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PU = Public PP = Restricted to other programme participants (incl. the Commission Services) RE = Restricted to a group specified by the consortium (incl. the Commission Services) CO = Confidential, only for members of the consortium (incl. the Commission Services)	PU

A. Abstract / Executive Summary

LENS has developed an ultrafast random access multi-photon microscope that, in combination with a voltage sensitive dye and a fluorescent calcium indicator, is used to simultaneously measure action potentials (APs) and calcium transients at multiple sites within the sarcolemma with sub-ms temporal and sub-µm spatial resolution in real-time. The plasma membrane of cardiac myocytes presents complex invaginations known as transverse-axial tubular system (TATS). Despite TATS's crucial role in excitation-contraction coupling and morphological alterations found in pathological settings, TATS's electrical activity and its coupled calcium-release have never been directly investigated in remodeled tubular networks. LENS found that the tight electrical coupling between different sarcolemmal domains is guaranteed only within an intact tubular system. In fact, acute detachment by osmotic shock of most tubules from the surface sarcolemma prevents AP propagation not only in the disconnected tubules, but also in some of the tubules that remain connected with the surface. This indicates that a structural disorganization of the tubular system worsens the electrical coupling between the TATS and the surface. The pathological implications of this finding were investigated in failing hearts. LENS found that AP propagation into the pathologically remodeled TATS frequently fails and may be followed by local spontaneous electrical activity. These findings shed new light on the relationship between abnormal TATS and asynchronous calcium release, a major determinant of cardiac contractile dysfunction and arrhythmias.

B. Deliverable Report

1 Introduction

The transverse-axial tubular system (TATS) is a complex network characterized by transverse (t-tubules, TT) and longitudinal (axial-tubules, AT) components running from one transverse tubule to the next (1-3). The TATS of a myocyte rapidly conducts depolarization of the surface sarcolemma (SS) to the core of cardiomyocyte. (4). The coupling between sarcolemmal Ca^{2+} entry during an action potential (AP) and Ca^{2+} release from sarcoplasmic reticulum (SR) promotes synchronous myofibril activation throughout the myocyte (5). Recent studies highlight that AP-relevant ion channels and transporters are expressed in TATS membrane with different densities and isoforms than in SS (6, 7). These findings, in combination with diffusional limitations in TATS's lumen (3, 8), raise the possibility that AP may differ among membrane domains. Further interest in the TATS AP stems from the recent finding of loss and disorganization of tubules in several pathological conditions, including heart failure (9-14). Since correlation between morphological TATS alterations and Ca^{2+}-release asynchronicity has been observed (14, 15), recording simultaneously AP propagation and Ca^{2+} transients in TATS can elucidate the fundamental mechanisms linking structural and functional anomalies.

Although the uniformity of the AP across the whole sarcolemma has been mathematically (16) and experimentally (17) proved, a potential alteration of the AP propagation in a disorganized TATS has never been investigated. Here, we develop an optical method to simultaneously measure APs in different sarcolemma domains allowing optical mapping of AP propagation within intact and pathologically remodelled TATS.

2 Objectives

We aim at investigating how the integrity of the TATS is essential to allow a simultaneous and spatially homogeneous release of Ca^{2+} within the myocyte. Since several cardiac diseases are characterized by loss or dysfunction of TATS (18), understanding the role of the single tubule on the Ca^{2+}-transients can disclose important insights in onset, development and treatment of pathologies.

