

Silicon Nitride Cantilevers for Muscle Myofibril Force Measurements

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Primary CNF Tools Used: GCA 5X Stepper, SUSS MA6-BA6 Contact Aligner, Photolith spinners, Oxford 81 ion etcher, Reynolds Tech KOH Hood, Heidelberg DWL2000, MRL E4 LPCVD CMOS Nitride

Abstract:

Measurement of nano-Newton forces produced by individual sarcomeres and isolated myofibrils is possible using custom silicon-nitride cantilever pairs [1]. Advanced imaging techniques, including phase-contrast and immunofluorescent microscopy, allow for the correlation of visible physiological features with mechanical properties – at the level of the sarcomere. Recent advances in our lab have enabled, for the first time, fluorescent labelling of the giant spring-like protein titin without compromising mechanical properties of the sarcomere. Thus, the purpose of this study was to 1: identify the position of select antibody labels on titin during sarcomere elongation and 2: confirm if eccentric calcium-activation causes titin-actin binding, as has been widely suggested [2]. Using myofibrils isolated from rabbit psoas, we performed labelling of titin using N2A (anti-TTN-N2A, Myomedix, Germany) and F146.9 (anti-TTN-F146.9B9, Myomedix, Germany) primary antibodies followed by AlexaFluor488 (A32723 ThermoFischer Scientific, Illinois, United States) and AlexaFluor647 (A21449, A21244, ThermoFischer Scientific, Illinois, United States) secondary fluorophore conjugated antibodies. Chosen N2A and F146.9 primary antibodies flank titin's extensible PEVK segment, which is primarily responsible for titin elongation at physiological sarcomere lengths. Simultaneous measurement of the length of titin's PEVK segment during sarcomere elongation showed similar behavior to non-simultaneous previous experiments [3]. Post eccentric activation, measured PEVK segments were ~50nm longer than isometrically activated controls, suggesting increased force on titin caused by a proximal binding site. In all experiments tested, antibody labels had no effect on mechanical force production compared to protocol-matched unlabelled controls. In conclusion, for the first time, we labelled the sarcomeric protein titin without compromising mechanical function of

the sarcomere. This novel labelling system allowed us to confirm both the behavior of titin's PEVK segment during sarcomeric extension and show evidence for titin-actin binding caused by eccentric activation.

Summary of Research:

Imaging of isolated myofibrils was performed using an inverted Olympus IX83 microscope, and analysis performed using CellSens® Dimensions software. Both phase contrast (PC) and fluorescent channels (AF488, AF647) were collected simultaneously, allowing for the identification of Z-lines (PC), M-lines (PC), TTN-N2A (AF488), and TTN-F146.9 (AF647) within myofibrils (Figure 1). Isolated myofibrils were attached to a glass needle and custom silicon nitride cantilever pairs. Stretching protocols were performed using the glass needle, moved using a piezoelectric motor. Force measurements were performed using optically measured

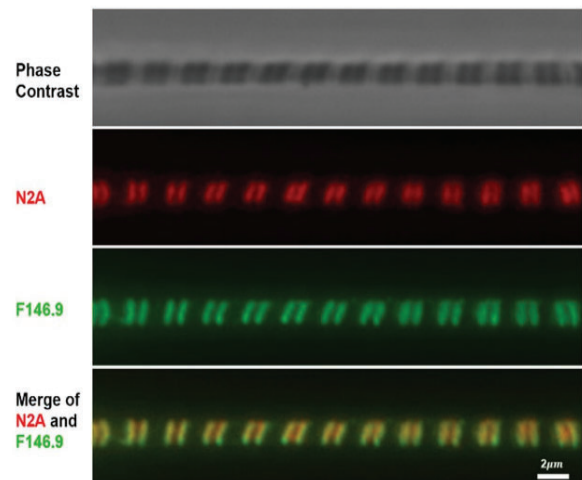


Figure 1: Isolated rabbit psoas myofibril visualized in phase contrast and fluorescent channels. Titin N2A labels are visualized in red (AlexaFluor647); Titin F146.9 labels are visualized in green (AlexaFluor 488). PEVK lengths were measured as the distance between Titin N2A and Titin F146.9 label centroids.

displacement of custom silicon-nitride cantilever pairs with known spring constants.

The introduction of titin labels (N2A and F146.9) did not compromise passive force production during passive sarcomere extension (Figure 2). Since titin is primarily, if not completely, responsible for the production of passive force during sarcomere extension, we concluded that our labelling system did not affect the mechanical behaviour of titin.

