Journal of Visualized Experiments

Single-cell optical action potential measurement in human induced pluripotent stem cell-derived cardiomyocytes

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61890R1
Full Title:	Single-cell optical action potential measurement in human induced pluripotent stem cell-derived cardiomyocytes
Corresponding Author:	Niels Voigt, M.D. University Medical Center Göttingen Göttingen, Lower Saxony GERMANY
Corresponding Author's Institution:	University Medical Center Göttingen
Corresponding Author E-Mail:	niels.voigt@med.uni-goettingen.de
Order of Authors:	Fitzwilliam Seibertz
	Martyn Reynolds
	Niels Voigt
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Goettingen, Lower Saxony, Germany
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the UK Author License Agreement (for UK authors only)
Please specify the section of the submitted manuscript.	Biology
Please provide any comments to the journal here.	

TITLE:

Single-Cell Optical Action Potential Measurement in Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes

AUTHOR AND AFFILIATION:

Fitzwilliam Seibertz[1,2], Martyn Reynolds[3], Niels Voigt[1,2,4]

[1]Institute of Pharmacology and Toxicology, University Medical Center Goettingen, Georg-August University, Goettingen, Germany
[2]DZHK (German Center for Cardiovascular Research), partner site Goettingen, Germany
[3]Cairn Research Ltd, Faversham, United Kingdom
[4]Cluster of Excellence "Multiscale Bioimaging: from Molecular Machines to Networks of Excitable Cells" (MBExC), University of Goettingen, Germany

Email Addresses of Co-Authors:
Fitzwilliam Seibertz (will.seibertz@med.uni-goettingen.de)
Martyn Reynolds (m.reynolds@cairn-research.co.uk)

Email Address of Corresponding Author
Niels Voigt (niels.voigt@med.uni-goettingen.de)

KEYWORDS:

voltage sensitive dye, fluorescence, action potential, induced pluripotent stem cell, cardiomyocyte, optical

SUMMARY:

Here we describe optical acquisition and characterization of action potentials from induced pluripotent stem cell derived cardiomyocytes using a high-speed modular photometry system.

ABSTRACT:

Conventional intracellular microelectrode techniques to quantify cardiomyocyte electrophysiology are extremely complex, labor intensive, and typically carried out in low throughput. Rapid and ongoing expansion of induced pluripotent stem cell (iPSC) technology presents a new standard in cardiovascular research and alternate methods are now necessary to increase throughput of electrophysiological data at a single cell level. VF2.1Cl is a recently derived voltage sensitive dye which provides a rapid single channel, high magnitude response to fluctuations in membrane potential. It possesses kinetics superior to those of other existing voltage indicators and makes available functional data equivalent to that of traditional microelectrode techniques. Here, we demonstrate simplified, non-invasive action potential characterization in externally paced human iPSC derived cardiomyocytes using a modular and highly affordable photometry system.

INTRODUCTION:

Electrophysiological modeling of cardiomyocytes and the construction of efficient platforms for cardiac drug screening is essential for the development of therapeutic strategies for a variety of arrhythmic disorders. Rapid expansion of induced pluripotent stem cell (iPSC) technology has produced promising inroads into human disease modelling and pharmacological investigation using isolated patient derived cardiomyocytes (iPSC-CM). "Gold standard" techniques for

electrophysiological characterization of these cells through patch-clamp (current-clamp) can quantify action potential (AP) morphology and duration, however, this method is incredibly complex and slow, and not well suited for high throughput data acquisition[1]. iPSC-CMs are regularly reported to have an increased diastolic membrane potential and increased leak current when compared to adult native cardiomyocytes[2]. It is suggested that smaller cell size and reduced membrane capacitance observed in iPSC-CMs may produce some systematic error when using the current-clamp technique, perhaps explaining these deviations[3]. In order to maximize the usefulness of an iPSC-CM platform, an additional method is valuable to increase throughput and ensure data accuracy when characterizing transmembrane voltage changes at a single cell level in iPSC-CMs.

Voltage sensitive dyes (VSD) have long been a proposed method to provide faster, non-invasive and equivalent analysis of cardiac AP kinetics comparative to those of traditional techniques[4]. A recent study has demonstrated the suitability of ratiometric voltage sensitive probe photometry to accurately quantify the cardiac AP[5]. Furthermore, the ability to readily scale up optical photometry approaches lends this technique to large scale cardiotoxicity screens critical in therapeutic drug development (e.g., CiPA). Development of standardized cardiotoxicity protocols in a blinded multi-site study using microelectrode array and voltage-sensing optical techniques has demonstrated the key value of this approach[6].