3 Results

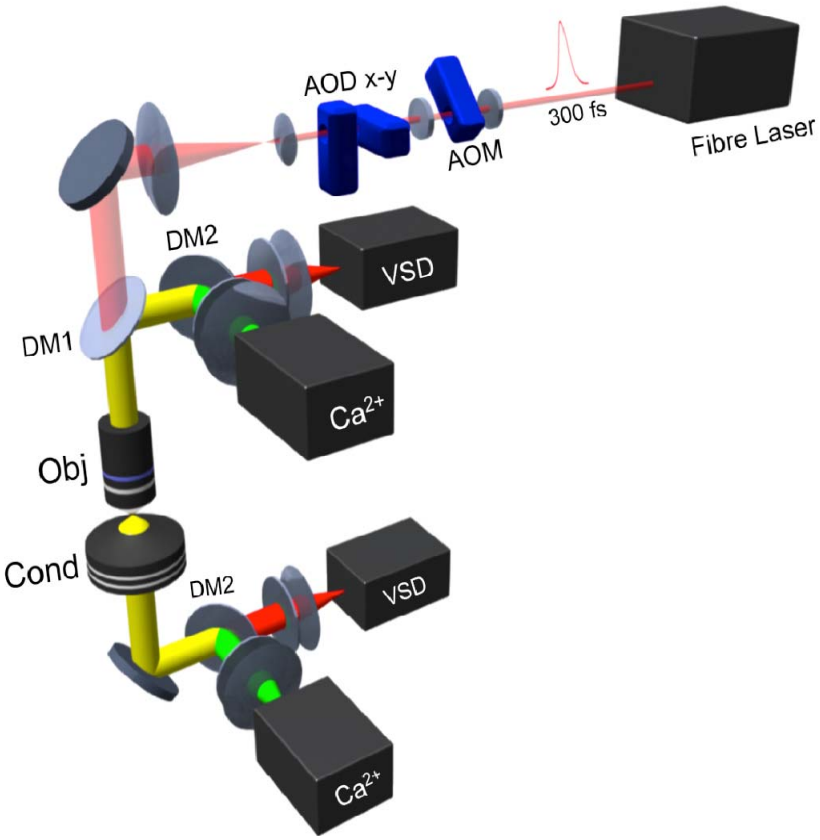

Fig. 1 Scheme of the Random Access Multi-Photon (RAMP) experimental apparatus. A 1064nm fiber laser provides the excitation light. The laser beam is adjusted for optimal linear polarization via a half-wave (λ/2) plate. Beam passes are made through 45° AOM for angular spreading pre-compensation. A second half-wave (λ/2) plate is placed after the AOM to optimize the diffraction efficiencies of the two orthogonally mounted AODs (AOD-x and AOD-y). The light is focused onto the specimen by the objective lens. The isotropic 2PE signal is collected in forward direction by an oil immersion condenser, and in backward by the objective; then the voltage signal and the calcium one are discriminated by using a dichroic mirror beamsplitter at 605 nm (DM1 and DM2). Then the signals are band-pass filtered and focalized into four GaAsP photomultiplier (PMT), two for the voltage signal and two for calcium. The excitation light beam is pumped by a high-power femtosecond pulsed fiber laser (FP1060-2s, Fianium; ~300fs pulse width, repetition rate 80 MHz, wavelength 1064nm).

We investigated the electrical properties and Ca^{2+}-transients of TATS in isolated rat ventricular myocytes labeled with di-4-AN(F)EPPTEA, a fluorinated voltage sensitive dye (VSD) and with a Ca^{2+} indicator (GFP-Certified™ FluoForte™ Reagent by Enzo Life Science). A custom-made random access (18, 19) multi-photon (20, 21) (RAMP) microscope was used to record membrane potential (Vm) and Ca^{2+}-release simultaneously from multiple sites in real-time (Fig.1). The RAMP acousto-optic deflectors rapidly scan linear segments of different membrane domains and perform multiplexed measurements of the two-photon fluorescence signal with sub-ms temporal resolution. Although the calcium and voltage indicators can be excited at the same wavelength, the large Stokes shift of the VSD emission allows us to use spectral unmixing to resolve the voltage and calcium responses. The experiments were conducted at room temperature in the presence of an excitation contraction uncoupler (Blebbistatin) and 1mM extracellular [Ca^{2+}]. A train of 10 stimulations was performed at 2 Hz to elicit APs. As previously shown at LENS (22), AP amplitude and time-course measured in TATS were not statistically different from those of SS. However, Ca^{2+}-release displayed a non negligible variation in the time-to-peak. In particular, this variation was observed both in the same site but at different stimulated events (beat-to-beat variation) and at different sites at the same single shot event (spatial variation). The

coefficient of variation, calculated as $CV=\sigma/\mu$ were 22 and 23% respectively. Interestingly, the averaging procedure cancelled out this variability highlighting the proper homogeneous Ca^{2+}-release across the whole cardiomyocyte in healthy control cells. The time to peak of the Ca^{2+}-release between SS and tubules was not statistically different.