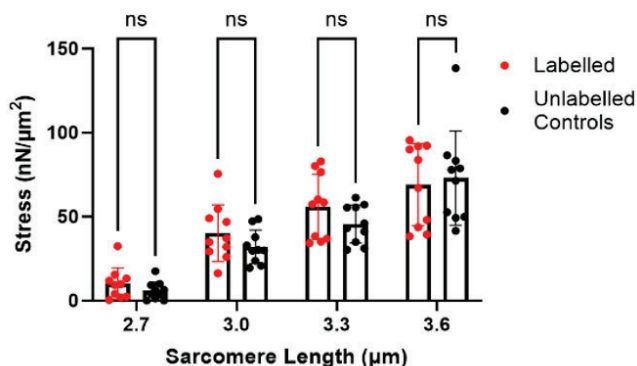


Figure 2: Passive stress production of Labelled myofibrils ($n=10$) is identical to unlabelled controls ($n=10$), (2-way ANOVA with Tukey's multiple comparisons test, $\pm SD$)

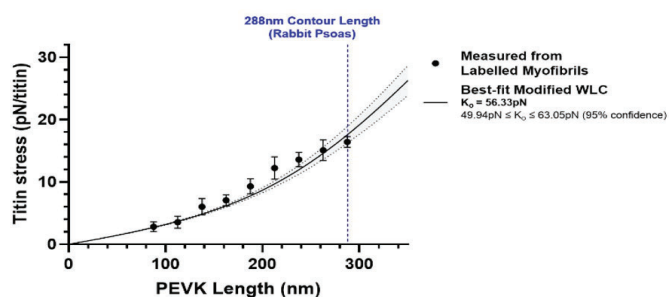


Figure 3: Stress-length relationship of titin's PEVK during passive extension. Data points are means of 25nm length bins, each containing ≥ 11 sarcomeres $\pm SEM$. Data fit using a modified WLC model showing 95% confidence interval.

We captured the passive extension of titin's PEVK segment in labelled myofibrils ($n=10$, Figure 3). Extension of the PEVK segment was well modeled using the existent modified worm-like chain model for titin's PEVK region [3]. Thus, we found that in-situ extension of titin's PEVK, when titin is in its natural configuration inside a sarcomere, largely reproduces previously shown extension of isolated titin molecules.

It has been long theorized that titin-actin binding may occur during eccentric activation of muscle, thus leading to 'over-extension' of titin, and sustained increased force production termed residual force enhancement [2]. In 26 sarcomeres, from $n=7$ myofibrils, we measured the length

of titin's PEVK post eccentric stretch. Titin's PEVK was significantly longer post-eccentric stretch compared to isometrically activated controls, thus providing strong evidence for titin-actin binding proximal to the PEVK region (Figure 4).

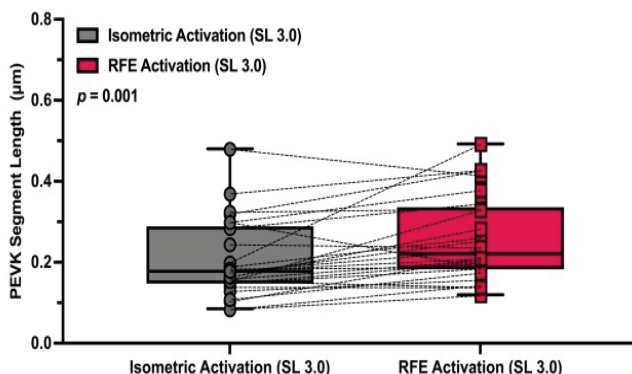


Figure 4: Titin's PEVK is significantly longer post-eccentric activation (RFE activation) compared to isometric controls (Isometric activation) ($p=0.001$, paired T-test).

Conclusions and Future Steps:

In conclusion, simultaneous measurement of forces produced by single sarcomeres and immunofluorescent imaging of titin allowed for the identification of in-situ behavior of the titin filament and showed strong evidence for titin-actin interactions during eccentric sarcomere activation. These findings support future advanced microscopy for subcellular biomechanics.

References:

- [1] M. E. Fauver, et al. IEEE Trans Biomed Eng 45(7):891-898, 1998.
- [2] Walter Herzog. J Exp Biol 15; 217(16): 2825–2833, 2014.
- [3] W. Linke et al. PNAS 95(14): 8052-8057, 1998.