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Vinculin Tension Probe in Neurons

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ABSTRACT

Vinculin is a known key regulator of focal adhesions; it undergoes tension in the locations of attachment to the extracellular matrix. In this study, we explore the use of a vinculin tension FRET probe to investigate vinculin tension within neurons. A critical component of neuronal growth is migration, which is dependent on the mechanical cues between the cells and the extracellular matrix. An understanding of tension variation within the neuron may help us understand mechanisms of neurogenesis. To study these forces, we use a previously developed molecular tension sensor, which consists of an elastic linker, TSMod, a 40-amino-acid-long peptide inserted between teal fluorescent protein (mTFP1) and mVenus. The vinculin tension sensor, VinTS, consists of TSMod embedded between the Vinculin head and tail. When under tension, VinTS will exhibit a lower fluorescence resonance energy transfer (FRET) efficiency between mTFP1 and mVenus. Cortical neurons were isolated from embryonic rat brains and cultured on glass coverslips coated with poly-D-lysine and laminin. The neurons were transfected with TSMod (the unloaded tension sensor) or VinTS. Neurons expressing TSMod are used as the experiment's control group since TSMod on its own is not affected by vinculin tension. The mean FRET efficiency of 171 TSMod and 127 VinTS expressing neurons was 27.08 ± 4.98%, and 22.86 ± 3.98%, respectively. The FRET efficiency of VinTS was significantly lower than that of TSMod ($p = 6.6e-15$ by Welch's t-test). These results support the feasibility of using the VinTS probe in neurons and provide a first assessment of VinTS FRET efficiency in neurons. The lower FRET efficiency of VinTS compared with TSMod suggests that VinTS may be under tension in neurons. However, additional studies are required to further characterize these results.

Keywords: Neurons, Mechanotransduction, FRET biosensor, FRET Efficiency, Vinculin, VinTS, TSMod

1. INTRODUCTION

Extracellular mechanical cues contribute to cell differentiation, proliferation and migration. Various diseases involve changes in the extracellular matrix stiffness which affects cellular mechanical cues. In immature neurons or in-vitro neuronal cultures, these mechanical cues play a role in dictating neuronal morphology and dendritic branching[1, 2]. Since vinculin is a known regulator in focal adhesions in epithelial cells, several investigations have been done to localize vinculin in neurons and find its effect in neuronal motility[3]. Focal adhesion kinase (FAK+) was shown to colocalize with vinculin in punctate distribution in growth cone body and filopodial tips[4]. To study the role of vinculin in neurite outgrowth, micro-scale Chromphore-Assisted Laser Inactivation (micro-CALI), a technique that uses light mediated acute protein ablation, was used to inactivate vinculin temporarily. A short-term loss of vinculin resulted in filopodial buckling and bending, which affects neurite outgrowth[5].

The long-term objective of this study is to sense forces along neurites using the VinTS (Vinculin Tension Sensor) molecular FRET probe [3]. Such measurements will help advance our understanding of the role of cellular mechanics in neuronal development and dendritic branching. VinTS is composed of a FRET tension sensor-TSMod, inserted between the vinculin head and tail. TSMod consists of a 40-amino-acid-long elastic peptide inserted between teal fluorescent protein(mTFP1) and mVenus [6] and is sensitive to molecular forces between 1 and 6 pN[3]. FRET Efficiency reports on the tension across the elastic linker in TSMod. The higher the force across the linker the lower the FRET efficiency. In

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this study we establish baseline measurements of FRET efficiency in neurons transfected with VinTS or TSMod. Our results show that between day-in-vitro (DIV) 5-8, the FRET efficiency of VinTS is significantly lower than that of TSMod in neuronal cells cultured on glass coverslips coated with poly-D-lysine and laminin, and suggest that vinculin may be under tension.