Many potentiometric dyes are commercially available, and ongoing synthetic development of new probes show exciting potential for streamlining their effectiveness across a variety of cardiac and neural constructs. The ideal VSD will have augmented kinetics and sensitivity, while displaying decreased capacitive load, photobleaching and cytotoxicity. The recently synthesized VF2.1Cl (e.g., FluoVolt) expresses many of these beneficial properties largely due to its novel wire-based molecular structure, shared by other members of the new VoltageFluor (VF) family[7]. In contrast to common electrochromic VSDs in which simple probes molecularly and electrically conjugate to the plasma membrane, this dye consists of a passively inserted, membrane-spanning synthetic wire which pairs an electron-rich donor with a modified fluorescein fluorophore (FITC). Mechanistic details are provided in **Figure 1**. This dye demonstrates excellent sensitivity to membrane voltage fluctuations, displaying a 27% change in emission intensity per 100 mV as opposed to ~10% seen in other common probes at comparable speeds[7]. In addition, wire-based PeT systems do not directly interact with the cellular electric field which produces minimal electrical interference and negligible changes in cellular capacitive load.

[Insert **Figure 1** here]

The FITC probe conjugated to VF2.1Cl ensures that it can be used effectively under standard and GFP filter configurations, and it only requires a single channel acquisition system, both of which are common features of fluorescent imaging platforms. Analysis of dense human iPSC-CM monolayers with this dye has been recently reported[8–11]. Our protocol differs to these studies due to our investigation of single, isolated iPSC-CMs, unperturbed by the electrical and paracrine influences of dense syncytial monolayers, and our use of an affordable and customizable photometry system as opposed to complex confocal or wide-field imaging arrangements.

Here, we describe our protocol for the rapid acquisition and analysis of robust optical APs from isolated human iPSC-derived cardiomyocytes and native cardiomyocytes (see **Supplementary**

File). We use VF2.1Cl coupled with a customizable state of the art platform for single cell photometry measurements.

PROTOCOL:

1. Cellular preparations

NOTE: Human iPSCs used in this protocol were derived from healthy donors and differentiated in monolayers using fully defined small molecule modulation of WNT signaling and lactate purification techniques as previously described[12–14]. iPSC-CMs were maintained every 2-3 days with a culture medium outlined below.

1.1. Prepare a culture medium of basal medium (RPMI 1640) and 2% supplement (B27). Store at 4 °C. Use at RT.

1.2. Prepare a plating medium of basal medium (RPMI 1640), 2% supplement (B27) and 1:2000 ROCK inhibitor. Store at 4 °C. Use at RT.

1.3. Coat sterilized 10 mm round glass #0 coverslips with 150 µL of 1:60 factor free basement membrane matrix and incubate at 4 °C for 4 h.

NOTE: Optimization of coverslip volume is necessary to ensure the entire glass is covered while maintaining adequate surface tension to prevent spillage. 150 µL is recommended for 10 mm round coverslips. Batch size, coverslip type, coverslip volume and culture plate type can be suited to the experimenters needs.

1.4. Begin iPSC-CM dissociation with an EDTA-based cell dissociation reagent. Ensure that the monolayer is completely detached by gently flushing with a 1,000 µL pipette.

1.5. Transfer the cellular suspension into a 15 mL tube and add double volume plating medium. Centrifuge for 10 min at 100 x *g*.

1.6. Resuspend the pellet with a desired volume (resuspension volume) of plating medium. Count cells manually or electronically.

1.7. Select optimal density per coverslip (15,000) which will allow for the isolated cellular analysis later.

1.8. Calculate the volume of 'active' cellular suspension (A) needed to plate all coverslips at this desired density. Apply the following formula and withdraw into a separate tube:

$$A\ [\mu l] = \frac{Resuspension\ Volume\ [\mu l]}{\left(\frac{Total\ Cell\ Count}{Desired\ Density}\right)} \times Coverslip\ number$$

1.9. Calculate the volume of extra plating medium (B) needed to accommodate each coverslip at a desired volume. Apply the following formula and add the resulting volume to the tube of active suspension:

$$B\ [\mu l] = (Coverslip\ number\ \times\ Coverslip\ Volume\ [\mu l]) - A\ [\mu l]$$

1.10. Remove the matrix from coverslips and apply the 'coverslip volume' of cell suspension (A+B) to each coverslip. Regularly resuspend in the tube to ensure even cellular distribution.