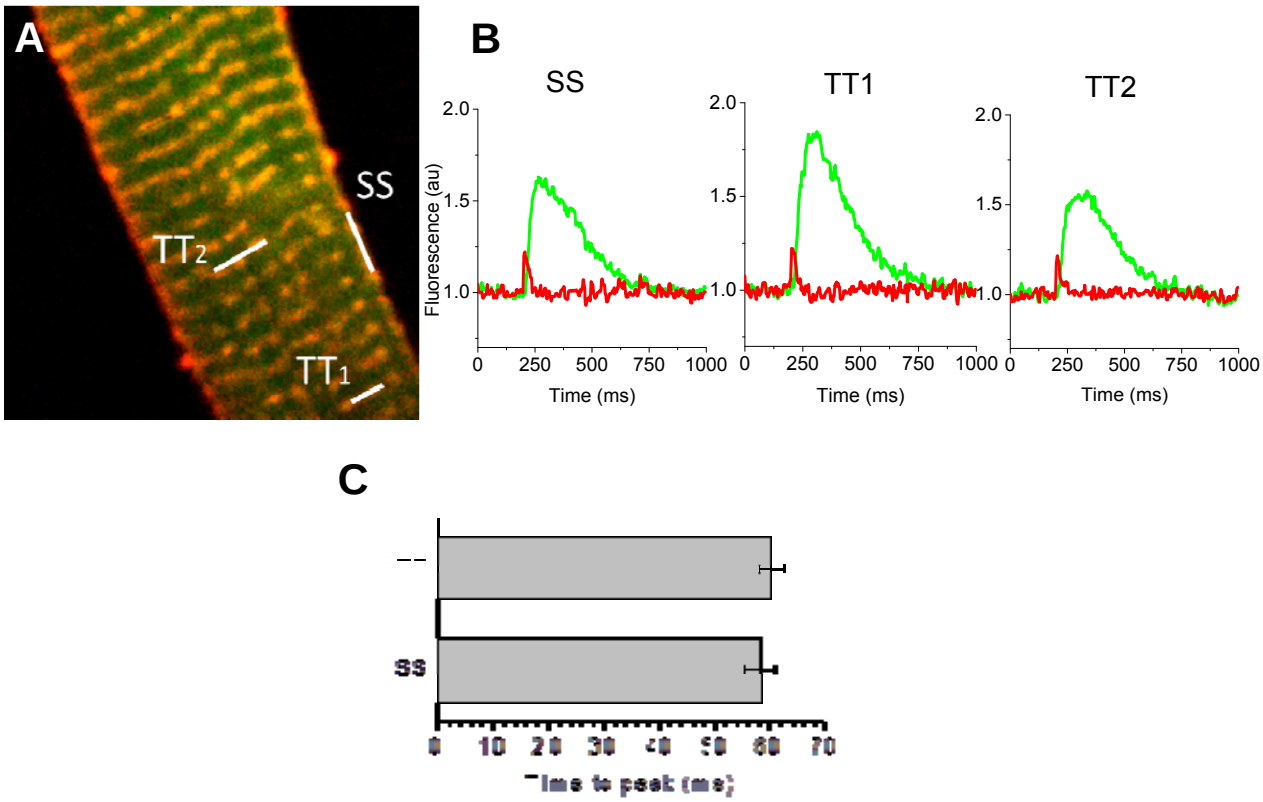

Fig. 2 Multisite optical recording in TATS. (A) Two-photon Fluorescence (TPF) image of a stained rat ventricular myocyte, Vm in red and Ca^{2+} in green. (B) Fluorescence traces from the scanned line indicated in panel A. AP was elicited at 200 ms. Vm signal in red and Ca^{2+} in green. (C) Histograms showing Ca^{2+}-release time-to-peak in SS and in tubules (TT). Each bar represents the mean ± standard error (SE).

