the Invariance of fluorescein and TAMRAs Excitation and Emission Spectra Shape. Unit area normalized excitation and emission cross-sections of single dye excitation-emission matrices from Figure 10A. Several spectra around the peaks of the excitation and emission bands (see Figure 10) showed little shape variance. Each excitation or emission spectra are plotted on top of each other with corresponding labels in the legend on the right portion of the graph. These spectra confirm the assumptions necessary to generate the FRET spectra from the averaged components in Figure 11A-B. Details on the independence of excitation and emission spectra are well established in the literature with few exceptions [55].
Fig. 13. Measured and Modeled Excitation-Emission Spectrum of a Mixture of fluorescein and TAMRA. A) Unmixing components detailed in Figure 11 were used to fit a 2uM mixture of fluorescein and TAMRA (B) with a linear least-squares model. The percentage weightings for each component (fluorescein=62%, TAMRA=38%, FRET=0%) are printed on the peaks of image. B) The measured mixture of 2uM fluorescein and TAMRA.
Fig. 14. Excitation Pairing Matrices. Excitation-emission recordings shown in Figure 10 were used to generate pairs of emission spectra at two different excitation frequencies: $F_1(\lambda_{ex1}, \lambda_{em}), F_2(\lambda_{ex2}, \lambda_{em})$, where $F_1$ is the spectra at excitation frequency 1, $\lambda_{ex1}$ the fixed excitation frequency 1, and $\lambda_{em}$ the entire emission spectrum. Each pair of spectra were used as inputs into the spectral unmixing algorithms (LuxFRET -A, sRET-B, and SensorFRET-C). Every possible paired input spectra were used to output an estimated FRET efficiency that is color coded in each matrix. Ideally, all pixels would register 0% FRET (green), however certain excitation pairs have poor signal to noise ratios. Each algorithm showed different sensitivity to the various signal to noise ratios and all failed at the red-edge of the spectrum.
Fig. 15. SensorFRET Unmixing of 2uM Dye Mixture using 1-Photon Spectral Imaging: fluorescein and TAMRA were mixed together at 1uM concentrations and imaged on glass slides using spectral mode. Pixels were averaged to form a single spectra per image. Alpha-Fit) Raw spectra (solid or dotted red) at two excitation frequencies ($F_{DA}^1$ and $F_{DA}^2$) were fit to the fluorescein component shape (solid or dotted blue). Beta-Fit) The magnitude of the direct excitation of TAMRA was estimated with the fitting of the TAMRA component shape (dotted red) with solid blue line defined in the legend. C) Using the Beta Fit, the direct excitation was subtracted from the raw spectra (red) to generate the corrected spectra (teal). D) Corrected spectra (teal) were unmixed from the fluorescein (solid blue) and Tamra (solid yellow) component shapes. The approximately zero magnitude acceptor component (solid yellow) yielded a FRET efficiency estimate of -0.02%.
Fig. 16. Estimation of the Fluorescent Lifetime of fluorescein: Lifetimes were captured on an inverted microscope using time-correlated single photon counting (TC-SCP). Stock fluorescein was diluted in sodium borate buffer (pH 9) to a final concentration of 50uM and imaged as a droplet on glass slides. A) global (256x256) or B) local (10x10) binning of lifetime images were used to aggregate photon counts into two separate decay traces (Photon Counts: $N_A=5.8E6$ and $N_B=4.4E5$). Non-linear least squares fitting a single exponential decay model (red) yielded a decay constant of A) 4.19ns or B) 4.13ns. The measured instrumental response function (IRF) is shown in black and the goodness of fit ($\chi^2$ statistic) measured A) 13.28 and B) 0.98 respectively. Fit residuals are plotted below each decay trace in blue.
Fig. 17. fluorescein and TRITC Fluorescent Lifetime and FRET Efficiency Distributions at 1-2μM Concentration: Lifetimes were captured on an inverted microscope using time-correlated single photon counting (TCSCP). A) Fluorescent lifetime distributions for fluorescein (FL-Blue), fluorescein and TRITC at 1μM (FL-TRITC-1μM-Green) and fluorescein and TRITC at 2μM (FL-TRITC-2μM-Red). Means of the lifetime distributions are shown as vertical dotted lines corresponding to the histogram color with averages printed in the legend. B) Inferred FRET efficiency for 1 and 2μM mixtures based on the lifetime distributions in A that were calculated according to equation 2.26.
Fig. 18. fluorescein and TRITC Fluorescent Lifetime and FRET Efficiency Distributions at 25-50uM Concentration:

Lifetimes were captured on an inverted microscope using time-correlated single photon counting (TC-SCP) A) Fluorescent lifetime distributions for fluorescein at 50um, pH=9 (FL-Blue), fluorescein at 50um, pH=7 (FL-Green), fluorescein and TRITC at 25uM (FL-TRITC-1uM-Red) and fluorescein and TRITC at 50uM (FL-TRITC-2uM-Purple). Means of the lifetime distributions are shown as vertical dotted lines corresponding to the histogram color with values printed in the legend. B) Inferred FRET efficiency for 25 and 50uM mixtures based on the lifetime distributions shown in A that were calculated according to equation 2.26
Fig. 19. fluorescein-only Fluorescent Lifetimes: Concentration vs pH. Lifetimes were captured on an inverted microscope using time-correlated single photon counting (TCSCP). Fluorescent droplets of fluorescein were diluted to 50uM in either PBS (pH7, Green) or sodium borate (pH9, Blue). In addition, fluorescein was diluted to 2uM in PBS (pH7, Red). Pixel-wise lifetime estimates were computed using a single-exponential decay model and aggregated into histograms using the ipython notebook.
Fig. 20. Colormap images of cells expressing the C32V FRET standard excited at 458nm with different levels of Gaussian image blurring. Row A) shows the peak intensity of the spectra for each pixel. Row B) shows the calculated FRET efficiency for each pixel. Row C) shows the expected standard deviation of the FRET efficiency for each pixel based on the residual.
Fig. 21. Comparison of experimental efficiency error to simulated behavior. A) Shows FRET efficiency vs normalized fit residual for all of the pixels in each of the images. B) Comparison of experimental and simulated standard deviations showing strong agreement between the two. The experimental standard deviation estimates are only valid where there is a significant number of pixels to estimate it with so corresponding histograms are provided on the secondary axis.
Fig. 22. Sensor FRET Unmixing of C32V, Two-Photon Spectral Imaging vs FLIM: Panel plot of the SensorFRET unmixing procedure on the C32V construct using 850nm and 920nm excitation frequencies (see Figure 15 for details on the fitting procedure). Alpha Fit) Donor fitting (blue) for 850nm (solid red) and 920nm (dotted red) raw spectra. Beta Fit) Fitting of the magnitude of the acceptor component (dotted red) with $F_{DA}^2 - \text{Alpha} \times F_{DA}^1$ (solid blue). Subtraction of Direct Excitation) Corrected spectra without direct excitation (teal) plotted with raw spectra (red). Unmixed Components) Linear unmixing of corrected spectra (teal) using donor(Cerulean-Blue) and acceptor(Venus-yellow) component shapes.
Fig. 23. Sensor FRET Unmixing of C5A, Two-Photon Spectral Imaging vs FLIM: Panel plot of the SensorFRET unmixing procedure on the C5A construct using 850nm and 920nm excitation frequencies (see Figure 15 for details on the fitting procedure). Alpha Fit) Donor fitting (blue) for 850nm (solid red) and 920nm (dotted red) raw spectra. Beta Fit) Fitting of the magnitude of the acceptor component (dotted red) with $F_{DA}^2 - Alpha \times F_{DA}^1$ (solid blue). Subtraction of Direct Excitation) Corrected spectra without direct excitation (teal) plotted with raw spectra (red). Corrected spectra (teal) curves are overlaid on top of the raw spectra (red). Unmixed Components) Linear unmixing of corrected spectra (teal) using donor(Cerulean-Blue) and acceptor(Venus-yellow) component shapes.
Fig. 24. Comparing FLIM and SensorFRET Pixels: Images were captured at 20X magnification using TCSPC and two-photon spectral imaging modes on the same field of view with approximately 50 to 100 cells measured for each construct. Five protein FRET standards (C5A, CTV, C32V, C17V and C5V) were imaged using live samples on a heated stage. SensorFRET and FLIM-FRET estimates are shown in red and blue boxplots respectively with box-widths displaying the interquartile range and center marking for the median. A) FLIM-FRET efficiency estimates used the published C5A lifetime as the donor-only reference [53]. B) FLIM-FRET efficiency estimates used the measured mean C5A lifetime.
Fig. 25. SensorFRET and FLIM using Comparative Images: Images were captured at 20X magnification using TCSPC and two-photon spectral imaging modes on the same field of view of live 3T3 fibroblasts. Pixels were converted to FRET Efficiency according to the SensorFRET unmixing algorithm or according to Equation 2.26. SensorFRET pixels were down-sampled using linear interpolation to match the image resolution of the FLIM-image and were registered using cross-correlation. **Top Row** Cells expressing CTV protein processed from spectral images using SensorFRET (Top-left) or FLIM traces using SPCI software (Top-right). **Bottom Row** Cells expressing C17V protein processed from spectral images using SensorFRET (Bottom-left) or FLIM traces using SPCI software (Bottom-right)
Fig. 26. SensorFRET and FLIM using Comparative Pixels: Images were captured at 20X magnification using TCSPC and two-photon spectral imaging modes on the same field of view of live 3T3 fibroblasts. FRET Efficiency pixels from registered SensorFRET (y-axis) and FLIM (x-axis) images (as shown in Figure 25) represent each point in the scatterplot. Each axis is reported in units of FRET Efficiency and point colors encodes the protein FRET constructs.
Fig. 27. Comparing FLIM and SensorFRET Pixels: Images were captured at 20X magnification using TCSPC and two-photon spectral imaging modes on the same field of view with approximately 50 to 100 cells measured for each construct. Five protein FRET standards (C5A, CTV, C32V, C17V and C5V) were imaged using live samples on a heated stage. SensorFRET and FLIM-FRET estimates are shown in blue and green boxplots respectively with errorbars displaying the median absolute deviation and bar heights representing the median FRET Efficiency. A) FLIM-FRET efficiency estimates used the measured C5A mean lifetime as the donor-only reference. B) FLIM-FRET efficiency estimates used the published mean C5A lifetime [53].
Fig. 28. Series of simulated FRET standard deviation vs Normalized Fit Residual curves for .1 FRET efficiency steps. At each simulated FRET efficiency, 1000 pixels were used to compute the standard deviation and the average normalized residual of the non-negative least squares fit. To generate each curve, Equation 2.1 was used and poisson noise was added based on the noise model shown in Figure 7
Fig. 29. SensorFRET analysis of MDCK cells expressing TV40 unimolecular FRET constructs incorporated into E-cadherin transmembrane proteins (denoted TS). A-E show analysis for the raw data while F-J show analysis results for the same data after a 5 pixel Gaussian blur. Blurring of the image has minimal effect on the mean FRET efficiency or load but significantly reduces the error associated with any given pixel. This allows differences to be discerned between regions within a single image.
CHAPTER 3
MEASURING FORCES APPLIED TO THE OUTER NUCLEAR MEMBRANE IN LIVE CELLS

3.1 Abstract

The nucleus of a cell has long been considered to be subject to mechanical force. Despite the observation that mechanical forces affect nuclear geometry and movement, how forces are applied onto the nucleus is not well understood. The nuclear LINC (linker of nucleoskeleton and cytoskeleton) complex has been hypothesized to be the critical structure that mediates the transfer of mechanical forces from the cytoskeleton onto the nucleus. Previously used techniques for studying nuclear forces have been unable to resolve forces across individual proteins, making it difficult to clearly establish if the LINC complex experiences mechanical load. To directly measure forces across the LINC complex, a fluorescence resonance energy transfer-based tension biosensor was generated for Nesprin-2G, a key structural protein in the LINC complex, which physically links this complex to the actin cytoskeleton. Using this sensor, the observed FRET changes show that Nesprin-2G is subject to mechanical tension in adherent fibroblasts, with highest levels of force on the apical and equatorial planes of the nucleus. In addition, the Nesprin-2G sensor was shown to respond to pharmacological treatments that modulate actomyosin contractility.

3.2 Introduction

Although a large body of evidence suggests the cytoskeleton exerts forces on the nucleus, there is a lack of understanding as to which proteins transmit this force
onto the nucleus. A specific understanding of the transmission of force from the cytoskeleton to the nucleus may help researchers resolve mechanical changes to the nucleus that potentially influence global gene transcription. Uncertainty around the force transmission from the cytoskeleton to the nucleus is largely explained by a lack of techniques capable of measuring these forces in living cells.

Recently, a genetically encoded, calibrated **Forster Resonance Energy Transfer (FRET)**-based tension biosensor (named TS-mod) was developed [46]. This sensor consists of a linear-elastic peptide spring flanked by a FRET compatible fluorescent protein pair. FRET is a fluorescent-based phenomena that is typically used as a 'spectroscopic ruler' in chemical and biological studies. It is highly sensitive to distance changes on a scale of 1 to 10nm [70].