2. METHODS

2.1 Neuronal Culture

The cortex was dissected from the embryonic brains at Day 18, trypsinized, and the neurons were dissociated and cultured. The neuronal culture maintained at 37°C and 5% CO_2 in neurobasal medium supplemented with B27, and Glutamax(Invitrogen)[7]. The neurons were cultured with a density of 50000 cell/cm^2 on glass coverslips coated with (Poly-D-Lysine) PDL and laminin. PDL is used to increase the number of positively charged sites for cell binding[8]. Laminin is a high molecular-weight protein of the extracellular matrix that mediates cell attachment, and motility. The PDL concentration was 0.0025 mg/cm^2. The laminin concentration was 0.001 mg/cm^2. Transfection with VinTS or TSMod was performed between DIV 4-6 so as to image neurons between DIV 5-8 when growth cones can be observed.

2.2 FRET measurement

To calculate the FRET efficiency between the donor and acceptor within our probes, we utilize the "three-cube" FRET fluorescence microscopy method. In our system, the three cube FRET consists of acquiring data in 3 channels: a teal fluorescent protein (mTFP1) channel with mTFP1 excitation and emission filters, a Venus channel with mVenus excitation and emission filters, and the FRET channel which is the resulting fluorescence with the mTFP1 excitation and mVenus emission filters. In the mTFP1 channel, the excitation and emission filter bandwidths are centered at 450nm and 485nm, respectively. The acceptor mVenus excitation and emission filter bandwidths are centered at 514 nm and 571 nm, respectively. I_{AA} is the acceptor channel (acceptor excitation, acceptor emission) signal. I_{DD} is the donor channel (donor excitation, donor emission) signal. I_{DA} is the FRET channel (donor excitation, acceptor emission) signal.

All images were acquired on an EM-CCD camera (Cascade 512B, Roper Scientific) with high sensitivity and high dynamic range. Imaging was focused on neurites. We used a 60x objective with NA= 1.4 and an OD=1.5 neutral density filter at the fluorescence excitation source to avoid photobleaching.

We calculate the FRET efficiency, E, based on the following equation:

$$E = \frac{F_C/I_{DD}}{G + F_C/I_{DD}} \quad (1)$$

F_c is the corrected FRET intensity after subtracting the donor and acceptor bleed-through as explained in Menaesse et al [9]. The G-factor [9] is a calibration constant for a specific imaging setup and fluorophores pair. The G-factor (= 2.08) was obtained in separate calibration experiments consisting of iBMK cells transfected with TSMod whose FRET efficiency was taken as 0.286 based on the data from Gates et al [10]. Combining the measurements of F_c and I_{DD} with the known FRET efficiency of TSMod, Equation.1 was used to obtain the G factor. Thus, in this study, the FRET efficiency of the untargeted TSMod probe is expected to remain close to 0.286 as our instrument calibration is based on this value. Since TSMod is a nontargeted protein, it is expected to be distributed throughout the cells and remain unloaded. Its FRET efficiency can be used as a reference to compare it with the FRET efficiency of VinTS which is expected to sense tension changes across vinculin.

2.3 Image Analysis

The images in the three channels were first registered, then background-subtracted and segmented to isolate the neurons from the background regions surrounding the cells. Segmentation was done using the SCIRD-TS

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method [11], which is designed for the segmentation of curvilinear structures. We applied the SCIRD-TS segmentation on the I_{AA} channel, which has the least noise and least auto-fluorescent signal. The resulting segmentation was then used as a mask for the images in all three channels.

3. RESULTS

A sample of the raw data of a VinTS expressing neuron, and the output mask from the SCIRD-TS segmentation, and Fc image are shown in Figure 1.

