SIMULTANEOUS HIGH-THROUGHPUT Ca^{2+}, VOLTAGE AND CONTRACTILE MEASUREMENTS WITH THE CYTOCYPHER MULTICELL

Introduction

A major concern in the drug development process is the proarrhythmic potential that comes with prolongation of cardiac repolarization. After a series of withdrawals of approved drugs starting in the mid-90s due to lethal Torsade de points (TdP) arrhythmias, new guidelines for preclinical cardiac safety evaluation were issued. Blocking cardiac hERG channels by many compounds has been identified as a major contributor to the potentially fatal prolongation of cardiac repolarization[1, 2], making the in vitro hERG channel assay the standard method in use today. However, cardiac electrophysiology relies upon a plethora of de- and repolarizing currents some of which, like I_{CaL} and I_{NCX}, are tightly coupled to Ca^{2+} release and reuptake[3]. Thus, cardiac safety testing in model systems that provide not only a representative composition of ion channels but also intact Ca^{2+} homeostasis, may detect potentially arrhythmic compounds that a simple hERG assay might miss. On the other hand, the attrition of safe compounds, like Verapamil, known to block hERG channels but due to concomitant effects on other channels does not carry significant proarrhythmic risk[4], may be avoided. With the introduction of new voltage sensitive dyes (VSD) and high-throughput systems capable of measuring hundreds of myocytes fully automatically, a more comprehensive cardiac safety testing regimen is now within reach. The proof-of-concept experiments presented here show the feasibility of this approach.

Methods

Cell isolation

Animal experiments were performed in accordance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and approved by the ethics committee of VU Medical Center, Amsterdam, the Netherlands.

Adult wild-type male Wistar rats (N=2) were euthanized, the chest was opened and the heart was injected with cold Tyrode's solution (composition: 130mM NaCl, 5.4mM KCl, 3mM sodium pyruvate, 25mM HEPES, 0.5mM MgCl2, 0.33mM NaH2PO4, 22mM glucose) containing 0.2mM EGTA (Tyrode's–EGTA) at pH 7.4.

The heart was mounted on a Langendorff apparatus and retrogradely perfused via the aorta for 2 min with Tyrode's-EGTA solution at 37°C to wash out remaining blood and fully arrest the heart. Thereafter the solution was switched to a collagenase containing Tyrode's solution supplemented with 50µmol Ca^{2+} and recirculated for 7 min.

After enzymatic digestion, the left ventricle was separated from the atria and the right ventricle, then cut into small chunks. Single myocytes were carefully dissociated by trituration with a plastic Pasteur pipette, followed by filtering the suspension through a 300 µm nylon mesh. Ca^{2+} was re-introduced before plating cells on Laminin-coated 35mm glass bottom dishes.

Before dye loading, non-attached cells were washed off with warm Tyrode's solution.

Loading protocol

To load cells with the Ca^{2+} indicator Rhod-2, Rhod-2/AM (ThermoFisher) was dissolved in DMSO to a final concentration of 1mM and kept as stock solution at -20°C. Rhod-2 stock solution was further diluted in 1ml Tyrode solution to a final concentration of 5 µmol/L.

In the same solution FluoVolt™ and PowerLoad™ (ThermoFisher) were diluted at 1:1000 and 1:100 respectively. Tyrode's solution was aspirated from the dish and replaced with the dye mixture. After 30 min incubation in the dark at 37°C, the dye containing solution was removed and replaced with warm Tyrode's solution, followed by 20 min de-esterification before mounting the dish onto the CytoCypher MultiCell microscope.

System setup

The optical system was based on the CytoCypher MultiCell microscope. The excitation light was supplied by a 470nm LED for FluoVolt and a 565nm LED for Rhod-2 powered by a dual OptoLED power supply unit (Cairn Research) and driven at 1A and 1.5A, respectively.

Excitation light was filtered through 470/40x and 570/20x single bandpass filters and combined by a 495 DC mirror. The excitation light was then reflected by a 405-488/568 dual bandpass polychroic mirror (Chroma) within the microscope and guided through a UPlanXApo 40x 0.95NA objective (Olympus) to the myocyte. A manually adjustable pinhole in the light path restricts excitation light to the myocyte in the region of interest.

The microscope condenser was equipped with IR-LEDs (>850nm) supplying light for contrast-based sarcomere measurements.

A 650 DC mirror reflected both fluorophore's emission while passing IR light for contrast-based sarcomere length measurements to the attached Myocam-S3 (IonOptix). The reflected emission light was further split by a 564 DC mirror. Emission from FluoVolt was filtered through a 535/36 bandpass filter and collected by a photomultiplier tube, or PMT (Electron Tubes). Emission light from Rhod-2 was filtered through a 609/34 bandpass filter and recorded with a red-shifted PMT (SensTech).