Using single molecule fluorescent techniques, it was shown that the TS-mod linker behaves as a rod-like, linear elastic spring [47]. This finding was based on calibration measurements where organic dyes ideal for single molecule FRET measurements were attached to the amino and carboxyl ends of TSmod. Using a laser trap to fix one end of the linker and a piezo-driven biotin coated polymer surface on the other, it was shown that the Cy3-TS-mod-Cy5 (F40) FRET changes inversely as a function of force through multiple loading-unloading paths (Figure 30). When the FRET efficiency of Cy3-TSmod-Cy5 was plotted against the applied force, a linear fit could approximate most of the dynamic range of linkers ranging from 25 to 50 amino acids (Figure 31).

It is important to note that these calibration measurements were made using organic dyes Cy3 and Cy5. In a live cell experiment, these donor and acceptor molecules are replaced with genetically encoded fluorescent proteins mTFP (Teal) and mVenus (Venus). While these organic dyes (Cy3 and Cy5) have a similar forster radius to teal and venus, they are not identical [69, 71]. The forster radius is a parameter in FRET experiments inherent to the donor and acceptor pair in a given
experimental micro-environment that determines the dynamic range of distances that can be accurately measured. It is also important to note that the physical dimensions of these organic dyes is considerably smaller than the fluorescent proteins. At short distances or low force measurements, it is possible that teal and venus experience steric hindrances that change the FRET force relationship as defined by the calibration curves. For example, if one assumes the minimum distance between teal and venus is approximately 5-6nm due to the physical dimensions of their beta-barrels, then the maximum transfer of energy between the fluorophores should be approximately 50% (assuming the forster radius is 5.5nm). However, the calibration curve for the linker F25 show average transfer efficiencies of roughly 70% at zero force (Figure 31). This example illustrates the total separation distance and transfer efficiency between the organic dyes and fluorescent proteins are not equivalent at zero force. Therefore, the zero-force FRET efficiency values cannot be inferred from the calibration curves shown in Figures 30 and 31. Instead, separate controls that contain teal-venus fluorophores should be used to determine the FRET efficiency value at zero force. Once the zero-force FRET efficiency is determined, the calibration curve intercept can be fixed and the force interpolated at each level of decreasing FRET efficiency (Figure 31).

After single molecule studies confirmed that TSmod could be used as a proxy for tensile forces on proteins, a series of different FRET biosensors were developed to measure mechanical strain across various proposed load bearing proteins in living cells. The first target was the focal adhesion protein vinculin. In this study, TSmod was embedded in between the head domain and tail domain of vinculin. A zero-force control was developed by removing the vinculin tail domain, disrupting binding with actin or paxilin. The FRET efficiency in the zero-force control showed significantly higher transfer efficiency than the full length tension sensor construct. The authors estimated that vinculin was under a resting tension of roughly 2.5pN [46]. Following
the first publication of the TSmod, a series of sensors were developed to measure protein tension across cell-cell contacts, such as VE-cadherin and E-cadherin [72, 73, 52, 74]. To date, no sensor has been developed to measure tension across proteins in the nuclear envelope or lamin in live cells.

To test whether the putative load bearing protein Nesprin2G is under significant myosin dependent tension, we developed a sensor that utilizes the TSmod FRET-force reporter. As predicted in the literature, we expected this sensor would respond dynamically to changes in cellular contractility. We also expected spatial variation in the measured tension, particularly in regions of highest curvature. Finally, a force strengthening response was predicted under conditions of high strain, as observed in focal adhesions.

3.3 Methods

Design of the Nesprin Tension Sensor:
The mouse mini-Nesprin-2G tension sensor was designed based on the previous mini-Nesprin-2G construct [75]. GeneArt Gene Synthesis (Life Technologies) was used to chemically synthesize the mini-Nesprin-2G linker, which consisted of the mini-Nesprin2G sequence, with an XhoI and NotI site linker between the 1-485 N-terminal actin binding CH region and the 6525-6874 C-terminal KASH domain. A previously characterized FRET-based tension sensor (consisting of mTFP1 and venus separated by a 40 amino acid elastic linker, flanked by XhoI and NotI) (13), was inserted between the XhoI and NotI sites of the mini-Nesprin-2G linker. The mini-Nesprin-2G linker was then moved into pcDNA 3.1 (+) using HindIII and EcoRI sites. The headless mini-Nesprin-2G linker was made by digesting the full-length tension sensor with XhoI and EcoRI (eliminating the 1-485 N terminal domain) and inserting into pcDNA 3.1 (-) using the XhoI and EcoRI sites.
Cell Culture:

NIH3T3 mouse fibroblasts were cultured in DMEM with 10% fetal calf serum. Primary human fibroblasts were cultured in DMEM with 10% fetal bovine serum. DNA plasmids were transfected into cells using Lipofectamine 2000 or 3000 (Life Technologies) per manufacturer instructions. In all experiments cells were allowed to adhere to fibronectin coated glass bottom dishes or coverslips overnight before imaging. In indicated experiments cells were treated with 1nM calceulin A (Cell Signaling Technology) and imaged 5 to 20 minutes later. In other indicated experiments cells were treated simultaneously with 10uM Y-27632 (R&D Systems) and 10 uM ML-7 (Sigma) to reduce myosin activity and imaged 30 to 45 minutes later. Additional experiments were performed with Rho Activators I and II (Cytoskeleton, Inc.) per manufacturer instructions. To examine nuclear positioning and TAN lines, confluent NIH3T3 fibroblasts were subjected to scratch wounding and lysophosphatidic acid (LPA) treatment as previously described [76].

FRET Image Acquisition

Images were acquired from cells grown on glass bottom dishes on an inverted Zeiss LSM 710 confocal using a 458nm excitation wavelength from an argon laser source. A plan-apochromat 63 oil NA 1.4 objective lens was used for all images analyzed. Live cells expressing either soluble teal (mTFP1) or venus were imaged in spectral mode using a 32-channel spectral META detector to record the spectral fingerprints of each fluorescent protein. After acquisition of the spectral fingerprints, cells expressing the Nesprin tension sensor were imaged using online-unmixing mode in the Zeiss Zen Software. Images were spectrally unmixed into teal and venus channels respectively during acquisition. In each experiment images were captured on the same day, with the gain and laser intensities fixed across all samples. During acquisition, images were captured in 16bit mode and averaged 4 times.
FRET Image Analysis:
First, all images were background subtracted in the teal and venus channels respectively to reduce noise. Second, all saturated pixels were removed. Ratio images were calculated by dividing the unmixed venus channel by the unmixed teal channel. To reduce FRET noise from edge artifacts, pixels with very large FRET ratios (≥20) were removed from analysis. To examine FRET pixels of interest, ratio images were multiplied with binary image masks that outlined the nuclear membrane. Pixels from each experimental group were aggregated and sorted by their fluorescent intensity. To remove the influence of acceptor bleed through on the fret ratio, only intensity-sorted fret ratio pixels were compared across all experimental groups. Sorted pixels were binned into discrete intensity ranges and the average fret ratio in a given intensity range were compared, using an algorithm based on Chen et al [41].

3-Dimensional FRET Analysis:
On a per cell basis, z-stack confocal images were acquired as spectrally unmixed teal and venus images using Zeiss Zen software. In each cell z-stack, top, middle, and bottom image planes were extracted. For each image plane, binary masks were manually created in ImageJ to exclude pixels outside of the cell nucleus as determined by the transmission channel. Ratio fret images were calculated by dividing unmixed venus images by unmixed teal images after background subtraction. The median of each ratio image was computed for each image plane (top, middle, and bottom). To determine the relative force distribution on a per cell basis, the fret indices were normalized by the average of the medians for the top, middle, and bottom image planes of each cell.

Immunocytohistochemistry:
Primary human fibroblasts or NIH3T3 cells were grown on fibronectin coated glass coverslips and fixed using 4% paraformaldehyde or methanol as indicated. Cells were
permeabilized with 0.1% Triton X and stained with rabbit anti-Nesprin2G (19) (gift of Gregg Gundersen), mouse anti-vimentin (Santa Cruz), or -tubulin (Iowa Developmental Hybridoma Bank, gift of Amanda Dickinson), and detected by Alexa Fluor secondary antibodies (Life Technologies). Actin was labeled using rhodamine phalloidin (Cytoskeleton). Nuclei were counterstained using Hoechst 33342 (Life Technologies). Slides were mounted and images collected using a Zeiss 710 LSM confocal microscope.

Statistics:
Sorted pixels, binned into discrete intensity ranges, were analyzed for statistical differences using t-test (for groups of 2) or ANOVA with NewmanKeuls post hoc test. Data was considered significant for p values less than 0.05. Data on graphs are presented as mean +/- standard error.

3.4 Results

3.4.1 Developing a mini-Nesprin 2G FRET-force tension sensor

Using the FRET tension sensor module described in section 3.2, we developed a Nesprin tension sensor that directly measures mechanical tension applied to the LINC complex. Nesprin-1 and -2 are the principal isoforms that connect actin to the LINC complex, and we hypothesized that a Nesprin-2 biosensor could be used to measure actomyosin forces applied across the LINC complex. The Nesprin-2 FRET-based tension sensor (Figure 32A) was based on a previously developed, artificially shortened form of Nesprin-2G, known as mini-Nesprin [75]. Mini-Nesprin 2G was previously shown to function similarly to endogenous Nesprin 2G (including supporting actin-dependent nuclear movement) [75, 76], supporting its suitability as an artificial sensor for measuring Nesprin-2G forces.
3.4.2 Validating the biological function of the Nesprin-2G FRET sensor

When expressed in NIH3T3 mouse fibroblasts the Nesprin tension sensor localized to the nuclear membrane (Figure 32C), similar to wild-type Nesprin (Figure 33A and stlund et al. (2009)). We also generated a headless control (Figure 32) in which the actin-binding domain of Nesprin was removed; this sensor also localized to the nuclear membrane. A major difference between the tension sensor and headless control was that only the tension sensor was organized into fibers in the cytoplasm (Figure 34). This expression pattern is consistent with endogenous Nesprin (Figure 33A) and suggests that the ability to associate with actin is preserved in the Nesprin tension sensor. Furthermore, we observed that actin was associated with the nuclear membrane in cells expressing the Nesprin tension sensor, whereas actin localization to the nuclear envelope was reduced in cells expressing the headless control (Figure 35) further indicating that the Nesprin-actin interaction is preserved in the biosensor.

To determine if over-expression of the sensor induced a dominant negative KASH (Klarsicht, ANC-1, Syne Homology) phenotype, in which nuclear-cytoskeletal connections are disrupted [77], we examined if expression of the tension sensor altered vimentin and -tubulin morphology at the nucleus. We did not observe any significant morphological changes in vimentin or -tubulin staining at the nucleus for cells expressing the Nesprin-2G tension sensor as compared to untransfected cells (Figure 36 and Figure 37); nevertheless, high levels of expression of the Nesprin-2G sensor may act like a dominant negative KASH, disrupting intermediate filament and microtubule interactions with the nuclear membrane. We also confirmed that the Nesprin-2G tension sensor rescues wound-induced rearward nuclear movement in fibroblasts depleted of endogenous Nesprin-2G (Figures 38A, 40,41) [78]. Finally, we observed that the Nesprin-2G tension sensor is incorporated into TAN lines in wounded fibroblasts (Fig-
ure 42A-B) (Luxton et al. (2010)). Because the Nesprin-2G tension sensor localizes to the nuclear membrane, interacts with actin, rescues nuclear movement, and incorporates into TAN lines we conclude it retains similar biological function to endogenous Nesprin-2G.

3.4.3 Measuring Nesprin-2G tension in live cells with modulated contractility

To establish a baseline level of tension, we examined the FRET of the Nesprin-2G tension sensor as compared to the headless control (Figure 32A). Initially we limited our analysis to the equatorial plane in all samples (the plane where the diameter of the nuclear ring was the largest). In NIH3T3 cells the Nesprin-2G tension sensor had significantly reduced FRET compared to the force-insensitive headless control (Figure 32B-C), indicating that the Nesprin2G sensor is subject to mechanical force. We also observed that Nesprin-2G sensor is subject to mechanical force in bovine aortic endothelial cells (unpublished observation).

Next we examined the distribution of force on the apical, equatorial, and basal planes of the nucleus. We observed that the FRET at apical and equatorial planes of the nucleus was significantly lower when compared to the basal plane on a paired cell basis (38B). There was no significant differences in FRET between the apical, equatorial, and basal planes on a paired cell basis when using the headless control (38C), suggesting that the tension sensor FRET differences are force-mediated. However, it should be noted that the same trend of decreasing FRET ratios from basal to apical regions of the cell was observed in the headless cells. Because each cell was individually background subtracted, FRET indices were normalized on a per cell basis. Thus, it is not possible to directly compare the FRET indices between tension sensor and headless in these experiments. Because the intensity of the sensor was greatest in the
equatorial plane (resulting in a better signal to noise ratio), only the FRET at the equatorial plane was measured in the remainder of the experiments.

