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# Assessing Membrane Micro-domain Physiology from the Inside-Out Using Confocal Microscopy

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Non-ratiometric $Ca^{2+}$-sensitive dyes that fluoresce in the visible range are commonly used to track cytoplasmic $Ca^{2+}$ transients in all cell types using confocal or wide-field microscopy. The fluo- or rhod-based dyes have an excellent dynamic range due to their minimal basal fluorescence under conditions where they do not bind $Ca^{2+}$ as well as a relatively high quantum efficiency. These properties allow for the imaging of fluo or rhod-based dyes within small cellular compartments or micro-domains to report the [$Ca^{2+}$], volume or local diameter using confocal microscopy. However, one problem with the use of non-ratiometric $Ca^{2+}$ dyes is the inability to quantify the actual $Ca^{2+}$ concentration being reported with the changes in fluorescence.

In skeletal muscle, contraction is regulated by tightly controlled changes in $Ca^{2+}$ levels within a muscle fiber. Movement of $Ca^{2+}$ into and out of the fiber occurs across the tubular (t-) system. The transverse tubules form a junction with the sarcoplasmic reticulum (SR), a highly specialized $Ca^{2+}$ storage organelle of muscle. It is therefore valuable to gather information on the spatial organisation and structure of this system and to describe quantitatively the $Ca^{2+}$ movements that are critical to muscle function. We can achieve this by trapping and calibrating non- ratiometric $Ca^{2+}$ dyes in the t-system of skeletal muscle fibers using the mechanically skinned muscle fiber preparation [1]. These measurements could only previously be made using reconstituted vesicles as the t-tubules are too deep to be patched and the fluxes are silent to conventional electrophysiology.

To perform measurements of micro-domain $Ca^{2+}$ movements, bundles of muscle fibers were isolated and exposed to a $Na^{+}$ based extracellular physiological solution containing Fluo-5N or Rhod-5N salt. Fibers were allowed > 10 min to equilibrate with the physiological solution and then individual fibers were isolated and mechanically skinned. Skinned fibers with t-system-trapped fluorescent dye were mounted on a custom-made chamber that used a coverslip as a base and bathed in a standard internal solution, which contained (mM): EGTA, 50; Hepes, 90; $K^{+}$, 126; $Na^{+}$, 36; ATP; 8; $Mg^{2+}$, 1; creatine phosphate, 10; $Ca^{2+}$, 6.7 x 10-4. [$Ca^{2+}$] in the standard internal solution was varied in the range 28 nM to 1.3 μM. Ca2+ was released from the SR using a similar solution in the nominal absence of $Ca^{2+}$, 0.01 mM Mg2+ and 30 mM caffeine. Mounted skinned fibers were imaged using an Olympus FV1000 confocal microscope equipped with an Olympus 0.9NA 40x Plan-Apochromat objective. Rhod-5N was excited with 543 nm HeNe laser and the emission was filtered using the Olympus spectra detector. For tracking $Ca^{2+}$ movements across the t-system membrane images were continuously recorded in xyt

mode with an aspect ratio of 256 x 512, with the long aspect of the image parallel with that of the preparation. Temporal resolution of imaging in this mode where the fluorescence signal from within the borders of the fiber was 0.8 s.

In animal models we have been able to evaluate changes in $Ca^{2+}$ in various health and diseased models. However, studies performed using human fibers provided an opportunity to describe basal $Ca^{2+}$ dynamics in health and disease for the first time. We determined the structure and $Ca^{2+}$-handling properties of the t-system in human skeletal muscle fibers isolated from needle biopsies of the mid *vastus lateralis* [2]. We found that in healthy skeletal muscle, the t-system is a dynamic membrane able to alter its structure in response to exercise and increased $Ca^{2+}$ levels.
To build upon the quantitative t-sys $Ca^{2+}$ measurements, we aimed to measure $Ca^{2+}$ leak that occurs from the calcium release channel (Ryanodine receptor). This $Ca^{2+}$ leak has implications not only for basic muscle physiology but also in muscle diseases where ryanodine receptor (RyR) $Ca^{2+}$ leak is increased, such as Malignant Hyperthermia (MH). By utilizing tetracaine, a reversible RyR blocker, during t-sys $Ca^{2+}$ measurements we were able to assess the basal $Ca^{2+}$-handling properties and RyR $Ca^{2+}$ leak in fibers from control and MH individuals. Interestingly, we found that MH-susceptible subjects display leakier RyRs and a greater capacity to extrude $Ca^{2+}$ across the t-system membrane when compared to control fibers [3].

This technique can be expanded by using dyes trapped in the t-system to examine other fluxes of interest across the tubular membrane, such as those responsive to $Ca^{2+}$ perturbations (e.g. reactive oxygen species or ROS). Ongoing work utilizes the t-sys/RyR leak technique to examine the effects of ROS on $Ca^{2+}$ signaling via dual loads of $Ca^{2+}$ and ROS indicating dyes and/or genetically encoded biosensors.

We have developed a confocal based technique that provides a unique approach to determine the basal $Ca^{2+}$ handling and t-tubular structure in skeletal muscle in health and disease, a method to characterize and quantify $Ca^{2+}$ leak in muscle disease and a platform for testing pharmacological inhibitors in the future.

References:

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