Cells were electrically stimulated by a biphasic pulse of 4 ms at 1Hz or 2Hz delivered by an IonOptix MyoPacer. Fluorescence signals were digitized at 1000Hz, whereas FFT-based sarcomere length data was calculated at 250Hz. Signals were recorded using IonWizard data acquisition software (IonOptix).

Results

Simultaneous recording of Ca^{2+} transients (CaT) and membrane voltage (Vm) with FluoVolt and Rhod-2

To test the separation of Rhod-2 and FluoVolt emission spectra, myocytes were loaded with either dye separately. Rhod-2 loaded myocytes excited at 565nm did not evoke a detectable signal in the FluoVolt emission channel (Fig. 1A). When loading myocytes with FluoVolt alone a fraction of the emission signal could be registered in the Rhod-2 detection channel, due to the wide red tail of FluoVolt's emission spectrum (Fig. 1B). The bleed-through of FluoVolt's emission artefact, however, was negligible when compared to Rhod-2's emission signal (<10 counts, or less than 5%, of typical Rhod-2 emission). Thus, we did not attempt to unmix signals for all other experiments presented below.

FluoVolt offers excellent signal to noise ratio

For VSDs, the signal to noise ratio (S/N) and the change in fluorescence intensity upon membrane depolarization is a major concern. Dual loaded myocytes exhibited ΔF of ~20% (Fig. 2A) for FluoVolt and peak F/F_0 of ~2 for Rhod-2. A peak S/N for FluoVolt without averaging or filtering of 12 was attainable, at the same time the S/N for RHOD2 was >20 S/N. Averaging data from 5 beats further increased the VSD S/N to >15.

Photo-damage/toxicity

Another concern when measuring OAPs with VSD is phototoxicity and artificial APD prolongation. Indeed, we found that in a subset of myocytes such APD prolongation became apparent after several seconds of measurement (Fig. 3B), whereas in the remaining myocytes 10 sec long recordings could be obtained without notable changes to the APD.

Discussion

A major challenge when attempting to measure two fluorophores simultaneously is registration of emission light from the first dye into the second detector due to an overlap in emission spectra or insufficient separation of emission light. In imaging applications several methods can be employed to tackle this issue[5]. For photometric approaches, these methods either slow down acquisition or are simply not applicable. Thus, in a first step we verified that crosstalk-free acquisition can be achieved with the proposed setup. Our experiment showed that combination of FluoVolt and Rhod-2 indeed satisfies the requirements for such measurements without the need to unmix signals or rapidly switch between light sources. This allowed us to recording OAPs and CaT at rates up to 2000Hz (Data not shown).

Another concern regarding the use of VSD is the change in fluorescence intensity upon the change in membrane potentials. With FluoVolt, a dye based on photo-induced electron transfer, we achieved ~20-30% change in fluorescence upon depolarization which is significantly higher than the well know VSD Di-4-ANEPPS (2-10%) and in line with newer generation electrochromic VSD, such as Di-2-AN(F)EPPTEA or Di-4-ANBDQBS[6]. FluoVolt's more pronounced ΔF greatly improves S/N and reduces the need for averaging or otherwise filtering the acquired signal, reducing the time needed to acquire resolvable data.

The well documented phototoxicity of VSD[6] makes shortening the recording time an absolute necessity in order preserve the naïve APD of isolated cardiac myocytes. Although FluoVolt's phototoxicity has been reported to be lower than other VSD[7], photodamage remains an issue that needs to be considered when recording OAPs.

Preliminary results suggest that improvements to the optical setup, such as employing higher NA oil-immersion objectives, could further improve upon S/N despite lower dye concentrations or reduction in excitation light intensity (data not shown), which could minimize the risk of phototoxicity.

Our proof-of-concept experiments demonstrate that simultaneous recordings of OAPs, CaT and SL in adult cardiac myocytes with the CytoCypher MultiCell system are feasible and reproducible. As a modular add-on to existing hardware, this setup configuration should allow measurements of OAPs from hundreds of myocytes per hour. This throughput would significantly improve the statistical power over lower throughput measurements, like patch clamping, and enable more comprehensive drug screening while simultaneously revealing changes in contractility and Ca^{2+} homeostasis.