In order to study the role of the single t-tubule in determining the local Ca^{2+}-release, we achieved a physical disconnection of TTs from SS (acute detubulation) with an osmotic shock technique (23). In brief, sudden increase of cell volume pulls t-tubules and detaches a large portion of them from the surface sarcolemma, thereby preventing electrical propagation of AP to the disconnected TATS elements. Staining was performed before detubulation (S/D), so that every tubule was labelled even if uncoupled from the surface. In fact, the osmotic shock procedure usually induces an incomplete detubulation; most TTs are disconnected while a sub-population maintains connections with SS. In this class of experiments, we found that after averaging 10 stimulations, those TATS elements that showed an AP, were characterized by a Ca^{2+}-release comparable to SS and control tubules. On the other hand, the tubules that didn't display an electrical activity exhibited a remarkable delayed Ca^{2+}-release.

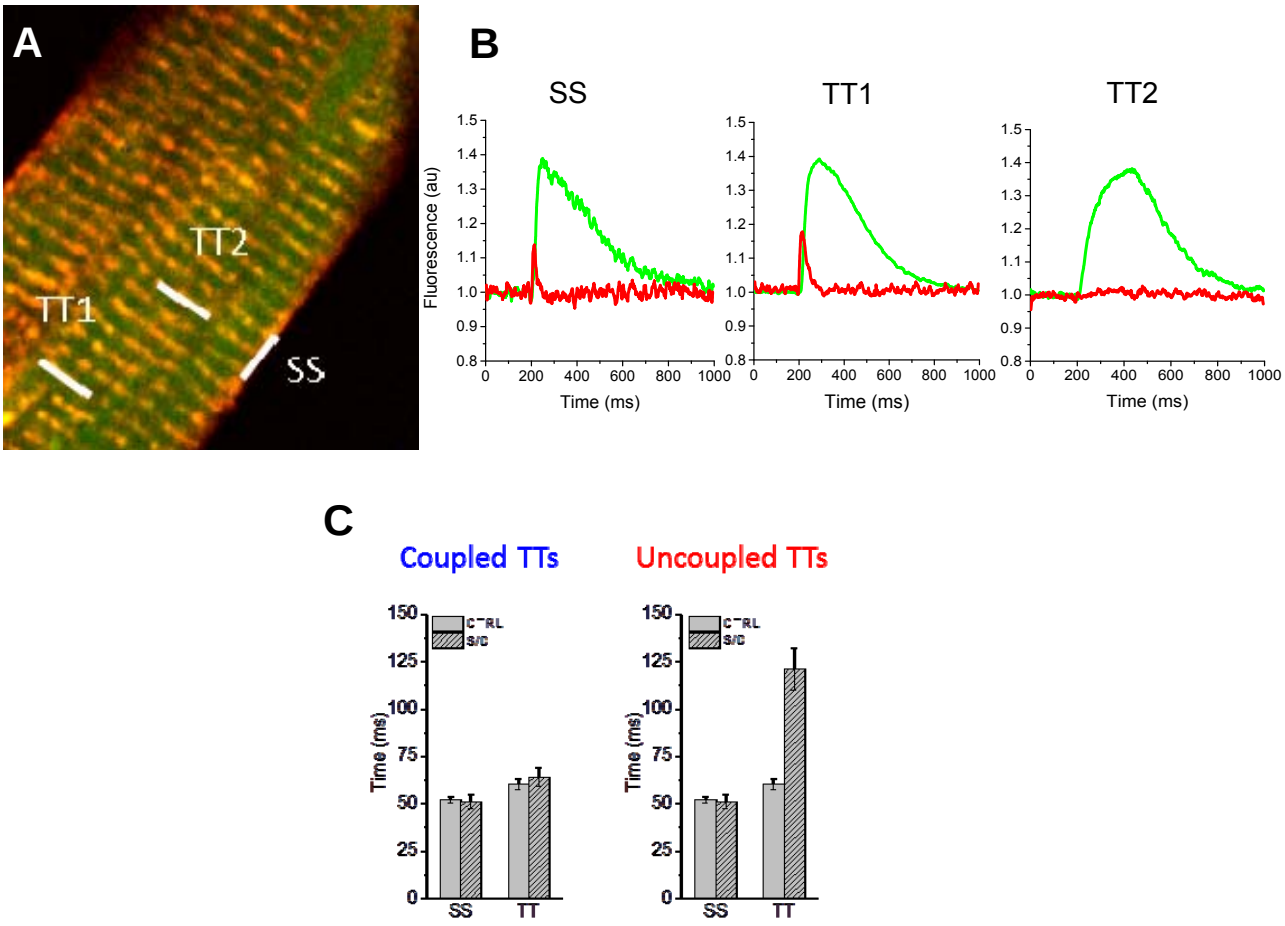

Fig. 3 Uncoupled tubules induce local Ca^{2+} delay. A) Two-photon Fluorescence (TPF) image of a stained rat ventricular myocyte, Vm in red and Ca^{2+} in green. After the staining the cell was detubulated with a formammide-induced osmotic shock procedure. (B) Simultaneous optical recording of voltage (red) and Ca^{2+} (green) performed the three different positions indicated in panel A surface sarcolemma (SS) and two t-tubules (TTi). Average of 10 consecutive APs. TT2 did not show electrical activation and AP were elicited at 200 ms. Vm signal in red and Ca^{2+} in green. (C) The two histograms compare the time to peak of Ca^{2+}-release measured in TT and SS of control (light grey) and detubulated cells (crossed grey) distinguishing the coupled and uncoupled tubules.

4 Conclusions

Combining the advantages of random access microscopy with the double staining approach, we developed an imaging method to simultaneously record APs and Ca^{2+}-release at multiple sarcolemmal sites with sub-ms temporal and sub-µm spatial resolution. We found that Ca^{2+} transients display the same kinetics in all probed sites. Control cells showed a stochastic variability in