To assess the contribution of actomyosin contractility to Nesprin-2G tension, we treated NIH3T3 fibroblasts with calyculin A. Calyculin A, inhibitor of protein phosphatase 1 and 2, results in increased myosin phosphorylation and contractility [79]. The Nesprin tension sensor in cells treated with calyculin A had significantly reduced FRET as compared to untreated cells (Figure 39A). Treatment of cells with calpeptin, an indirect activator of Rho kinase [80], or a cell-permeable peptide that directly activates Rho kinase [81] also reduced FRET (Figures 43A-D). To reduce resting actomyosin tension, we treated cells simultaneously with Y-27632 and ML-7, inhibitors of Rho (Uehata et al. (1997)) and myosin light chain (Saitoh et al. (1987)) kinases. Cells treated with Y-27632 and ML-7 showed an increased FRET ratio relative to untreated cells (Figure 39A,B). Blebbistatin, a more direct inhibitor of myosin activity, is autofluorescent and phototoxic at 468nm excitation, therefore could not be used in combination with the tension sensor [82, 83].

3.5 Discussion

Using a novel Nesprin-2G FRET-based biosensor, the first direct evidence of mechanical forces being applied across a nuclear membrane protein was demonstrated (Figure 32). It was also shown that force across Nesprin-2G is sensitive to perturbations to actomyosin tension (Figure 39). Because Nesprin-2G has been shown to be one of several Nesprin isoforms that serve as physical linkers between the LINC complex and the actin cytoskeleton [20], our data indicate the LINC complex, and by extension the nuclear membrane, are subject to mechanical loading.

The existence of mechanical force on the nucleus is supported by a number of previous studies that have observed nuclear deformation in response to applied forces.
([84, 85, 86, 87, 88, 77]). The tension sensor approach extends upon these prior findings by identifying a specific protein on the nuclear membrane subject to force. The observation of mechanical tension across Nesprin-2G is in agreement with prior work that reported reduced nuclear deformation in cells with disrupted LINC complexes [77], impaired nuclear movement in cells depleted of Nesprin-2G [76], as well as a recent article showing that Nesprin-2 couples myosin force generation to nuclear translation [89]. The direct measurement of force with a protein-specific biosensor offers a number of advantages over previous methods to study nuclear forces. First, it allows for measurement of forces of cells in their native state, without the use of mechanical perturbations used by other nuclear force estimation methods. Second, the approach avoids the use of gene knockdowns and overexpression of dominant negative KASH proteins; it is possible that knocking down LINC proteins not only disrupts physical connections between the cytoskeleton and nucleus but also a host of intra-cellular signals. Third, the biosensor approach provides protein-specific resolution. Force biosensors can readily be developed for other nuclear proteins to map force transmission onto and within the nucleus. Finally, the sensor can be used to identify spatial differences in Nesprin-2G force (Figure 38B).

Because the FRET sensor is sensitive to the molecular conformation, our results are consistent with a model in which FRET changes of the sensor can be interpreted to represent changes in the mechanical force across Nesprin-2G, albeit in a highly non-linear relationship. Changes in FRET could also be attributed to changes in Nesprin dimerization (creating intermolecular FRET [90] or a loss of sensitivity due to overexpression of the sensor that may result in a high background of zero force sensors). These different competing effects are not straightforward to parse. While changes in dimerization cannot be ruled out to affect FRET measurements, it is
hypothesized that the mini Nesprin-2G sensor has limited potential to dimerize because it lacks many of the spectrin repeats shown to promote Nesprin oligomerization [91]. To avoid dimerization, the sensor was expressed at low levels to prevent loss of sensitivity. This is supported by histogram analysis of FRET which indicated one discernible peak of FRET for each cell (Figure 44). A significant unloaded FRET sensor population would likely result in a second peak on the aforementioned FRET histogram. Furthermore, experiments showed a relationship between FRET and actomyosin contractility, further support the FRET-force relationship of this sensor. Based on these results, the changes in FRET are likely the result of mechanical forces across Nesprin-2G.

Nesprin-2G is believed to primarily interact with actin; therefore these results likely reflect actin-mediated forces applied to the LINC complex. In addition to Nesprin-2G, a number of other isoforms of Nesprin-2 are expressed. Because some of these other isoforms lack either the actin binding domain or the SUN-binding KASH domain, they likely experience different levels of force or no force at all. In addition Nesprin-1 is also connected to actin and may transfer actin-generated forces onto the LINC complex. Intermediate filaments and microtubules, which bind Nesprin-3 and -4, respectively, could also contribute mechanical forces to the LINC complex. Intermediate filaments were shown to mediate nuclear positioning [92], nuclear volume [93], nuclear mechanical homeostasis [87], and nuclear movement during 3D migration [94]. Similarly designed Nesprin-3 and -4 sensors would allow for measurement of intermediate filament and microtubule-based nuclear forces.

Our Nesprin-2G sensor was designed based on the shortened mini-Nesprin-2G, which lacks many of the spectrin repeats that promote Nesprin oligomerization [91], as well as the kinesin binding domain [95]. Gundersen and colleagues have shown that mini-Nesprin-2G behaves similar to endogenous Nesprin-2G, in that it is able to
rescue TAN (transmembrane actin-associated nuclear) lines and nuclear movement in Nesprin-2G depleted cells [75, 76, 96]. This suggests that oligomerization and kinesin-binding are not essential for nuclear-cytoskeletal interactions, and data presented above further demonstrate they are not required for force. The Nesprin-2 sensor could be modified to incorporate the kinesin binding domain to assess the contribution of kinesin to force.

It is anticipated this newly developed biosensor will be an important tool to assess the role of nuclear forces in regulating cellular functions, providing answers to a number of long-held fundamental questions, most notably if changes in Nesprin force can regulate gene expression or nuclear transport. Development of additional tension biosensors for other nuclear proteins that interact with lamin or DNA may be a useful tool to determine if nuclear forces are transmitted inside the nucleus, possibly onto chromosomal DNA. Because nearly every cell contains a nucleus, it is anticipated the ability to measure forces on and within the nucleus will directly enhance the fundamental understanding of cellular biomechanics and mechanobiology, with wider application to the advancement of human health and disease.
Fig. 30. Donor (Cy3-green) and acceptor (Cy5-red) TIRF fluorescent intensity line traces through cyclic loading with force-time plot below. During peak forces, the donor signal reaches a maximum and the acceptor a minimum. Figure from [47]
Fig. 31. A) FRET-force calibration curves for TSmod and various linker lengths. Linker lengths are specified by the number of amino-acids per linker (green-yellow-25, purple-red-40, and pink-blue-50) C) Measured compliances as a function of linker lengths. Figure from [47]

Fig. 32. Nesprin tension biosensor (A) Schematic of Nesprin-2G tension sensor and headless control. (B) The tension sensor had significantly reduced FRET at the nucleus as compared to the force-insensitive headless control, t-test, $p < 0.01$. Bar graphs represent FRET pixels from discrete intensity ranges collected across a minimum of 20 cells per condition. Similar results were obtained for three independent experiments. (C) Nesprin-2G tension sensor and headless control localized to the nuclear membrane in NIH3T3 fibroblasts. The FRET at the nuclear envelope was reduced for the tension sensor as compared to the headless control.
Fig. 33. (A) Nesprin-2G localization in normal (non-diseased) and HGPS fibroblasts. Normal and HGPS fibroblasts were grown on fibronectin-coated glass coverslips, fixed, and stained for endogenous Nesprin 2G. Nuclei were stained with Hoechst. (B) When examined across multiple cells the intensity of Nesprin-2G at the nuclear membrane in HGPS cells was reduced relative to normal cells.

Fig. 34. Live cell images from NIH3T3 cells overexpressing Nesprin-2 tension sensor or headless control. The tension sensor, but not the headless control, organized into linear filament-like structures in the cytoplasm.
Fig. 35. Immunocytochemistry staining of paraformaldehyde fixed NIH3T3 cells showed that actin-nuclear interactions are maintained in Nesprin-2 tension sensor expressing cells but not in cells expressing the headless control. Transfected cells are indicated with white arrows.

Fig. 36. Immunocytochemistry staining of paraformaldehyde fixed NIH3T3 cells expressing Nesprin-2 tension sensor. Immunocytochemistry staining of methanol fixed NIH3T3 cells showed that intermediate filaments as shown by vimentin were similar between tension sensor expressing (green expressing) and untransfected cells.
Fig. 37. Immunocytochemistry staining of paraformaldehyde fixed NIH3T3 cells expressing Nesprin-2 tension sensor. Immunocytochemistry staining of methanol fixed NIH3T3 cells showed that microtubules as determined by alpha-tubulin staining were similar between tension sensor expressing (green cell) and untransfected cells.

Fig. 38. A) Nesprin-2G tension sensor rescues rearward nuclear movement in Nesprin-2G depleted cells subjected to scratch wounding and lysophosphatidic acid (LPA) treatment. (B) The tension sensor relative FRET index per cell at various cross-sections of the nucleus (apical, equatorial, and basal). Significantly reduced FRET was observed for apical and equatorial planes compared to basal (ANOVA, NewmanKeuls post-hoc test, $p < 0.01$). (C) No significant differences were observed for the headless control at various cross-sections of the nucleus (apical, equatorial, and basal).
Fig. 39. (A) NIH3T3 cells expressing the Nesprin tension sensor were treated with either 1nM calyculin A or both 10 μM Y-27632 and 10 μM ML7 and were compared to unstimulated cells (B) Statistical analysis of each condition showed that calyculin A treatment significantly reduced FRET whereas Y27632 and ML7 treatment increased FRET, ANOVA NewmanKeuls post-hoc test, \( p < 0.01 \). Bar graphs represent FRET pixels from discrete intensity ranges collected across a minimum of 20 cells per condition. Similar results were obtained for two independent experiments.
Fig. 40. Images of Nesprin-2G depleted NIH3T3 cells subjected to scratch wounding and lysophosphatidic acid (LPA) treatment. Cells expressing Nesprin-2G tension sensor, but not GFP or headless control, exhibited rearward nuclear movement.

Fig. 41. A) Quantification of nuclear and centrosome movement after wounding. B) Western blot to confirm knockdown of endogenous Nesprin-2G.
Fig. 42. A) Images showing Nesprin-2G tension sensor incorporation into TAN lines, in contrast to the headless control. TAN lines were induced by scratch wounding and LPA treatment. B) Quantification of scored cells that are positive for TAN lines.
Fig. 43. (A) NIH3T3 cells were treated with Rho Activator I or II per manufacturer instructions (Cytoskeleton, Inc.). (B) Both activators of Rho resulted in significantly decreased FRET, ANOVA, NewmanKeuls post-hoc test, \( p < 0.01 \). Bar graphs represent FRET pixels from a discrete intensity range collected across a minimum of 15 cells per condition. Similar results were obtained for two independent experiments.
Fig. 44. Stacked histogram of FRET images from Nesprin-TS population. Each colored histogram represents a single image of a Nesprin-ts expressing nuclei. Data extracted from images shown in Figure 32
CHAPTER 4

MEASURING TENSILE FORCES ON NESPRIN-2G IN SIMULATED PHYSIOLOGICAL STATES.

4.1 Abstract

Using a validated FRET-force biosensor, mechanical tension on Nesprin-2G was observed in patterned fibroblasts, uniaxially and biaxially strained fibroblasts, and in fibroblasts derived from patients with Hutchinson-Gilford progeria syndrome (HGPS). Highly elongated fibroblasts reported lower average FRET and a higher implied tension across Nesprin-2G independent of the curvature of the examined nuclei. Using a novel 3-D printed cell stretcher, 3T3 fibroblasts subject to bi-axial strain had decreased FRET efficiency and increased implied tension on Nesprin-2G that varied with the degree of applied strain. Uni-axial strain did not reveal a consistent relationship between the degree of applied strain and the measured FRET. Finally, HGPS fibroblasts seeded on glass had a higher average FRET ratio relative to control cells, implying decreased tension on Nesprin-2G molecules in these diseased cells.

4.2 Introduction

As described in 3.4, a dynamic, FRET-based tension sensor embedded in Nesprin2G was developed and validated [arsenovic2016Nesprin]. This sensor showed that tension on Nesprin 2G responds to changes in the contractility of the cytoskeleton, as expected. Additionally, the sensor revealed that apical tension was higher than equatorial or basal tension on Nesprin2G, consistent with previous observations [97, 98, 7]. While the development of a nuclear tension sensor with protein-level res-
olution is novel, the observation of increased tension in cells with increased global contractility was previously reported [6, 97]. However, to date no direct protein-level force measurements on Nesprin2 have been made while simultaneously manipulating the cytoskeleton or nucleoskeleton in a living cell. One way to rearrange the entire cytoskeleton is to constrain cell-matrix adhesions to a patterned substrate. Alternatively, the cell-substrate can be deformed by seeding cells on a flexible membrane subject to varying levels of strain. Analogously, to perturb the nucleoskeleton, cells can be transfected with progeria-type lamins. Progeria lamins are a shortened splice-variant of laminA that is known to stiffen the nucleoskeleton and cause an altered nuclear envelope phenotype (known as blebbing)[17, 99, 100]. How these alterations of the cytoskeleton and nucleoskeleton affect Nesprin2 tension is not known.