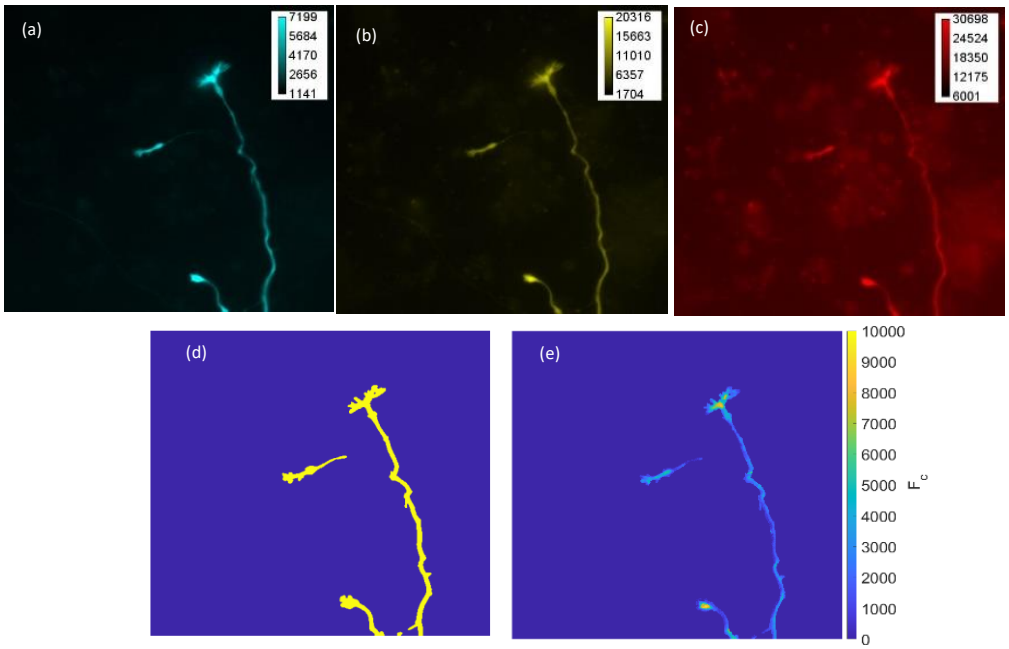

Figure 1.Raw images without any preprocessing a) I_{AA} b) I_{DD} c) I_{DA} d) SCIRD-TS mask obtained from I_{AA} e) F_c. The field of view is 128×128µm.

To measure the average FRET efficiency of each sample, the intensity range of the Fc and I_{DD} images is divided into 100 bins, and the occurrence of combinations of intensities is shown on a two-dimensional histogram (Figure 2). In Figure 2, the x-axis represents the I_{DD} intensity bins, and the y-axis the Fc intensity bins. The color scale represents the numbers of pixels falling in each two-dimensional bin located at a given (I_{DD}, F_c) coordinates. We fit a line through this histogram with y=ax and take the fitted slope as the Fc/I_{DD} ratio. This ratio is then used together with the calibration G-factor (see Methods) in Equation 1. to calculate the average FRET Efficiency, E, for each sample. The TSMod expressing neurons give a more confined 2D histogram compared with VinTS expressing neurons. Since VinTS is a tension sensing probe, more variation in FRET efficiency (and therefore the ratio of F_c/I_{DD}) may occur along the neurite where VinTS senses areas of tension along the neurite, unlike the control TSMod plasmid which is expected to have the same FRET efficiency along the neurite. This is done for all the TSMod (n=171) images and VinTS (n=127) images and used to compare the mean FRET efficiency in each of the two conditions. The mean (+/- standard deviation) FRET efficiency of the VinTS transfected neurons was 22.86 ± 3.98% and was significantly lower than the mean FRET Efficiency of TSMod transfected neurons, which was 27.08 ± 4.98% (p = 6.6e-15 by Welch's t-test). A box and whisker plot of the average FRET efficiencies of TSMod and VinTS samples is shown in Figure 3.