1.11. Incubate at 37 °C for 1 h. Gently fill the well with plating medium.

1.12. After 24 h, exchange media with normal culture medium and maintain every 2-3 days.

2. Experimental setup

2.1. Equip an inverted epifluorescence microscope with a 40x magnification, high numerical aperture lens (N.A: > 0.75) to conduct experiments.

2.2. Couple a fast switching warm white LED to the transmitted illumination port of the microscope. Insert a simple red 660 nm filter into the transmitted light path.

NOTE: This light can be activated throughout photometry experiments to observe the sample without contaminating the green fluorescent signal.

2.3. Mount a fast switching 470 nm LED head for photometry recording. Insert a 470/40 excitation filter at the epifluorescent port of the microscope to clean-up the light generated by the LED.

NOTE: For optimum signal quantification an illumination system with high speed feedback control of optical output is recommended.

2.4. Insert a microscope cube containing a 495 nm long pass beam splitter in the mirror unit carousel within the microscope.

2.5. Fit a detection arm containing an adjustable field diaphragm to the microscope C-mount port to allow region of interest selection.

2.6. Separately couple a photomultiplier detector (PMT) and a USB camera to the microscope. This will form the basis of the emission detection system.

2.7. Insert a filter cube containing a 565 nm long pass beam splitter and a 535/50 emission filter into the PMT port. This splits emission light between the two detectors.

NOTE: A camera attached to the transmitted port of the emission detection system can detect transmitted light under brightfield throughout all experiments.

2.8. Couple the PMT to a power supply and a PMT amplifier. Connect the PMT amplifier output to an analogue input pin of a data acquisition system.

2.9. Filter analogue data from the PMT at 1 kHz or higher.

2.10. Digitize data at a frequency that is at least double that of the highest frequency present in analogue signal (2 kHz or higher) to fulfill Nyquist criteria and prevent aliasing.

3. Cellular loading with VF2.1Cl

NOTE: All steps involving this dye must be carried out in low light conditions.

3.1. Prepare a Tyrode's bath solution of (in mM): 140 NaCl, 10 HEPES, 10 Glucose, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, pH = 7.35 and warm to 37 °C.

3.2. Prepare an aliquot of loading solution in a microcentrifuge tube by mixing 5 µL of 1,000x VF2.1Cl and 50 µL of 20% solubilizing poloxamer solution.

3.3. Apply 5 µL of the loading solution to 5 mL of warmed Tyrode's solution (total 0.1x dye concentration) in a 20 mm Petri dish.

NOTE: Final dye concentration is 0.1x. This is $1/10^{th}$ that suggested by the manufacturer. This conserves resources, ensures negligible cytotoxicity, and importantly, still retains clear optical signals from loaded cells with high signal to noise ratios.

3.4. Add a single iPSC-CM cover slip to the dish and incubate at 37 °C for 20 min.

3.5. Assemble a heated live cell imaging chamber. Mount onto the microscope stage and fill with 500 µL of fresh Tyrode's solution.

3.6. Wash the coverslip with fresh Tyrode's solution at 37 °C.

3.7. Carefully apply the iPSC-CM coverslip to the pre-warmed bath chamber using fine point forceps.

NOTE: Ensure the chamber and its contents are always heated at physiological temperatures. If desired, commence with continuous perfusion of warmed Tyrode's solution.

4. Electrical field stimulation

NOTE: External triggering of iPSC-CM is optional but useful for standardization of cellular dynamics and experimental parameters. It increases the ease of analysis and, also allows for the investigation of frequency-dependent effects.

4.1. Attach a stimulation insert with two platinum electrodes spaced 5 mm apart into the recording chamber.

4.2. Connect an external stimulator to the stimulation insert. Set to 5 ms bipolar field pulses at 0.5 Hz.

4.3. Determine optimal stimulation voltage by increasing the stimulus from 1 V upwards. Threshold stimulus is defined as the lowest voltage at which cells begin to contract. Apply voltages roughly 25% above this threshold. Normal range is between 1 V and 30 V.

4.4. Fix stimulation frequency with the external stimulator or trigger it with acquisition software.

5. Optical action potential acquisition

NOTE: This protocol uses a commercial software for acquisition and analysis.