In this chapter, these cytoskeleton/nucleoskeletal manipulations were investigated while recording FRET measurements from the Nesprin-2G tensions sensor. We are not aware of any physical models that predict whether Nesprin2 tension will increase or decrease if 1) the cytoskeleton is constrained/elongated or 2) if the nucleoskeleton is globally stiffened.

It was expected that elongated patterned cells would lead to increased tension on Nesprin2G, particularly at the areas of highest curvature. A similar response was expected for cells placed under uniaxial strain. This was based on prior observations that polarized fibroblasts have an increased number of stress fibers parallel to the long-axis of the cell [unpublished data, 93]. However, an equally plausible hypothesis is that stress fibers that wrap over the apical nucleus exert compressive forces on Nesprin2G due to normal forces perpendicular to the apical surface [6, 93]. Both of these predictions are dependent on the molecular orientation of Nesprin in relation to the actin fibers that terminate on the LINC complex, however these orientations cannot be resolved with standard fluorescent-based imaging techniques.
because they are below the diffraction-limit resolution of conventional fluorescent microscopes. (≈200nm). For cells transfected with progeria-lamins, it was predicted the force on Nesprin would decrease relative to controls. Progeria nuclei show an altered phenotype that suggests a defective nucleoskeletal organization. It was hypothesized that a disorganized nucleoskeleton may disrupt some of the connections between the cyto-nucleoskeleton at the LINC complex resulting in a population of unloaded Nesprin-2G tension sensors.

4.3 Methods

Cell culture:
NIH3T3 mouse fibroblasts were cultured in DMEM with 10% fetal calf serum. Primary human fibroblasts were cultured in DMEM with 10% fetal bovine serum. Normal fibroblasts (catalogue GM00316) and Hutchinson-Gilford Progeria Syndrome (HGPS) fibroblasts (catalogue AG11498) were obtained from the Coriell Cell Repository (Camden, NJ). DNA plasmids were transfected into cells using Lipofectamine 2000 or 3000 (Life Technologies) per manufacturer instructions. In all experiments cells were allowed to adhere to fibronectin coated glass bottom dishes or coverslips overnight before imaging.

Micropatterning:
Stamps of lines with varied widths: 5, 20, 40 m and 1 cm in length were made with PDMS. A coverslip was prepared with a thin layer of PDMS where it was to be stamped. The stamps were then coated with a layer of fibronectin and hand pressed onto the PDMS layer on the coverslip or membrane. Substrates were rinsed and then treated with Pluronic F-127 solution to block cell adhesion to regions without fibronectin. Transfected cells were seeded onto the surface of the substrate and cultured overnight before imaging. Unpatterned controls consisted of fibronectin that
was directly pipetted onto the substrate and allowed to adsorb.

*Immunocytohistochemistry:*

Primary human fibroblasts or NIH3T3 cells were grown on fibronectin coated glass coverslips and fixed using 4% paraformaldehyde or methanol as indicated. Cells were permeabilized with 0.1% Triton X and stained with rabbit anti-Nesprin2G [76] and detected by Alexa Fluor secondary antibodies (Life Technologies). Actin was labeled using rhodamine phalloidin (Cytoskeleton). Nuclei were counterstained using Hoechst 33342 (Life Technologies). Slides were mounted and images collected using a Zeiss 710 LSM confocal microscope.

*Strain Experiments*

Either transfected or stable-expressing cells were seeded onto an assembled, pre-tensioned cell stretcher on .005” glossy PDMS membrane (Specialty Manufacturing, Inc. (SMI)). To retain cell media, a custom made round PDMS chamber was placed on top of the membrane and liquid-sealed against the membrane using either vasoline or silicone grease. Silicone grease was found to cause membrane swelling, therefore only vasoline was used in bi-axial strain experiments. Cells were allowed to adhere to the membrane 24hrs before imaging.

4.4 Results

4.4.1 Measuring tension forces on Nesprin2G in elongated, micropatterned cells with a geometrically constrained cell matrix

We sought to understand how cell elongation affects force across Nesprin-2G. To achieve uniform elongation, cells were grown on glass micropatterned with 20 micron wide lines of fibronectin or unpatterned fibronectin. As expected, we observed that cells grown on 20 micron lines had significantly decreased Nesprin-2G FRET
compared to cells grown on unpatterned surfaces (Figure 45A-B). We also observed that 5 micron wide lines similarly decreased Nesprin-2G FRET, whereas 40 micron lines did not (Figure 45C). In addition we observed increased staining of endogenous Nesprin-2G in cells on 20 micron lines (Figure 46). Interestingly, Nesprin-2G FRET ratios were homogeneous along the perimeter of the nucleus and were not significantly different at the regions with highest curvature (Figure 45A). Unexpectedly, we saw an increase in FRET on 40 micron lines (Figure 45C). This increase in FRET may be explained by a highly rounded nucleus for cells patterned on 40 micron widths. Khatau et al. 2009, reported higher aspect ratios for cells on 40 micron widths relative to un-patterned cells [6]. It is generally held that round cells and nuclei are correlated with lower actomyosin tension [101, 102]. Stress fibers that attach to the nucleus are longer and larger in diameter in patterned cells (Figure 47A). Actin fibers in un-patterned cells appear more diffuse, randomly oriented, and localized to the cortical region (Figure 47B). However, published data on the relationship between the geometry of cells and the measured tension in the actin cytoskeleton is lacking.

4.4.2 Measuring tensile forces on Nesprin2G in fibroblasts derived from Hutchinson-Gilford progeria syndrome (HGPS) patients

We sought to understand how Nesprin-2G tension is affected by the disease Hutchinson-Gilford progeria syndrome (HGPS), a rare genetic disorder in which young patients present clinically with premature aging. HGPS is caused by a mutation in lamin A that alters the splicing of the lamin A transcript, resulting in the loss of 50 amino acids of the protein and permanent farnesylation [100, 103]. Nuclei of HGPS patients, as well as normal cells expressing the lamin A truncation, have nuclei with altered morphology (blebbing) and altered mechanical stiffness [17]. We examined Nesprin-2G FRET in fibroblasts from an HGPS patient compared to a nor-
mal non-diseased patient. Unexpectedly, we observed increased Nesprin-2G FRET in HGPS fibroblasts (Figure 48A-B). We did not observe significant differences in the fluorescent intensity of the Nesprin-2G sensor at the nuclear envelope between HGPS and normal cells (Figure 48C). We also investigated if the localization of endogenous Nesprin-2G at the nucleus was altered in HGPS cells. While we did not observe any dramatic changes in Nesprin localization in HGPS cells, when examined across multiple cells, the intensity of Nesprin 2G at the nuclear membrane was reduced relative to normal cells (Figure 33A-C).

4.4.3 Measuring tensile forces on Nesprin2G under uniaxial and biaxial substrate strains

It was reported that fibroblasts on a patterned substrate have greater average Nesprin2G tension than unpatterned cells ([arsenovic2016Nesprin] and section 4.4.1) Conversely, patient-derived progeria fibroblasts had reduced tension on Nesprin2G [arsenovic2016Nesprin]. In both of the aforementioned experiments, experimental cells had time to equilibrate to these mechanical perturbations on the cytoskeleton and/or nucleoskeleton. It is likely those cells re-arranged their structural components to adapt to the mechanical stress imposed by these conditions. Since cells are not accurately modeled as passive elements, cytoskeletal remodeling may significantly influence the tensile forces on Nesprin2G. It remains unknown how tensile forces are redistributed on Nesprin2G nearly instantaneously (< 1 minute) after inducing strain in the cell substrate.

To determine how strain on the cell substrate strain relates to strain on Nesprin, it is possible to image the fluorescent emission from the Nesprin tension sensor in cells plated on an optically clear flexible membrane. However, in practice there are a number of technical hurdles to overcome in order to image a cell after an immediate
application of strain. First, it requires that cells adhere to a flexible membrane that can be submerged in cell media. Second, it requires a flexible elastic membrane that is optically clear and thin enough to be within the working distance of high magnification objectives (≈ 100-300um tolerance for 40x or 63x lenses). Thirdly, the stretch device must impart significant strain without slipping or relaxing. Fourthly, either the imaging software or device must compensate for vertical or horizontal translations that result from the membrane strain. After several design iterations, we developed a device that met these requirements that is less costly than commercially available equivalents by an order of magnitude.¹

The cell stretcher was designed with CAD software and 3D-printed at the VCU machine shop using ABS plastic. The bottom frame of the stretcher fits into the base plate of a Carl Zeiss 710 inverted microscope (Figure 60A-C). A roller at the end of the stretcher rotates by meshing with a worm-gear that bolts to the side frame (Figure 60C). A polydimethylsiloxane (PDMS) flexible membrane wraps around a rotating roller which applies membrane strain. The PDMS membrane is an elastomeric substance that can be accurately modeled as a linear-elastic solid within a reasonable range of strain magnitudes [104]. To verify the linearity of the PDMS membrane, a stress-strain analysis was performed. A simple bench-top weight scale was used to measure the applied tension on the PDMS membrane through a series of worm gear rotations. Markings on the membrane were tracked by a stationary camera during each tuner rotation. Images of the marked membrane were converted to binary images and the markings were tracked using particle tracking software integrated into the FIJI ImageJ open source software platform. The stress-strain analysis showed a

¹ Electron Microscopy Services has a comparable device with motorized control that costs in excess of 50,000 dollars. Other strain devices that do not have cell tracking, such as flex cells, cost approximately 5000 dollars.
linear response of the PDMS membrane up to 100% strain (Figure 61-A:dotted red line). The estimated modulus in this linear region of the loading curve was 1.1 MPa from linear least squares fitting (slope of dotted red line in Figure 61A). From 100-250 % strain, the membrane stress was approximately linear with a modulus of 1.6 times the first linear fit (Figure 61A-dotted green line). The stress-strain curve showed a nearly identical response during the unloading of tension, confirming the PDMS is linear and elastic.

While the data support that the PDMS membrane is linear elastic, it is possible the PDMS chamber which holds liquid media around the cells on top of the membrane creates a stress shielding effect. To determine the extent of stress shielding potentially caused by the PDMS media chamber, an experiment was performed to measure the strain on the membrane inside the media chamber filled with water. The strain was measured by tracking a circular marking on the membrane and quantifying the change in area of that marking (Figure 61C). Images of the membrane marking at 20x magnification showed a linear response up to at least 50% strain (Figure 61B). While the strain response of the membrane appeared linear, it cannot be ruled out that regions of the membrane experience non-homogeneous strain or non-linear strain.

After characterizing the response of the membrane, live-cell experiments were performed using Madin-Darby Canine Kidney (MDCK) epithelial cells. The goal of these experiments was two fold. First, to determine if the fluorescent emission from the tension sensor could be measured accurately enough to estimate FRET. Secondly, to verify the position of strained cells could be tracked before and after stretching the membrane. Rather than measure the Nesprin tension sensor in these experiments, a previously published E-Cadherin tension sensor was used for validation [52].

The E-cadherin tension sensor (ECAD-TS) was used in place of the Nesprin tension sensor for two reasons. First, ECAD-TS was cloned into a stably expressing
MDCK cell line. These cells stably express ECAD-TS, showing bright fluorescence emission in nearly every cell. This greatly simplifies the preparation of cells when compared to cell lines that require lipid-mediated transfection (all 3T3 fibroblast experiments with the Nesprin tension sensor required lipid-mediated transfection). Stable cell lines are also much easier to image because most cells emit measurable fluorescent signal. Second, the E-cadherin tension sensor was previously shown to instantly respond to strain when applied by micropipette aspiration \[52\].

Unlike Nesprin-TS, ECAD-TS measures the tension on the junction protein E-cadherin. Since ECAD-TS is located at cell-cell junctions, measurements of this sensor require densely seeded cells to ensure that junctions form. In the first set of validation studies a stable expressing MDCK monolayer was seeded onto an optically clear PDMS membrane imaged at 40X magnification. The cell monolayer was strained roughly 50% while the same focal point on the membrane was tracked. Since vertical, horizontal, and axial translations occur during strain application, the focus and stage was adjusted on the fly to maintain the monolayer position. This experiment generally required four pairs of hands. Cells were tracked to directly compare strained junctions before and after strain application. To get an idea of the average strain on these MDCK cells, the summed fluorescent intensity images (Figure 49A-B) were thresholded to produce binary images (Figure 49C-D). These binary images create an approximate mask of each cells area and shape, albeit with some distortions due to image noise and variable fluorescent intensities. After masking these images, the particle analysis function ("Analyze Particles") from ImageJ was used to quantity the cell strains. As expected, the distribution of strained cells showed a large drop in circularity (leftward shift) and an increase in pixel area (rightward shift) (Figure 49E-F).