1. De Bruin ML, Pettersson M, Meyboom RH, Hoes AW, Leufkens HG. Anti-HERG activity and the risk of drug-induced arrhythmias and sudden death. *Eur Heart J* 2005;**26**:590-597.
2. Roden DM. Cellular basis of drug-induced torsades de pointes. *Br J Pharmacol* 2008;**154**:1502-1507.
3. Bers DM. Cardiac excitation-contraction coupling. *Nature* 2002;**415**:198-205.
4. Zhang S, Zhou Z, Gong Q, Makielski JC, January CT. Mechanism of block and identification of the verapamil binding domain to HERG potassium channels. *Circ Res* 1999;**84**:989-998.
5. Zimmermann T, Marrison J, Hogg K, O'Toole P. Clearing up the signal: spectral imaging and linear unmixing in fluorescence microscopy. *Methods Mol Biol* 2014;**1075**:129-148.
6. Warren M, Spitzer KW, Steadman BW, Rees TD, Venable P, Taylor T, Shibayama J, Yan P, Wuskell JP, Loew LM, Zaitsev AV. High-precision recording of the action potential in isolated cardiomyocytes using the near-infrared fluorescent dye di-4-ANBDQBS. *Am J Physiol Heart Circ Physiol* 2010;**299**:H1271-1281.
7. Salerno S, Garten K, Smith GL, Stolen T, Kelly A. Two-photon excitation of FluoVolt allows improved interrogation of transmural electrophysiological function in the intact mouse heart. *Prog Biophys Mol Biol* 2020;**154**:11-20.