5.1. Visualize the myocytes in brightfield view using the transmitted light path and the USB camera.

5.2. Select an isolated cell and tightly crop its optical path with the field diaphragm ensuring only light from the cell of interest is monitored.

5.3. Activate the PMT amplifier and set the PMT supply to 750 V.

5.4. Run the stimulation protocol (see step 4) along with the acquisition software and simultaneously activate the 470 nm excitation light. The latter can be done via a remote panel or automated at a fixed intensity (TTL signal).

5.5. Adjust gain and offset of the PMT amplifier to make sure the signal does not saturate and is optimized for the detection range of the recording system.

5.6. Record 10 sweeps ensuring stable action potentials are detected.

5.7. Continue recording and immediately move the microscope stage to briefly acquire background signal from a region devoid of cells. Turn off the excitation light.

NOTE: This background value (F_{offset}) will be used to account for any background fluorescence.

5.8. If desired, locally perfuse reference drugs such as 1 µM nifedipine to identify cellular responses to pharmacological manipulation.

5.9. In a sequential fashion, repeat steps 5.2–5.7, each time selecting a new cell. Swap out coverslips if desired to ensure high experimental turnover in a single sitting.

6. Data analysis

6.1. Open a saved recording with the analysis software and average 10 sweeps containing stimulated action potentials from a single cell.

6.2. Take a mean of the baseline signal representing F_{offset} and subtract this from the averaged trace.

6.3. Calculate ΔF/F0 with the following formula (where F is measured fluorescence and F0 is diastolic fluorescence):

$$\Delta F/F_0 = \frac{F - F_0}{F_0}$$

6.4. Identify the trace baseline (diastolic) and area of interest (AP) and measure the desired cardiac action potential parameters. This includes but is not limited to the decay time for 50% (APD_{50}) and 90% (APD_{90}) repolarization.

6.5. Export the data from this single cell to a spreadsheet software.

6.6. Repeat steps 6.1 – 6.5 for all recordings. Assess results with appropriate unpaired tests or analysis of variance.

NOTE: Loading and image acquisition protocols are described in **Figure 2.**

[Insert **Figure 2** here]

REPRESENTATIVE RESULTS:

[Insert **Figure 3** here]

A high signal to noise ratio was regularly observed in our samples, despite the reduced field of view when focusing on a single cell. More noise was observed in smaller iPSC-CMs, but that did not hinder analysis subsequent to ensemble averaging. AP morphology is clearly defined, giving a thorough overview of cellular electrophysiological function and repolarization mechanics across cardiomyocyte constructs. Of note, here is the previously described[15] sharp upstroke velocity and pronounced phase 1 features of murine cardiomyocytes (APD_{50}: 58.34±17.98 ms, APD_{90}: 160.9±30.15 ms, n=7; **Figure 3A**) which are morphologically distinct from human iPSC-CM optical signals (APD_{50}: 170.1±11.18 ms, APD_{90}: 317.5±15.56 ms, n=48; **Figure 3B**). We observed a higher rate of photobleaching and cellular toxicity after optical investigation in native cardiomyocytes compared with cultured human iPSC-CMs. Protocols for preparation and dye loading in native cardiomyocytes are included in the **Supplementary Material**.

Human iPSC-CM are responsive to pharmacological manipulation with nifedipine (1 µM). A known L-type Ca^{2+} channel ($Ca_V1.2$) antagonist, nifedipine is expected to decrease the AP duration in cells with physiological function. During continuous drug application, a 41.5% decrease in APD_{90} was observed (n=5), suggesting both the physiological expression of $Ca_V1.2$ channels in these cells and the functionality of VF2.1Cl-based imaging as a platform for prospective high throughput cardiac drug screening studies (**Figure 3C**).