Once it was visually clear the monolayer was under significant strain, FRET on
the ECAD strain sensor (ECAD-TS) was measured using a spectral image detector (Zeiss Quasar). The Quasar spectral detector can capture emission signals from 400-720nm with 9.7nm spacing between each channel. Each pixel in the spectral image can be considered a fluorescent emission spectrum in the visible light range. Integrated intensity images showed visible strain in select junctions (Figure 50A-D). In this particular example, a statistically significant increase in donor fluorescence was observed in the selected cell junction (Figure 50C-E). This indicates higher strain and higher force on ECAD-TS in that particular junction (Figure 50E-F). Spectral images were transformed into band-pass images by integrating the intensities of the donor and acceptor emissions into two channels. The ratio of the donor and acceptor images were computed as \( \frac{\text{acceptor}}{\text{donor}} \) to create a FRET index. The strained junction image had a lower average ratio (Figure 50F). An evaluation of multiple highly strained (n=13) junctions showed a statistically significant decrease in the average FRET (Figure 50G).

4.4.3.1 Measure Tension on uni-axially strained epithelial cells on a patterned membrane

It was hypothesized that increasing the strain on cell-matrix adhesions would transmit to the LINC complex rapidly and increase tension on the Nesprin2G FRET sensor. This was based on the theory that actin stress fibers can be modeled as non-linear, viscoelastic elements [105]. Assuming a viscoelastic model, the elastic strain component should cause an immediate increase in tension on LINC complexes if you assume as a first order approximation: 1) stress fibers run directly and independently (limited cross-linking) from focal adhesions to the LINC and 2) they align parallel to the strain axis. Without knowing the time-constant for the viscous element, it is entirely possible that stress-relaxation could occur on the order of seconds. This
would have the effect of dampening the tensile force on Nesprin as a function of time. However, even with stress relaxation occurring on a timescale much faster than the rate of strain application, it is unlikely that stress relaxation lead to the complete decay of tensile force. This can be illustrated by a simple example where a single stress fiber is considered a Voigt-body.

The Voigt-body model holds that the mechanical behavior of the fiber can be modeled as a spring and dashpot (dampening element) in parallel. With a parallel configuration, the forces across the Voigt-body are additive. The force on the spring element follows Hooke’s law such that the force is equivalent to the product of the spring constant and the element’s displacement. The dashpot element depends only on the rate of displacement. Summing the two together forms the following relationship (Equation 4.1):

\[
\sigma(t) = E \varepsilon(t) + \eta \frac{d\varepsilon(t)}{dt} \tag{4.1}
\]

Where \( E \) is the elastic modulus, \( \varepsilon(t) \) is the strain as a function of time, \( \eta \) is the dashpot constant, and \( \frac{d\varepsilon(t)}{dt} \) is the strain rate. If Equation 4.1 is integrated and solved with respect to time, then the following function is obtained:

\[
\varepsilon(t) = \frac{\sigma_0}{E} \left(1 - e^{-\frac{E}{\eta} t}\right) \tag{4.2}
\]

Where \( \sigma_0 \) is an instantaneously applied stress at time 0. Equation 4.2 is sometimes referred to as a creep function which describes the time-dependent deformation behavior of a mechanical body as a function of the applied stress. If a one dimensional stress fiber that acts as a voigt body is placed under uni-axial strain, Equation 4.2 shows that without any knowledge of the stress relaxation rate, the displacement at some time \( t \), where \( t \gg \frac{E}{\eta} \), is approximated by the ratio of the applied stress and the
elastic modulus. Conversely, the stress on the hypothetical voigt body stress fiber is equivalent to the ratio of the strain divided by the elastic modulus at some time \( t \), where \( t \gg \frac{E}{\eta} \). If the stress relaxation constant is on the order of the timescale of the applied strain, then the measured stress will 1) vary depending on the time delay of the measurement and 2) have a magnitude at least as large as the ratio of the applied strain divided by the elastic modulus. This simplified model of an actin stress fiber illustrates that without knowing the visco-elastic parameters, stress fibers would be predicted to deform some measurable amount after a near instantaneously applied stress.

To test the theory that intracellular protein deformations are measurable after a quick ramping of the substrate strain, homogeneously aligned MDCK cells were micropatterned on narrow (10\( \mu \)m) strips of fibronectin. Micropatterning constrains cells into an elongated phenotype where stress fibers run parallel to the long-axis of the cell (see Figure 47A). Cells were micropatterned to reduce the variability in the orientation of the stress fibers. To confirm the feasibility of FRET measurements with patterned cells on a flexible membrane, stable expressing E-cadherin-TS MDCK cells were seeded onto a flexible micropatterned pdms membrane (Figure 51-top-row). Cells were stretched under increasing levels of strain and their junctions were tracked (Figure 51-right to left columns). The cells adhered to the constrained fibronectin lines as expected. These experiments confirmed the feasibility of FRET measurements in strained, patterned cells however the average change in FRET was not consistent among repeated trials.

In each stretch experiment using patterned MDCK cells the maximum strain of the membrane was approximately 60\% strain. The first trial showed a statistically lower FRET ratio average in the max strain condition compared to the initial zero strain FRET reference (Figure 52). However, the subsequent experimental trials
showed no statistically significant differences in the FRET ratio between the max strain and zero strain reference group (Figures 62,62,62). In the second trial, junctions under maximum strain showed less variance and a slightly lower median FRET ratio (Figure 62A). Using a pairwise analysis, the difference of the FRET ratio between maximum and zero strain was computed (Figure 62B). If the force were to increase across E-cadherin, then the difference between the 0 strain and maximum strain FRET ratios would have positive magnitude. Figure 62B showed this difference was both positive and negative, indicating both increases and decreases in force across E-cadherin-TS. A third replicate experiment showed a general decrease in FRET with increasing substrate strain that was not statistically significant (Figure 62C). Pairwise analysis of these junctions showed that all but one junction showed decreased FRET from zero to maximum substrate strain (Figure 62D). A fourth replicate experiment showed nearly the opposite, where the median FRET ratio increased upon increased substrate strain (Figure 62E). In this experiment two steps of increasing strain were applied to the cell substrate (called midstrain and maxstrain). Pairwise analysis of these junctions showed that changes in the FRET ratio were correlated to the region of the monolayer examined (Figure 62F:Image1vsImage2). Some junctions had uniformly increased FRET at medium strain while other junctions on the same substrate had uniformly decreased FRET (Figure 62F). These results suggest the force across E-cadherin junctions likely depends on the geometry of the monolayer and this geometry varies spatially. At maximum applied strain, the average junction FRET ratios showed little change relative to medium strain, however the variance decreased (Figure 62E).

Next, stable expressing Nesprin-TS MDCK cells were seeded on a patterned uniaxial stretcher and placed under high strain. Individual cells were tracked such that paired observations could be made before and after applied strain. The FRET
ratio of patterned nuclei appeared to decrease in regions of lowest strain (Figure 53A-B). However, areas of initially low FRET in the pre-strain condition did not appear to respond to strain (Figure 53A-B). A bulk analysis of the normalized histograms of all 20 nuclei did not show a significant difference between the strained and unstrained patterned nuclei (Figure 54). However, there was a large difference in the FRET ratio of patterned and unpatterned nuclei (Figure 54). It is important to note that patterned cells have greater signal intensity due to an increased amount of Nesprin molecules (section ). FRET ratios can be prone to intensity artifacts, where low intensity pixels tend to have higher FRET ratios because the acceptor signal behaves like a random signal with the constraint that only positive magnitudes are measured (the FRET ratio in this context is defined as $\frac{\text{acceptor}}{\text{donor}}$). If the FRET efficiency is low, the FRET ratio as defined will also be low magnitude. At some point, the signal to noise ratio of the acceptor signal will be lost to noise and the FRET ratio will be biased higher. The ratio is higher because thermal noise on the detector pushes the pixel intensity higher in the absence of signal.

To illustrate this principle, the noise properties of the FRET ratio can be replicated in simulation where the donor and acceptor spectra are simulated as two Gaussian spectra with equivalent standard deviations but different peak locations (Figure 55A). Intensity distributions of the donor and acceptor can be simulated as two random distributions with mean values ($\bar{A}, \bar{D}$) that are proportional to the FRET efficiency by their average ratio ($\frac{\bar{A}}{\bar{D}}$) (Figure 55B). Under an assumption of high FRET efficiency, the mean value of the acceptor will be high relative to the donor mean and vice versa. Assuming a poisson noise model, the variance of the these underlying intensity distributions should be proportional to the square root of their mean value. In addition, a baseline level of variance is added to the simulated intensity distributions which represents the minimum thermal noise on the detector, independent of
the brightness of the sample. After the underlying intensity distributions are simulated, the donor and acceptor spectra are scaled by their respective intensities. Figure 55C shows a subsample of these scaled spectra where the mean donor pixel is 80% of the total signal magnitude. These scaled spectra represent individual noiseless pixels separated into their donor and acceptor components respectively. To generate a noisy pixel, their components are summed together and a mixture of poisson/gaussian noise is added. The result of this simulation is a collection of noisy pixel spectra similar the plot shown in figure 55D. If these noisy spectra were recorded at the microscope and unmixed according to the built in Zeiss linear unmixing algorithm, then each spectra is decomposed into two coefficients that represent the magnitude scaling of the underlying donor and acceptor components. This unmixing model is based on a least-squares approach which iteratively minimizes the residual of the linear mixture of the components in the model. The constraint of the unmixing model is that the coefficients cannot be negative since negative intensity magnitudes cannot be measured on the detector. A large collection of these noisy pixels \(n=500\) can be simulated to produce a distribution of \(\frac{\text{acceptor}}{\text{donor}}\) ratios. The mean value of this simulated noisy distribution should be the same as the mean values of their underlying intensity ratios. In the simulation shown in Figure 55, the ratio of the true acceptor to donor ratio is \(0.25\) \((\frac{\text{A}}{\text{D}} = \frac{20}{80} = 0.25)\). Using this simulation framework where the donor and acceptor means are 80 and 20% of the total signal respectively, it can be shown the mean and median noisy FRET ratio are very close to the true ratio (Figure 56B). However, this accuracy is significantly degraded when the acceptor and donor are 5 and 95% of the total signal respectively (Figure 57). Both the median and the mean over-estimate the true FRET ratio significantly in percentage terms (Figure 56A). If a series of different FRET efficiencies are simulated, it becomes clear that the error in the FRET ratio is non-linear and increasingly significant as the FRET efficiency de-
creases (Figure 56C). These simulations show that the FRET ratio error depends on the level of FRET efficiency in the biosensor which presumably worsens as the acceptor signal competes with thermal noise as the level of transfer efficiency approaches 0%. It is also likely these simulations under estimate the total error in actual measurements due to the unmixing errors caused by autofluorescence which are generally ignored. The autofluorescence error is difficult to quantify since its spectral shape is not invariant, depends on the excitation frequency, and changes across different cell types and/or conditions. In summary, the FRET ratio should be viewed with caution particularly when there is a large intensity mismatch between groups and a low FRET efficiency. Thus, it is possible that the difference between the patterned and unpatterned cells results from fluorescent intensity and not strain on the sensor.

The lack of responsiveness of the low FRET regions of the patterned nuclei led to the hypothesis that the sensor was strained beyond its dynamic range. This could happen if the fluorophores are separated by a distance that is twice the Forster radius (≈ 10nm). To determine whether the sensor was beyond its dynamic range in patterned nuclei, 3T3 cells were patterned onto glass and the FRET efficiency of their nuclei were measured using the SensorFRET algorithm (for details on the SensorFRET see section 2.4.1). The FRET efficiency from seven nuclei showed an average FRET efficiency of 8% (Figure 58). The average spectra from these nuclei was nearly the same as the teal spectra, indicating the sensor was close to the end of its dynamic range for high forces (Figure 59-Alpha Fit and Unmixed Components). The FRET efficiency tended to be lower at the nuclear envelope and higher on the periphery of the nuclei (Figure 58-inset image). These low levels of FRET efficiency are not inconsistent with the theory that some biosensors are strained beyond their dynamic range. An alternative theory is that the acceptor fluorophores fail under high loads and irreversibly unfold. This alternative theory could not be tested with
the available equipment.

4.4.3.2 Measuring Tension on Nesprin-TS and ECAD-TS on biaxially strained epithelial cells

Uniaxial stretch measurements from section 4.4.3.1 revealed the complexity of predicting the magnitude and direction of strain on the E-cadherin tension sensor in a 2D monolayer. The strain behavior of the E-cadherin molecules did not appear to follow a consistent trend after several replicate experiments where the substrate was uni-axially stretched to approximately 60% strain. One explanation for the lack of consistent measurements with the uni-axial cell stretcher is that cell junctions are highly anisotropic. It is also possible that in a monolayer of cells uniaxial strain leads to compression and tension on junctions depending on their location in the monolayer and the geometry of the localized cell junctions. Similarly, the Nesprin-TS sensor did not yield robust differences in FRET between unloaded and loaded MDCK nuclei.