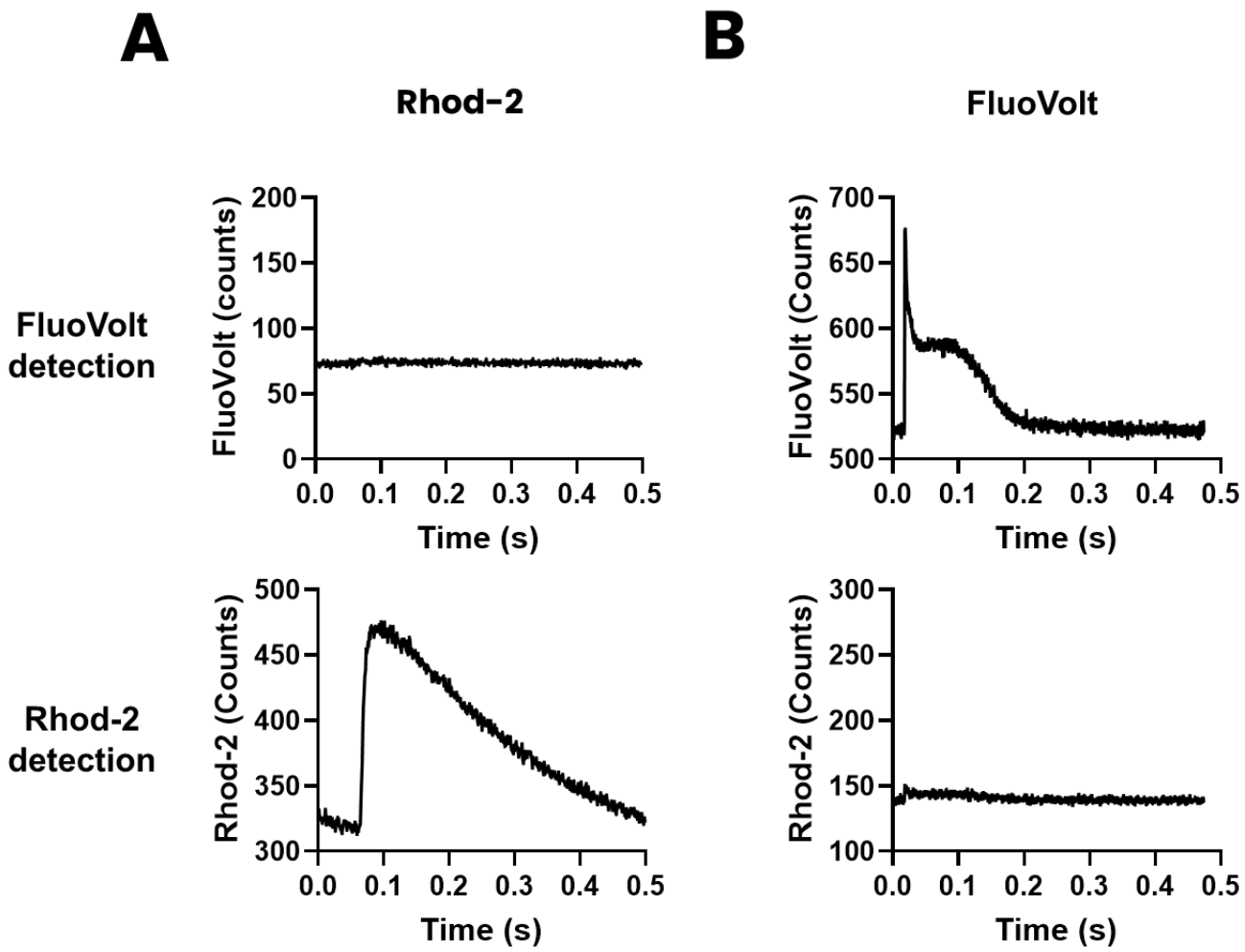

Figure 1. Sufficient separation of Rhod-2 and FluoVolt for simultaneous recoding. (A) Myocyte loaded with Rhod-2. No signal in the FluoVolt detection channel (top). High S/N Ca^{2+} transient in Rhod-2 channel (bottom). **(B)** FluoVolt loaded myocyte with corresponsing Vm signal (top) and minimal crosstalk in the Rhod-2 detection channel (bottom). Average of 20 beats recroded at 2Hz.

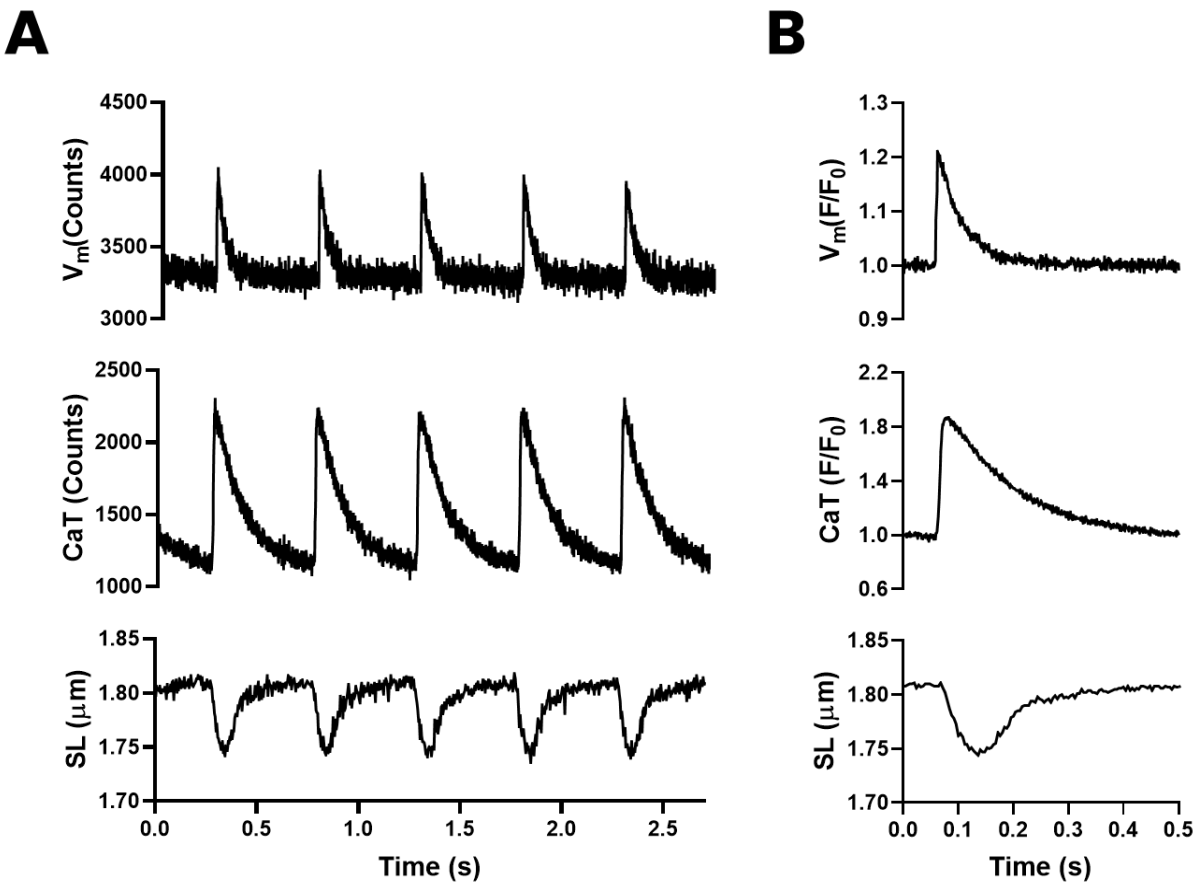

Figure 2. Simultaneous measuremtents of Vm, CaT and SL. From top to bottem: FluoVolt (Vm) signal, Rhod-2 (CaT) signal and FFT based sarcomere length (SL). **(A)** Raw unfiltered traces recorded at 2Hz **(B)** Averaged traces