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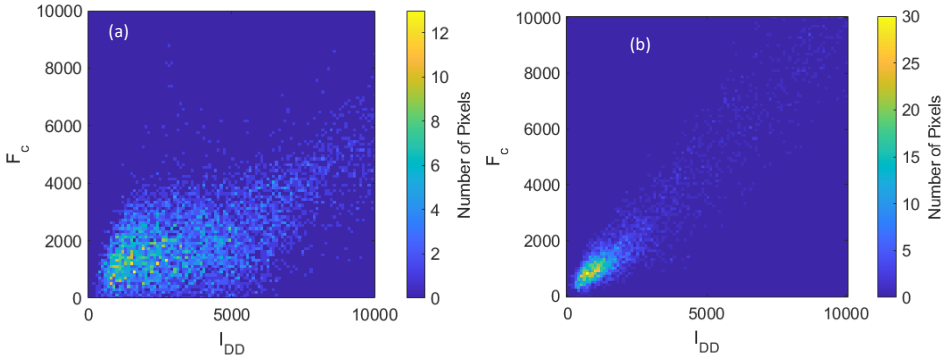

Figure 2. 2D histogram of F_c vs. I_{DD} a) VinTS expressing neuron. b) TSMod expressing neuron which shows more confined pixel values than the VinTS expressing sample.

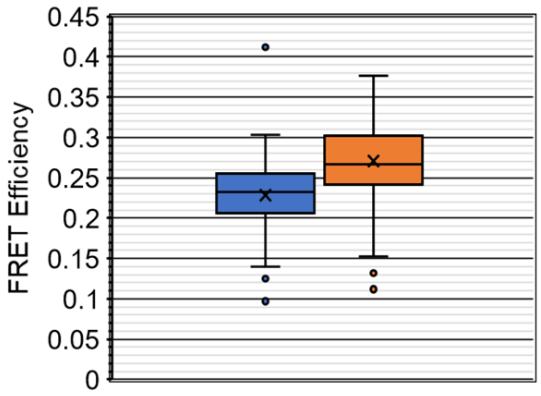

Figure 3. Distribution of FRET Efficiencies values of VinTS and TSMod transfected neurons.

Calculation of FRET Efficiency using Equation 1 at each pixel yields a FRET efficiency image depicting the spatial distribution of the FRET signal. Representative FRET images of VinTS and TSMod neurons are showed in Figure 4.

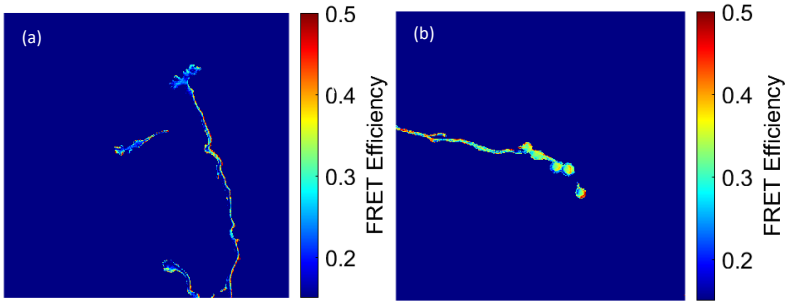

Figure.4. FRET efficiency a) VinTS Sample b) TSMod Sample

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4. DISCUSSION

Since the FRET efficiency of VinTS is lower than that of TSMod, our results suggest that vinculin is under tension in isolated neurons at DIV 5-8. Compared to results in the literature, the average FRET efficiency of VinTS (~23%) in our neurons is higher than the FRET efficiency of VinTS at the focal adhesions of epithelial cells. For example, the FRET efficiency reported at focal adhesions in mouse embryonic fibroblasts (MEFs) was 21.8% for VinTS compared with 28.6% for TSMod[10]. Unlike epithelial cells which exhibit localization of VinTS at focal adhesions, we did not always find vinculin to be localized at specific points in the neurons. Instead, VinTS was typically distributed throughout the neurites at the time of imaging between DIV 5 and 8. Future analysis will include investigation of the spatial distribution of VinTS FRET efficiency along the neurites and as a function of DIV, as well as studies on extracellular matrices with varying stiffness.

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