The lack of consistent strain measurements with the uni-axial cell stretcher led to the development of a biaxial stretcher which could apply strain equally in all directions. An advantage of the biaxial stretcher is that strain in the center of the membrane is applied in x and y directions such that any possible orientation effects on the loading of the sensor will be minimized.

The biaxial stretcher is designed similarly to the uni-axial stretcher described in section 4.4.3, except it has twice the number of worm gears attached. These worm gears are connected by a pulley and cam system such that the strain across the x and y dimensions are equivalent (Figure 60D). Since the x and y axes stretch equivalently, the horizontal and vertical translations are significantly reduced near the center of the stretcher when compared to the uni-axial stretcher. In theory, the dead center of the membrane will not translate in the x-y plane. In practice, cells of interest
are typically not located dead center and small horizontal/vertical translations occur. This requires visual tracking of the cells as strain is applied.

To test whether inconsistent strain measurements from section 4.4.3.1 resulted from uni-axial loading, stably expressing ECAD-TS and Nesprin-TS MDCK cells were seeded on clear flexible pdms membranes and strained biaxially. The FRET efficiency was measured using the SensorFRET algorithm. For Nesprin-TS expressing cells, two large increases in strain were applied to tracked nuclei (n=3). The strain increments were defined in degrees of rotation (90,135) of the membrane roller which roughly correspond to 40 and 60% membrane strain. Before applying strain, the median FRET efficiency of the Nesprin TS sensor was 18% (Figure 63A). At 90 degrees, the median FRET dropped to 13% (Figure 63A). At maximum strain (135 degrees), the median FRET decreased to 8% (Figure 63A). The same group of nuclei were then unloaded to the same approximate strains as the loading path. The change in the median FRET efficiency of nuclei during each unloading increment was diminished relative to the loading portion (Figure 63B). However, the median FRET efficiency increased as the strain decreased as expected.

The experiment above was replicated with a larger sample size (n=8) and the maximum strain was decreased to 90 degrees before unloading the membrane. The maximum strain increment was decreased to test whether large strain increments lead to sensor failure. It was predicted that the initial FRET efficiency would fully recover if a lower maximum strain increment was imparted on the pdms membrane. As predicted with increasing strain the average FRET efficiency decreased (Figure 64A). The median FRET efficiency increased roughly 5% after the membrane was unloaded from 90 degrees to 0 (Figure 64B). The median FRET of the initial and final membrane strain was nearly identical, suggesting full recovery of the sensor position (Figure 64B). This was in contrast to the previous experiment, where only
a partial recovery was observed (Figure 63B). These results suggest that under high strains the neprin sensor can fail under load. It is currently unclear how the sensor may be failing, but two different scenarios are possible. Under high tension the flagelliform linker may break, separating the fluorophores to a distance greater than 10nm. Alternatively, the venus or teal fluorophore could be irreversibly unfolding. In either case of failure, the FRET efficiency would drop to zero, lowering the average FRET in a mixed population of intact and broken sensors.
Fig. 45. (A) FRET ratio images of micropatterned 3T3 fibroblasts expressing Nesprin2G-TS on 20 micron wide fibronectin rectangular patterns or unpatterned fibronectin coated glass. (B) Statistical analysis of each condition showed that cells grown on lines had reduced average FRET compared to cells grown on the un-patterned surface. Bar graphs represent FRET pixels from a discrete high-intensity range of pixels collected across a minimum of 15 cells per condition, ttest p-value < .05. Similar results were obtained for three independent experiments. (C) Nesprin-2G FRET of NIH3T3 cells grown on micropatterned lines (width of 5, 20, or 40 m) or unpatterned FN. All conditions are significantly different from each other (ANOVA, NewmanKeuls post-hoc test, p < 0.01).
Fig. 46. Antibody staining of Nesprin-2 expression in NIH 3T3 fibroblasts on (A-left image) 20uM micro-patterned fibronectin lines or (B-right image) unpatterned fibronectin coated glass. B) Quantification of anti-Nesprin-2 fluorescence using histogram analysis. Images were acquired with identical power and gain settings.

Fig. 47. Phalloidin (Actin) and Hoescht (Nuclei) staining of 3T3 fibroblasts on A) 20uM-wide or B) unpatterned fibronectin coated glass. Thick stress fibers run parallel with the long-axis of the cell in (A)
Fig. 48. (A) The Nesprin tension sensor was expressed in normal and HGPS primary fibroblasts. (B) There was a significant increase in FRET for HGPS cells as compared to normal cells, t-test, \( p < 0.01 \). Bar graphs represent FRET pixels from discrete intensity ranges collected across a minimum of 20 cells per condition. Similar results were obtained for two independent experiments. (C) Histogram analysis of fluorescent intensity of the Nesprin sensor for normal and HGPS cells.
Fig. 49. Integrated fluorescent intensity images of confluent stable expressing E-cadherin-TS MDCK cells on a (A) pre-tensioned pdms membrane and (B) 60% uni-axial strained pdms membrane. Binary thresholded intensity images (C) and (D) using (A) and (B) as inputs for morphological analysis. Histogram analysis of (E) cell circularity and cell pixel area (F) of a confluent layer of MDCK cells before and after strain application.
Fig. 50. (A) Pre- and (B) post uni-axial strain spectral images of MDCK cells expressing E-Cadherin-TS with an individual junction highlighted with a magnified inset images of a single junction (C) and (D). (E) Average spectra of highlighted junction in (C) and (D) before and after application of strain. A 95% boot-stapped confidence interval of each mean spectra with teal and venus emission peaks shown with vertical lines. (F) Average FRET ratio of spectra shown in (E) and distributions of the FRET ratio of individual junctions (G).
Fig. 51. Fluorescent intensity, thresholded binary image, and FRET ratio images \( \frac{\text{acceptor}}{\text{donor}} \) of patterned MDCK cells expressing E-Cadherin-TS under (High), (Medium) and (Low) uni-axial strain. Each image contains the same cells on an equivalent intensity and ratio scale.
Fig. 52. Mean FRET ratio ($\frac{\text{acceptor}}{\text{donor}}$) of patterned MDCK E-Cadherin-TS expressing cells shown in Figure 51. Errorbars represent the 99% bootstrapped confidence interval about the mean FRET ratio.
Fig. 53. NesprinTS MDCK pattern: Patterned MCDK cells that stably express Nesprin-TS A) before strain and B) after strain. Each cell in image A) corresponds to the same cell in image B) where their relative positions are identical. A Gaussian blur ($\sigma = 1$) was applied to the unmixed images before computing the ratio. Pixels are color-coded to the smoothed FRET ratio defined as $\frac{\text{acceptor}}{\text{donor}}$. Both images are color-coded on an identical ratio scale.
Fig. 54. Patterned MDCK expressing NesprinTS: The normalized FRET ratio histograms of patterned MCDK cells stably expressing NesprinTS. Patterned (blue or red) and unpatterned (black or green) cells were measured before and after strain application. The membrane was strained roughly 30% however nuclei strain varied per cell. The histogram frequency was bootstrapped by the mean value at each FRET ratio shown as a 95% confidence interval band abound the mean line.
Fig. 55. FRET Ratio simulation: A) Simulated Donor and Acceptor emission spectra using Gaussian distributions with means centered at $\lambda_{em}(490)$ and $\lambda_{em}(550)$ and equivalent standard deviations. B) Intensity distributions for Donor and Acceptor signals with means centered at 80 and 20 respectively with standard deviations equivalent to the square root of the amplitude. C) Noiseless donor and acceptor spectra sampled from the intensity distributions shown in B. D) Simulated FRET signal with thermal and poisson noise added (purple), non-negative least squares (nnls) fitting (black), and the component fittings of the nnls fit (blue and green dotted lines).
Fig. 56. FRET Ratio Error Simulation: A and B) Histogram of ratios of acceptor/donor computed from the nlls fitting of simulated pixels from Figure 57 or Figure 55, with the true(solid-red), median(dotted-black), and mean ratio (dotted-green) shown as vertical vertical lines. C) The absolute % error of the computed FRET ratio ($\frac{\text{true ratio} - \text{computed ratio}}{\text{true ratio}}$) for each simulated level of FRET efficiency.
Fig. 57. FRET Ratio simulation Low FRET:A) Simulated Donor and Acceptor emission spectra using Gaussian distributions with means centered at \( \lambda_{em}(490) \) and \( \lambda_{em}(550) \) and equivalent standard deviations. B) Intensity distributions for Donor and Acceptor signals with means centered at 95 and 5 respectively with standard deviations equivalent to the square root of the amplitude. C) Noiseless donor and acceptor spectra sampled from the intensity distributions shown in B. D) Simulated FRET signal with thermal and poisson noise added (purple), non-negative least squares (nnls) fitting (black), and the component fittings of the nnls fit (blue and green dotted lines).
Fig. 58. 3T3 NesprinTS FRET Efficiency: (Inset Image) 20uM micro-patterned 3T3 fibroblasts with color-coded FRET Efficiency. FRET Efficiency pixels were thresholded by excluding normalized residuals errors greater than 6%. (Outer Image) Histogram of error thresholded FRET Efficiency pixels with the median value (solid red line)
Fig. 59. SensorFRET unmixing of Nesprin-TS: Patterned 3T3 fibroblast on glass expressing Nesprin-TS shown in Figure 58. (Alpha Fit) teal fitted raw spectra. (Beta Fit) Fitted Venus Direct Excitation. (Subtraction of Direct Excitation) Removal of direct excitation of Venus. (Unmixed Components) Fitting of the corrected Nesprin-TS spectra.
Fig. 60. (A-C) 3D renderings of uni-axial, 3D-printed cell stretcher controlled by a single (A) or double (B,C) independent worm gear mechanism. (D) Biaxial cell stretcher: CAD rendering of biaxial cell stretcher designed for a cruciform PDMS membrane. The PDMS membrane attaches to four cylindrical rollers fixed to four worm gears. The worm gears are meshed together by a pulley above the rollers producing equivalent strain in the x-y plane at the center of the membrane. The base of the stretcher fits into stage plate of a Carl Zeiss inverted microscope.
Fig. 61. (A) Stress strain analysis of clear .005” PDMS membrane with fitted modulus at low (red-line) or high (green-line) strains. (B) Precision and slippage of the uni-axial cell stretcher as shown by the axial membrane strain as a function of manual worm gear rotation. (C) Image analysis of membrane strain through each manual gear rotation.
Fig. 62. Second Trial: (A) boxplot distribution of the mean junction FRET ratio for tracked cell junctions at zero and maxstrain. (B) Change in the FRET ratio of junctions analyzed from data shown in (A). Third Trial: (C) Boxplot of mean FRET ratios for zero and maxstrains for tracked cell junctions and (D) change in FRET ratios per junction analyzed. Fourth Trial: E) Boxplot of mean FRET ratios for zero, mid, and max strain distributions and F) Change in the mean FRET ratio per condition of strain and grouped by image.
Fig. 63. Loading curve: (A) Distribution of FRET efficiencies of unloaded (0deg-blue), 30% (90deg-green) and 60% (135deg-red) bi-axially strained MDCK cells expressing Nesprin-TS determined by SensorFRET. Unloading curve: (B) Distribution of FRET efficiencies of (135deg-blue) 60%, (90deg-green) 30%, and 0% (0deg-red).
Fig. 64. Recovery: (A) FRET efficiency histograms of MDCKs expressing Nesprin-TS after 30% biaxial strain. (B) Change in FRET efficiency histograms of MDCK cells expressing Nesprin-TS from 0% to 90% biaxial strain. FRET efficiency histograms were determined using SensorFRET.
Fig. 65. Model of Nesprin-2 orientation in response to applied force. (A) Nesprin-2 is oriented radially for non-polarized cells. (B) In elongated cells, in which actin stress fibers are oriented parallel to the longitudinal axis, there is more force applied to the LINC complex, as well as additional Nesprin-2 molecules recruited to the nuclear membrane. Nesprin-2 forces are spatially homogeneous around the periphery of the nucleus, suggesting that Nesprin-2 orients in the longitudinal direction.
CHAPTER 5

DETERMINING HOW FORCES ON THE NUCLEAR ENVELOPE EFFECT MESENCHYMAL STEM CELL DIFFERENTIATION ON VARIOUS SUBSTATES.

5.1 Abstract

The extent to which mechanical forces on the cell nucleus impact cellular function is not well understood. Despite this uncertainty, mutations in nuclear proteins such as lamin-A are known to cause a variety of diseases known as laminopathies that tend to disproportionately alter mesenchymal tissue. How these mutations directly contribute to the observed laminopathies and whether force transduction plays a role remains unclear. To better understand how forces on the nucleus alter mesenchymal stem cell function, bone-marrow derived mesenchymal stem cells (MSCs) were genetically modified to over-express KASH (Klarsicht, ANC-1, Syne Homology) domain proteins (DN-KASH) that effectively disconnect the cytoskeleton from the nucleus by saturating the binding domains of sun proteins embedded in the nuclear envelope. MSCs treated with DN-KASH preferentially differentiated into bone cells at a higher rate than MSCs exposed to osteogenic growth factors. This osteogenic preference due to DN-KASH treatment was independent of the surface topology on titanium surfaces and did not significantly alter integrin expression. However, the tendency to differentiate into osteocytes was dependent on the substrate stiffness. Preliminary data as determined from traction force microscopy reveal that MSCs expressing DN-KASH have much higher stress on their integrins relative to controls. Overall, the data imply that an intra-cellular force-dependent mechanism connected to the cell
nucleus strongly influences MSC differentiation.