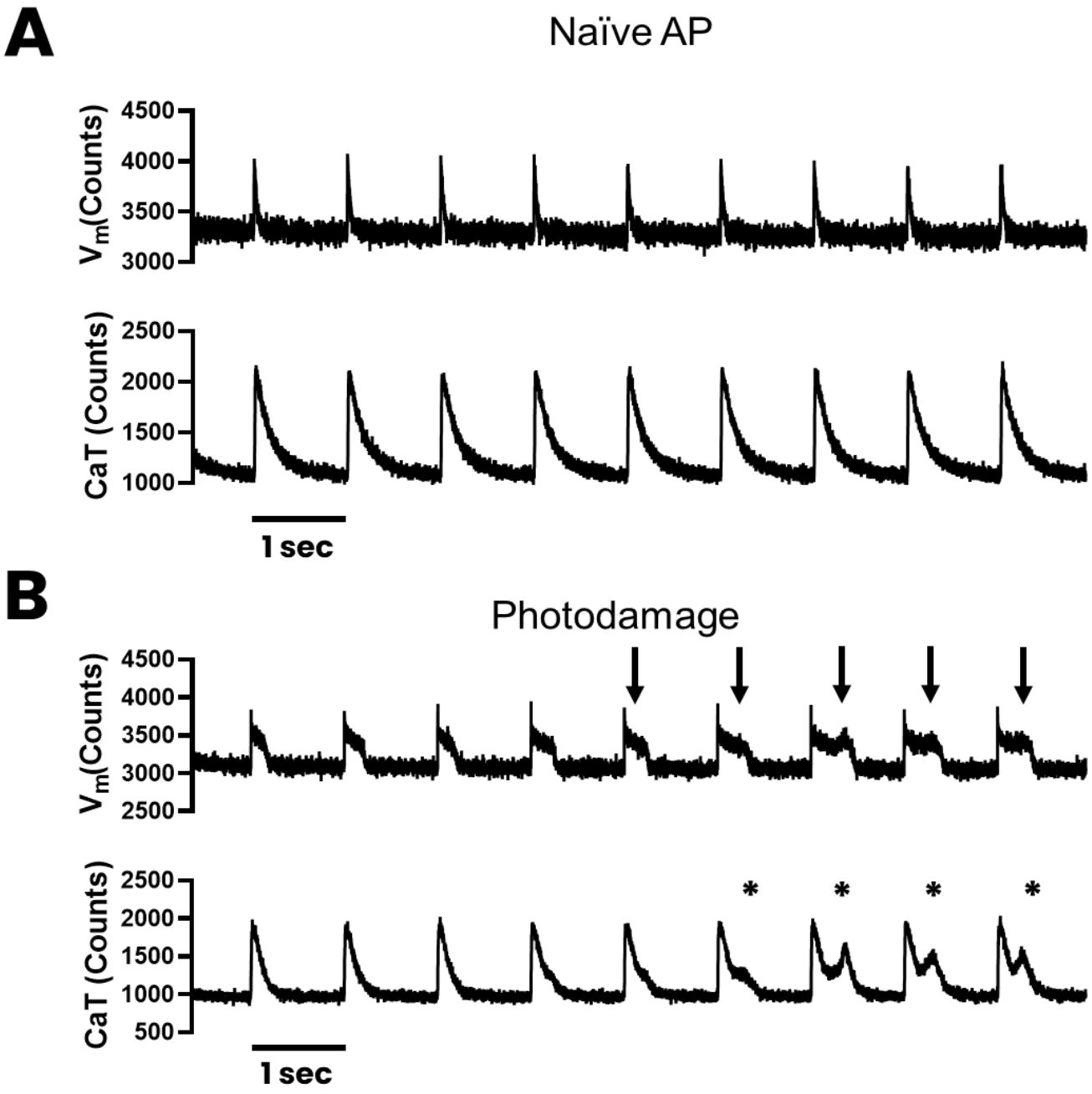

Figure 3. FluoVolt phototoxicity evokes EADs in rat myocytes. (A) Myocyte without apparent changes to the action potential duration (APD) or CaT kinetics. **(B)** Myocyte with clear APD prolongation and EADs (arrows)**,** CaT inline with ICaL reactivation (*).

Patrick Schönleitner, MD
Research Scientist
Office: +353 (0)1 6854800
Email: patrick@ionoptix.com
Web: www.IonOptix.com