FIGURE LEGENDS:

Figure 1: Chemical, spectral and mechanistic parameters of VF2.1Cl dye. (**A**) Chemical structure of VF2.1Cl. Molecular features to note include multiple alkyl groups within the phenylene vinylene molecular wire which facilitate insertion into the plasma membrane. A negatively charged sulfonic acid group conjugated to the FITC probe ensures fluorophore stabilization on the extracellular surface and aids near perpendicular insertion relative to the electrical field of the lipid bilayer. (**B**) A simplified schematic of perpendicular VF2.1Cl embedding into the plasma membrane of a target cell. (**C**) Absorption and emission spectra of VF2.1Cl dye. Spectra is identical to that of standard FITC and GFP probes. (**D**) Depiction of the mechanistic mode of action of VF2.1Cl. In resting conditions (hyperpolarized), negative intracellular voltages drive free electrons towards the rostral fluorophore. Electron abundance ensures photo-induced electron

transfer (PeT) is favored as a pathway out of the excited state after the optical excitation, effectively quenching fluorescence. In contrast, a depolarized membrane potential influences downward electron movement favoring fluorescence upon optical excitation. The resulting fluorescent response is linearly related to membrane voltage and can be precisely utilized to gather detailed temporal information on cellular electrophysiological kinetics. (**E**) Representative brightfield (upper) and fluorescence at 470 nm (lower) images of leporine cardiomyocytes loaded with VF2.1Cl. (**F**) Z stack of a single loaded cardiomyocyte. Arrows indicate areas of clear localization of VF2.1Cl to the cellular membrane. Images were acquired with a spinning disk confocal system consisting of a X-lightv3 spinning disk confocal head with a 50 µm pinhole pattern; LDI-7 illuminator (89 north); Prime95B camera and a PlanApo Lambda 100x objective. Scale bar: 20 µm.

Figure 2: Loading and image acquisition protocols (**A**) Flow chart of complete VF2.1Cl loading protocol for iPSC-CMs and native cardiomyocytes. (**B**) A simplified schematic of beam splitter (BS) and filter configurations used in this protocol for excitation and detection of VF2.1Cl emission in response to changes in transmembrane voltage.

Figure 3: Optical action potential (AP) profiles of isolated native cardiomyocytes and human induced pluripotent stem cell derived cardiomyocytes (iPSC-CM). (**A**) Representative optical AP of a single murine cardiomyocyte (center) with Mean ± SEM of APD_{50} and APD_{90} (n = 7, right). (**B**) Representative optical AP of a single human iPSC derived cardiomyocyte (center) with Mean ± SEM of APD_{50} and APD_{90} (n = 48, right). (**C**) Pharmacological manipulation of iPSC-CM AP (center) with 1 µM nifedipine. Mean ± SEM of APD_{90} alteration after nifedipine application (n=5). $^{**}p < 0.01$.

Table 1: A brief list of common VSDs and their major fluorescent properties.

Supplementary Material: Native murine cardiomyocyte preparation for voltage imaging.

DISCUSSION:

Here we describe a basic protocol to easily acquire detailed AP profiles from isolated iPSC-CMs suitable for electrophysiological modelling and cardiac drug screening. We detect regular, robust APs from our sparsely seeded iPSC-CMs which suggests both indicator functionality and methodological fidelity.

Due to the wide spectrum of commercial methodologies for iPSC reprogramming and lack of standardization for cardiac differentiation protocols, iPSC based models can show immense variability in their function and morphology[16]. This can also hinder the effectiveness of cardiotoxicity studies. We report generally robust responses to extended experimental investigation with minimal indicator-induced cytotoxicity at low excitation light intensities. Standardization of basic dissociation and coverslip seeding is critical to ensure consistent quality of iPSC-CM preparations. Loose coverslips, which can be easily inserted and replaced inside the imaging platform, are incredibly useful for rapid data acquisition and characterization of single cardiomyocytes. It should be noted however, that we do observe a slight decline in cell viability after extended periods (i.e., more than 4 weeks) of sparse culture on glass coverslips.

The modular construction of a high-speed photometry system outlined in step 2 is of critical importance to this protocol. Many optics-based setups are commercially available and can be

optimized for a wide range of signal recording requirements. These range from quantitative imaging using high resolution, large area cameras through to photometry measurements of total signal from a defined area. Using the latter, we quantify fluorescence at high speed from a single masked area using a photomultiplier (PMT). Combined with fast switching illumination components, this allows for thorough dissection of fast action potential components with extremely high temporal resolution (analogue signal up to 1 kHz). High sampling rates are required to ensure reliable measurements of action potential upstroke in cardiomyocytes and can be critical in other excitable constructs (i.e. neurons) with extremely fast excitation kinetics. Further, this flexible system is beneficial because 1) it allows for thorough investigation of single cell electrophysiology with field diaphragm selection, 2) digitization of the analogue signal is not integrated or pre-processed and is therefore directly under experimenter control, 3) the modular nature of the photometry instrumentation allows simple reconfiguration to enable simultaneous optical measurement with other indicators or microelectrodes. Addition of a second photomultiplier channel, with appropriate filter sets to avoid spectral overlap, will enable true simultaneous measurement of the fluorescent intracellular calcium indicator CAL-590 alongside VF2.1Cl. It is also important to note that this multipurpose system can be used alternatively for deep assessment of intracellular calcium handling as previously described[17–20].