5.2 Introduction

In the previous chapters 1 through 4, a methodology of measuring force on the Nesprin-2G biosensor was established. Quantitative methods for accurately measuring the FRET efficiency were developed. Using these methods it was shown that Nesprin-2G is under higher forces in patterned fibroblasts and under less force in patient-derived fibroblasts with the progerin mutation. While these observations are interesting from a modeling perspective, they do not offer much incite into biologically relevant diseases or potential therapies for diseases.

To determine whether forces on the nuclear envelope could be manipulated for a biologically useful purpose, a series of experiments with mesenchymal stem cells (MSCs) were conducted. Mesenchymal stem cells are precursors for bone, muscle, fat and certain neuronal cell types. The ability to control the differentiation tendency of these cells offers great promise for stem cell therapy and tissue engineering applications. With the overall goal of gaining a better understanding of MSC differentiation, the specific purpose of these experiments was to determine if forces on the LINC complex influence mesenchymal stem cell differentiation. It was already shown previously by Engler et. al.[1] that mesenchymal stem cell differentiation could be manipulated solely through the stiffness of their substrate. It remains unclear how the stiffness of the cytoskeleton or nucleoskeletal may play a role in this process.

To determine if the nucleoskeleton plays a role in MSC differentiation, a series of experiments were conducted by genetically modifying the nucleoskeleton to disconnect actin attachments to the nuclear envelope. In all of these experiments, the connectivity to the LINC complex was disrupted by over-expressing a protein domain that binds to SUN proteins anchored in the nuclear envelope. This over-expressed protein
domain is named Klarsicht, ANC-1, Syne Homology (KASH) domain. When over-expressed, KASH domains saturate Sun proteins thereby displacing Nesprins from the nuclear envelope. The net effect of KASH over-expression (referred to as dominant negative KASH (DN-KASH) is a de-coupling of Nesprins from the nuclear envelope such that force transmission from Nesprins to the LINC complex is removed. By abolishing force transmission from Nesprin to the nuclear envelope, any mechano-sensitive signaling through Nesprins should be diminished or eliminated.

5.3 Methods

Cell culture:
Mesenchymal stem cells (MSCs) derived from patient bone marrow from (company x) were cultured in MSC specific media (company x) until passage 7. Cells were maintained in an atmospherically controlled incubator at 37 degrees celsius and 5% CO2 atmosphere. Media were changed every other day.

Cell Transfection
Low passage (3-5) MSC cells were plated onto tissue culture plastic 6-well dishes and infected with an adenovirus expressing either cytosolic GFP or DN-KASH-mCherry. Due to large differences in the infectivity of GFP and DN-KASH adenoviruses, dosages were based on the emission intensity of each construct after 48hrs of infection. For DN-KASH infection, each 6-well were dosed with 1mL of DN-KASH-adenovirus, and GFP infections were dosed 50uL of GFP-virus. Due to a dependence of the transfection efficiency on the substrate stiffness, dosages were scaled back by 10 fold on the soft surface (8kPa, 0.5kPa).

Quantifying MSC differentiation on different titanium surfaces
Uninfected, GFP or DN-KASH transfected MSCs were plated onto plastic, smooth, or rough titanium surfaces after 48hrs of viral mediated transfection. Cells were allowed
to grow and differentiate on these surfaces for 5 days. Get rest of protocol from Dr. Olivares.

*Quantifying MSC differentiation on different pdms with varying stiffnesses*

Uninfected, GFP or DN-KASH transfected MSCs were plated onto plastic, 0.5kPA, 8kPa, and 32kPa pdms surfaces after 48hrs of viral mediated transfection. Cells were allowed to grow and differentiate on these surfaces for 5 days. Get rest of protocol from Dr. Olivares.

*Traction Force Gel Preparation*

40uL of 8kpA polyacrylamide solution embedded with 1micron diameter fluorobeads were pipetted onto functionalized 35mm glass coverslips and sandwiched between two coverslips. One coverslip was treated with APTES to functionalize the surface, allowing the gels to firmly attach to the ”sticky” coverslip. After gels were cast for 1 hr, they were stored in PBS until cell seeding (stored for up to 5 days). Sandwiched coverslips were seperated by razer blade and the gel surface was functionalized with fibronectin using a sulfa-sampah solution (1:20:400 parts Sanpah:HEPES:DI H20) with coating time of 1hr and 10 minutes under direct UV light (365nm). After rinsing, gels were coated with fibronectin (30ug/mL) and MSCs were seeded into 35mm ibidi-u glass bottom slides at a density of 5000 cells per dish. 4hours after seeding to the surface of the gel, 500uL of DNKASH virus and 125uL of GFP virus were added to each dish.

*Traction Force Imaging*

Z-stack images of gel surfaces below GFP or DN-KASH expressing cells were captured with 0.8µ z-axis resolution (8-10 planes) using 60x magnification before the addition of high strength trypsin. Using a flow chamber, trypsin solution was gently added to the media chamber while a reference marker was tracked to ensure the cell location remained in view. 5 and 10 minutes after the addition of trypsin, two more z-stacks
were acquired at the same locations. Using ipython, stacked images were projected by sum method. Images were registered using an affine transformation to account for translation and rotation after the addition of trypsin. Using a nearest-neighbor algorithm with trackpy package, bead centroids were tracked to determine bead trajectories. Only trajectories that persisted in all projected images were included in the analysis. Initial and final locations of the beads were used to determine bead displacement magnitudes which were linearly interpolated on the image grid to avoid sampling artifacts.

5.4 Results

To determine how DN-KASH influences MSC differentiation, low passage patient derived MSCs were plated onto tissue culture plastic (TCP) and transfected by an adenovirus coding DN-KASH. The DN-KASH protein domain was tagged with mCherry on the amino terminus to track the relative expression of KASH domains. As a negative control, cells were transfected with an adenovirus coding for soluble GFP. The relative fluorescent intensity was used as a proxy for the level of infection of the adenovirus and dosed according to the emission intensity. Approximately 96hrs post infection, cells were harvested and analyzed by PCR and by enzyme activity for markers of bone differentiation. Examination of alkaline phosphatase (AP) activity (an early bone cell marker) showed increased enzyme activity in cells expressing DN-KASH compared to a GFP control. The normalized levels of osteocalcin (a late bone cell marker) were also elevated in DN-KASH cells compared to GFP cells on TCP. When compared with cells treated with osteogenic media, cells expressing DN-KASH had higher levels of osteogenic markers. These results suggested that the connectivity of the cytoskeleton to the nucleoskeleton was related to MSC differentiation and could influence bone cell differentiation with greater magnitude than osteogenic
growth factors.

To determine whether this connectivity was correlated with substrate dependent MSC differentiation, these bone cell markers were examined on substrates with varying surface roughness. The surface topology of titanium substrates has been shown to influence integrin-mediated signaling and can enhance bone cell differentiation without the need for growth factors in the media [106]. Therefore, a second experiment was conducted that examined MSC differentiation on stiff surfaces (titanium or plastic) with varying surface roughness treated with DN-KASH or GFP adenovirus. Control MSCs (GFP-infected) showed increased AP activity on surfaces with increasing roughness as previously reported (Figure 66A). In contrast, MSCs infected with DN-KASH showed elevated levels of AP activity independent of the surface roughness (Figure 66A). Similarly, the normalized levels of osteocalcin were elevated in MSCs expressing DN-KASH on all surfaces (Figure 66B). Conversely, GFP infected cells showed decreased osteocalcin on smooth or TCP surfaces (Figure 66B).

Collectively, these results led to the hypothesis that the linkage between the cytoskeleton and nucleoskeleton play an important role in MSC substrate mechano-sensing. To determine whether this proposed substrate sensing could be extended to other surface properties, MSCs with and without DN-KASH were seeded onto pdms substrates of varying stiffness and bone differentiation markers were monitored. This experiment was designed to replicate the surfaces used by Engler et al.[1] where three different substrate stiffnesses (.1-1kPa, 8-17kPa, 25-40kPa) were used. Rather than fabricate polyacrylamide gels by hand and verify their stiffness, pre-fabricated pdms gels with stiffnesses of .5, 8, and 32kPa were purchased. MSCs expressing DN-KASH were seeded onto pdms surfaces coated with bovine serum protein and compared to control cells infected with soluble GFP and untransfected cells. Interestingly, the adenovirus infectivity depended on the substrate stiffness. The softer substrates
showed significantly higher expression of soluble GFP. While this observation was unexpected and interesting in its own right, it led to an extremely high expression of GFP 96hrs post infection. High expression levels of GFP were correlated with much lower cell viability presumably due to the high concentration of adenovirus in the cells. For these reasons the dosage of GFP virus was reduced by 8-fold compared with the surface roughness experiment.

Using PCR, MSCs expressing DN-KASH showed similar integrin expression profiles to control with elevated levels of integrins A1-A2 with increasing stiffness. Expression of integrins A6,AV, B1 and B3 were all attenuated and independent of the surface stiffness in both DN-KASH and controls. One noticeable exception was integrin A5, which was elevated in control cells on 32kPa surfaces and largely attenuated in DN-KASH cells. In addition to integrin profiles, osteogenic expression markers (SP7, RUNX2, and BGLAP) were also quantified by PCR. Large differences between DN-KASH and control cells were observed for all osteogenic markers on medium stiffness surfaces. Within the DN-KASH treatment group, large differences were observed on TCPS and 0.5kPa relative to 8 and 32kPa surfaces. On medium stiffnesses (8kPa, 32KPa), DN-KASH cells showed much higher levels of SP7, RUNX2, and BGLAP relative to controls.

The aforementioned experiments suggested that MSCs expressing DN-KASH maintain a preference toward bone cell differentiation that is 1) independent of substrate topography, 2) dependent on substrate stiffness and 3) largely independent of integrin activation. Since integrin expression profiles were similar in DN-KASH and control cells, the different expression profiles of osteogenic markers are likely explained by a mechanically-linked cell signaling. While the integrin profiles were similar in control and DN-KASH cells, it is possible that forces or the average stress on integrins at focal adhesions are dependent on nucleoskeleton connectivity. Since
focal adhesions are known to be activated by forces, this could explain the differential osteogenic markers of DN-KASH compared to control. To test this hypothesis, traction forces from MSCs infected with soluble GFP or DN-KASH were measured on polyacrylamide gels embedded with fluorescent beads. Significantly higher or lower traction forces in DN-KASH cells would support the theory that these osteogenic markers are controlled by a mechanically-dependent cell signaling mechanism.

To measure traction forces, 8kPa polyacrylamide gels were casted with 1µm diameter fluorescent beads to use for traction force microscopy (TFM). In TFM, the cell traction deformation of the gel substrate is determined by displacements of fluorescent beads that are embedded in the gel. In these experiments, MSCs were seeded onto TFM gels and infected with soluble GFP or DN-KASH-mCherry. Bead displacements were determined by imaging TFM gels before and after the addition of trypsin to remove the cells from the surface of the gel (Figure 68). Rather than measure true traction forces from the bead displacements (due to computational complexity), the net bead displacements were determined under each cell (Figure 68). Since the polyacrylamide gel can be considered a linear system when displacements are small (≈ 1µm)[107], the magnitude of the bead displacements should be proportional to the traction forces exerted by the cell so long as most displacements are less than 1 µm. This approach simplified the image analysis and relative quantitative comparisons could be made between DN-KASH and the GFP control.

Bead displacement magnitudes were generally higher in cells expressing DN-KASH (Figure 68). Because the fluorescent beads are distributed randomly in the gel, there is no guarantee the number of displacements under each cell is the same. To remove potential sampling artifacts displacements were linearly interpolated on a grid. Heatmaps of interpolated displacements showed increased magnitudes under DN-KASH expressing MSCs (Figure 69A-B). It has been shown that cells with higher cell
spread area have higher traction forces [108]. To account for this tendency, the total displacement magnitude per cell was plotted against cell spread area. As expected, the linear least squares fit of these parameters showed a positive relationship in both DN-KASH and GFP expressing cells (Figure 69C). However, the slope of DN-KASH expressing MSCs was approximately 5 times higher than the GFP control (Figure 69C). While the sample size of this study was small (N=7), the data support a model where DN-KASH increases traction forces in MSCs, independent of their cell spread area.

5.5 Discussion

As previously discussed in sections 1.1 and 5.4, Englar et. al. demonstrated that MSC differentiation is at least in part determined by a cellular force sensing mechanism that is currently poorly understood. In this chapter, a series of experiments showed this form of force-driven MSC differentiation depends on the attachment of the nucleus to the cytoskeleton. On tissue culture plastic, MSCs are driven toward an osteogenic phenotype when the cytoskeleton and nucleus are decoupled at the LINC complex. This decoupling increases the osteogenic propensity of MSCs more than osteogenic growth factors. This observation alone could facilitate improvements in the efficacy of differentiation growth media.