Ca^{2+}-release kinetics, both among the probed sites and among the consecutive . are a Notably, the average of 10 stimulations discloses the fact that, surface sarcolemma and tubular Ca^{2+} transient overlap perfectly without significant variation in the Ca^{2+} transient time to peak. This result underlines the relationship between the voltage space-clamp of the tubular system and the uniformity of Ca^{2+} release. Since the AP propagates through an electrical coupling between the surface sarcolemma and the tubular system network, we disconnected the tubular system from the surface thus preventing the tubular depolarization. In order to that, we used an osmotic-shock based technique. In this condition we found that the elicited AP is clearly visible in the surface sarcolemma but it is absent in some tubules. We observed that tubules showing AP cause a Ca^{2+} transient kinetically comparable to surface sarcolemma while electrically uncoupled tubules show slower Ca^{2+} rise. By separating the two populations of tubules, with and without AP, we found that while the Ca^{2+} transient time to peak of the disconnected tubules is statistically increased in comparison to

the surface sarcolemma, the connected tubules show a time to peak identical to the surface membrane. This result indicates that local Ca^{2+} transient is determined by the single tubule activity. This is an important finding, suggesting that the sporadic AP failures and spontaneous activity that we have found in heart failure (22) may produce spatial heterogeneities that cannot be overridden by functional healthy neighboring tubules. This indicates that structural integrity of the TATS is crucial to maintain a functional electrical coupling between the TATS and SS.

The simultaneous optical recording of AP and Ca^{2+}-release from different membrane domains in real-time not only reveals sarcolemma voltage space-clamp at unprecedented spatial and temporal resolution but also allows the first observation of single failure events occurring in structurally altered tubular systems. It is our perspective to investigate the pathological implications of this finding in failing hearts.

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