Whilst the temporal resolution of our system brings many advantages, it should be noted that parameters requiring analysis of the spatial distribution of fluorescence signals, such as excitation patterns or conduction velocity, are not suitable for investigation by the photometry techniques that we use here and are in the realms of optical mapping. The hardware described here can be readily converted to an optical mapping configuration by exchange of the photomultiplier for a suitable specialist camera with high frame rates and large well capacity. Our described configuration can offer extremely detailed temporal information at a fraction (up to 1/10th) of the commercial price of advanced large area cameras with equivalent capabilities. Confocal line scan techniques are also more spatially detailed than our method, however limitations in *z* restrict fluorescence acquisition from multiple transverse planes at once. This is not ideal for imaging membrane bound reporters held by iPSC-CMs with typically heterogeneous morphologies. Photometry measurements using a standard epifluorescence microscope avoid this issue by accessing the entire cellular surface instantaneously, again at a much lower cost per data point.

We highly recommend VF2.1Cl for conducting voltage assays with excitable cells. Currently, many VSDs are available that operate under a multitude of different mechanisms, each with their own inherent limitations. Common electrochromic styryl dyes like di-8-ANEPPS or di-4-ANBDQBS display fast responses to transmembrane voltage however are hindered by low sensitivity and high capacitive load[21,22]. Genetically encoded voltage indicators (GEVIs) utilize the fusion of fluorescent proteins to molecular voltage sensing domains[23] or microbial opsins[24] and provide highly sensitive dissections of cellular voltage dynamics, but are hindered by reduced kinetics and nonlinear responses. A list of common VSDs and their basic dynamic properties are included in **Table 1**. PeT probes like VF2.1Cl offer a good compromise, displaying fast kinetics and decent sensitivity with minimal cellular disruption. However, this VSD is limited because, unlike ratiometric styryl dyes[21], it cannot be used to resolve absolute membrane potential. This inherent disadvantage highlights the unfortunate consequence of maintaining data accuracy at higher throughput when assaying cellular electrophysiology.

[Insert **Table 1** here]

We note that excitation-contraction uncouplers such as blebbistatin or 2,3-butane-dione monoxime (BDM) are often used in optical voltage measurements to reduce motion artefact by suppressing cardiac contraction[35]. However, previous reports have shown that both compounds significantly prolong AP duration and flatten AP restitution in whole perfused hearts[36,37]. In addition, blebbistatin has fluorescent properties which overlap with the spectra of VF2.1Cl and therefore may not be appropriate for use with this dye[38].

Our versatile protocol requires minimal adjustments for optical investigation into a variety of two-dimensional and three-dimensional excitable constructs. This method allows for rapid and precise quantification of repolarization mechanics, which can provide valuable insights into cellular ionic abnormalities. In our hands, this protocol delivers clear optical signals with good signal to noise ratios even in smaller cells. Our simple and effective platform can be applied for non-invasive validation of cardiac drug safety and high throughput screening studies using patient specific iPSC-CMs.

ACKNOWLEGEMENTS:
The authors would like to acknowledge Cairn Research Ltd. for their kind financial contribution which covered production costs of this publication. In addition, we thank Ms. Ines Mueller and Ms. Stefanie Kestel for their excellent technical support.

The authors' research is supported by the German Center for Cardiovascular Research (DZHK), the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation, VO 1568/3-1, IRTG1816 RP12, SFB1002 TPA13 and under Germany's Excellence Strategy - EXC 2067/1-390729940) and the Else-Kröner-Fresenius Stiftung (EKFS 2016_A20).

DISCLOSURES:
Cairn Research Ltd supported this publication by covering production costs of the video file.