While the intracellular mechanisms that control force-driven differentiation remain elusive, this data suggests that integrin-mediated stem cell differentiation through the variation of surface topology depends on the connectivity of the LINC complex. This was supported by the observation that DN-KASH eliminated any differences in osteogenic markers (Alkaline Phosphatase and Osteocalcin) between smooth and rough surfaces (Figure 66). This dependency appears to be largely independent of the composition and expression of integrins as determined by an examination of 7
types of integrin levels using PCR post-differentiation. Since the composition and amount of integrins do not appreciably change after treatment with DN-KASH, it is possible that the force or stress on integrins is the major intra-cellular signal which propagates from focal adhesions to the nucleus. This theory is supported by the observation that MSC osteogenic differentiation increased on stiffer surfaces which correlate with higher traction forces. Unlike surface topology experiments, the effect of DN-KASH and the surface stiffness were synergistic. Bone markers SP7 and RUNX2 increased 1.5 and 2-fold respectively from the softest to stiffest surfaces in control cells (Figure 67C). In contrast, MSCs treated with DN-KASH expressed SP7 and RUNX2 with 15 and 25-fold increases in expression when comparing .5kPa with 32kPa. If this expression of osteogenic markers were due to a force-mediated process, then the traction forces in DN-KASH treated cells should be different than control MSCs. Preliminary data from traction force microscopy experiments suggests that the average integrin stress increases roughly 5-fold relative to controls (Figure 69).

Collectively, these experiments strongly indicate that the LINC complex and forces on the cytoskeleton may control MSC differentiation and osteogenesis. To characterize this dependency, the Nesprin-TS sensor and other FRET-force sensors will likely be useful in determining how much force on the nucleus is required to influence MSC differentiation.
Fig. 66. Bone cell marker assays on different surface topographies: (A) alkaline phosphatase activity on tissue culture plastic (TCPS), smooth, and rough titanium surfaces for untransfected, GFP, and DN-KASH expressing (MSCs) Mesenchymal Stem Cells. (B) osteocalcin transcription activity on tissue culture plastic (TCPS), smooth, and rough titanium surfaces for untransfected, GFP, and DN-KASH expressing (MSCs) Mesenchymal Stem Cells.
Fig. 67. Bone cell marker assays on different surface stiffnesses: (A) Integrin expression on tissue culture plastic (TCPS), or PDMS gels of varying stiffness (0.5kPa, 8kPa, and 32kPa) for control GFP or (B) DN-KASH infected patient derived MSC cells. Osteogenic marker expression on tissue culture plastic (TCPS), or PDMS gels of varying stiffness (0.5kPa, 8kPa, and 32kPa) for control GFP or DN-KASH infected patient derived MSC cells.
Fig. 68. Traction Force Bead Trajectories: Tracked fluorescent bead trajectories shown as colored vectors with cell outlines shown in white for A) DN-KASH and B) GFP adeno-virus treated cells.
Fig. 69. Traction Displacements for DN-KASH expressing MSCs: (A) Interpolated traction displacement map for MSC cells infected with DN-KASH or (B) cytosolic GFP. (C) Cell area adjusted traction stresses for GFP (green) or DN-KASH (red) expressing cells on 8kPa PDMS gels.
CHAPTER 6

CONCLUSION

In this dissertation, the development and validation of a novel FRET-force biosensor (Nesprin-TS) that measures mechanical tension on Nesprin-2G was documented. In order to validate this FRET-force sensor, a new method (SensorFRET) to measure sensitized emission FRET in unimolecular biosensors using spectral imaging microscopy was developed. In the process of validating SensorFRET, a zero-FRET standard dye pair (Fluorescein-TAMRA) was developed and used to characterize the absolute precision of experimental FRET efficiency estimates. SensorFRET was developed out of a practical need for fast, accurate FRET efficiency estimates that were compatible with a flexible acquisition routine suitable for low-intensity live cell imaging.

Using novel methods to generate FRET efficiency images, the Nesprin-2G biosensor was shown to respond to modulating cellular contractility, cell elongation, and biaxial strain. To determine how Nesprin-TS responded to induced cellular strain, two novel, 3-D printable cell strain devices were developed. With these devices, a methodology to track cells or monolayers after ramping and/or de-ramping of the substrate was developed. To demonstrate a biomedical use of Nesprin-TS, cells derived from patients with HutchinsonGilford progeria syndrome (HGPS) were shown to have less average force on Nesprin2 molecules.

Finally, to demonstrate how forces on the nucleus may impact the physiology of medically useful stem cells, mesenchymal stem cells (MSC) were modified by over-expression of KASH domain proteins to decouple nucleo-cytoskeletal connections.
These experiments showed that MSCs over-expressing KASH domain proteins preferentially differentiate into bone cells and lose the ability to sense varying surface topology. Small sample size traction force experiments revealed that DN-KASH infected MSCs have higher stress on integrins independent of cell spread area. Collectively, these data suggest that an intracellular force-dependent mechanism that is connected to the cell nucleus has a strong influence on MSC differentiation.

In chapter 1, an overview of the cytoskeleton was provided as well as a justification to develop tools to investigate force transmission from the cytoskeleton to the nucleus. Two major justifications were 1) mesenchymal stem cell differentiation can be influenced by stress on integrins which are connected to the nucleus through the cytoskeleton [1] and 2) defects in structural proteins of the nuclear envelope (laminA) lead to clinical pathologies that are correlated with mesenchymal tissue disruption and are likely related to impaired force transmission between the cytoskeleton and the nucleus. To determine whether justifications 1 and 2 were related required a tool that could measure intra-cellular force transmission originating from integrins and terminating on structural proteins at the nuclear envelope. Before starting this project, a tool or biosensor capable of making these measurements did not exist and thus formed the basis of this dissertation.

It was decided early on in this project that the proposed genetically encoded force-sensor would sense small changes in distance by a FRET-based mechanism since a FRET-force module was already validated [46]. Given this design constraint, previously established methods to quantify relative changes in FRET were implemented while the biosensor was developed. It quickly became apparent that simple ratio imaging would not be sufficient in resolving small FRET changes that are relevant to force-sensing with TSmod, even though this is the standard practice for measuring relative changes in unimolecular FRET sensors [42]. One of the reasons small changes
could not be detected consistently was due to the variance of the FRET ratio as a function of the signal intensity. This relationship was reproduced in simulations and found to be particularly relevant for low FRET signals (< 10% efficiencies) where the acceptor signal is difficult to detect above noise (see section 4.4.3.1). A quick solution to this problem was to sort all ratios as a function of their intensity such that any errors related to this dependency could be controlled. While this technique increased the resolution of ratio imaging and was used for a number of experiments in sections 3 and 4, it had a number of shortcomings. First, ratio imaging could not be used as an absolute measure of force or strain on the biosensor. This was due to the problem that ratio-imaging FRET is an instrument dependent measure that cannot be related to FRET efficiency without robust, time-consuming calibrations [109, 45]. Second, sorting ratios by their total fluorescent intensity required that comparative samples have similar intensity distributions which was not always guaranteed with lipid-mediated transfections. The obvious solution to this problem was to implement a method to estimate the actual FRET efficiency (not ratio) in the biosensor, forming a basis for chapter 2.

The implementation of SensorFRET enabled estimates of the actual FRET efficiency in the FRET-force biosensor and provided information on the absolute precision of each measurement. This method was an improvement over existing spectral-based methods because the acquisition routine offered flexibility in the adjustment of laser power and detector gain, allowing FRET efficiency imaging of dim and bright samples during each experiment. During the validation of the SensorFRET methodology, fluorescent lifetime imaging (FLIM) was used to independently verify sensitized emission-based FRET efficiency estimates between fluorescein and TAMRA fluorescent dyes. For this dye-pair, SensorFRET and FLIM estimates were in agreement. However, this was not the case for the FRET protein standards. While FLIM and
SensorFRET efficiency estimates were correlated, this correlation tended to break down at either high or low levels of transfer depending on how the donor lifetime was computed. In either case, the variance of the donor-alone lifetime was so high that small changes in FRET could not be accurately measured with FLIM using the protein standards. In contrast, the variance of the sensitized emission was very small in the donor-only sample. This observation is in contrast to the dogma in the FRET literature claiming FLIM is the most accurate measure of FRET. In the absence of any confounding variables, FLIM measurements in theory should be a highly accurate measure of FRET since the signal to noise ratio is excellent due to the large number of collected photons and supposedly independent of concentration. However, in practice many independent variables inside the cell may influence the fluorescent lifetime, such as pH, autofluorescence, viscosity, quenchers or any other unobservable factor [68]. It is possible that in the specific case of the protein FRET standards, the high sensitivity of FLIM to any non-FRET based parameter increases the baseline level of variance to a greater extent than fluorescent emission measurements. Determining the source of this variance in FLIM measurements could have important implications into the type of measurements employed for any type of quantitative FRET study involving fluorescent proteins.

Using SensorFRET in combination with ratio imaging, chapter 3 revealed that a modified, truncated Nesprin-2G (mini-Nesprin-2G) with TSmod could function similarly to endogenous Nesprin-2G as measured by 1) TAN line incorporation, 2) rearward nuclear movement, and 3) an indistinguishable gross cytoskeletal morphology revealed by intermediate filament, microtubule, and F-actin staining relative to controls. Additionally, a headless control was developed where the actin binding domain was removed to put the tension module under minimal resting tension. The headless construct reported a higher FRET on average relative to Nesprin-TS in 3T3 fibrob-
lasts, implying some resting tension on the Nesprin-TS construct in these cells. It should be noted that the implied resting tension is substrate and cell type dependent. MDCK cells that stably express Nesprin-TS show at least 2-3 fold higher FRET on pdms when compared with fibroblasts on glass. After validating the biological function and force-sensing capabilities of the Nesprin-TS sensor, tension measurements could be conducted on cells in a variety of conditions that mimic the in-vivo microenvironment or relate to disease specific mutations. These measurements formed the basis of chapter 4.

Using 3T3 fibroblasts, Nesprin-TS measurements revealed that micro-patterned cells with large aspect ratios have higher average tension on Nesprin-2G. This correlated with an increased diameter and frequency of stress fibers around the nucleus relative to un-patterned cells (see Figure 47). This observation implies that patterned cells are more contractile and report greater tension on the LINC complex when elongated. Interestingly, the FRET ratio or FRET efficiency did not vary with the curvature of the examined nuclei as expected. This could be explained by low FRET pixel resolution or by a rotation of Nesprin-2 molecules such that they align with the major strain axis of the cell (Figure 65). Using a bi-axial cell stretching device (see Section 4.4.3) attached to a fluorescent microscope, Nesprin-TS consistently reported strain-dependent tension on the LINC complex. Uni-axial strain did not report a consistent strain-dependence on Nesprin-2. Micropatterning failed to improve the consistency of these measurements. Uni-axial measurements may have failed to report a strain dependency due to the larger time-lag between strain increments when compared with bi-axial strain. This time-lag is due to cell-tracking error. With the uni-axial stetcher, tracking error is large and difficult to prevent due to the magnitude of translations of the membrane which is position dependent. Because actin is a viscoelastic material, there is likely stress-relaxation and possibly remodeling that
takes place on the time scale of the measurement acquisition [110]. Improvements to the cell stretcher design may alleviate these potential confounding factors. In addition to orientation and strain dependence, Nesprin-TS reported lower average force on Nesprin-2 in fibroblasts derived from patients with HGPS (progeria). It is unclear how this may play a role in the progression of the disease, however there is anecdotal evidence from chapter 5 (see section 5.4) that suggests a relationship. Recent studies have shown that the progeria mutation (a truncated Lamin-A protein) in MSCs leads to premature osteoblast differentiation [23, 111] and a well characterized deterioration in mesenchymal tissue in patients with HGPS [112]. In chapter 5, it was shown that MSCs treated with DN-KASH differentiate into osteoblasts. These observations may be related since Lamin-A is thought to be connected to Nesprin2 via sun proteins that span the peri-nuclear space (see Figures 3 and 4). Assuming Nesprin-2 force measurements were accurate in the HGPS fibroblasts, the progeria mutation reduced force transmission between LaminA and Nesprin2-dependent cytoskeletal connections because the average stress on Nesprin2 was lower. Impaired force transmission may explain why MSCs prematurely differentiated into osteoblasts, as shown by the DN-KASH experiments (see section 5.4) in which the cytoskeleton was decoupled from the nucleus. Future studies on how the force on Nespin2G may correlate with MSC differentiation would be a good test of this hypothesis. Thanks for reading my thesis!
Appendix A

ABBREVIATIONS

VCU  Virginia Commonwealth University
RVA  Richmond Virginia
Fig. 70. fluorescein Tamra fluorometer 50uM.
REFERENCES


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[52] Nicolas Borghi et al. “E-cadherin is under constitutive actomyosin-generated tension that is increased at cell–cell contacts upon externally applied stretch”.

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