REFERENCES:

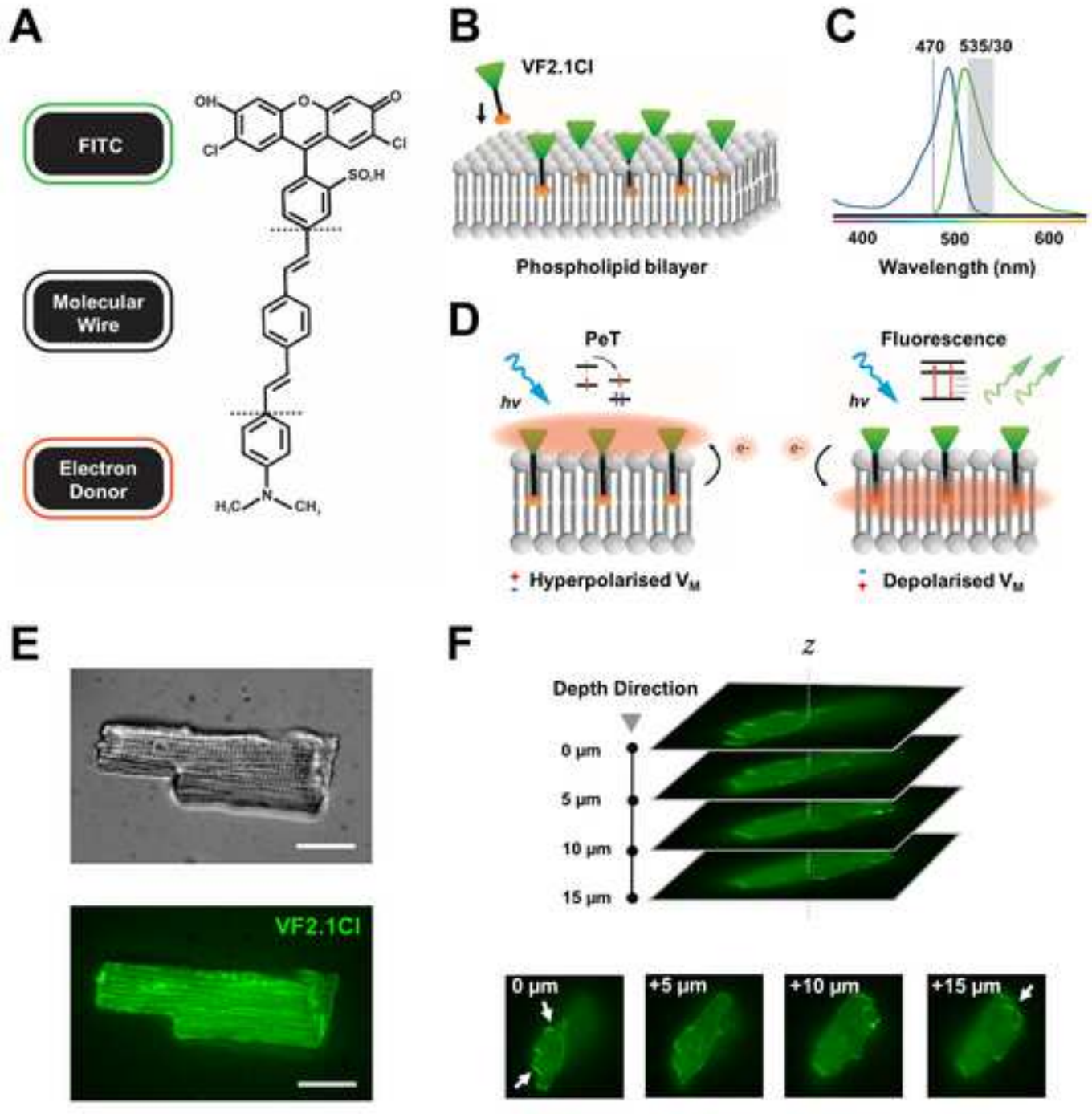
1. Miller, E. W. Small molecule fluorescent voltage indicators for studying membrane potential. *Current Opinion in Chemical Biology*. **33**, 74–80 (2016).
2. Liang, P. et al. Drug screening using a library of human induced pluripotent stem cell-derived cardiomyocytes reveals disease-specific patterns of cardiotoxicity. *Circulation*. **127** (16), 1677–1691 (2013).
3. Horváth, A. et al. Low resting membrane potential and low inward rectifier potassium currents are not inherent features of hiPSC-derived cardiomyocytes. *Stem Cell Reports*. **10** (3), 822–833 (2018).
4. Salama, G., Morad, M. Merocyanine 540 as an optical probe of transmembrane electrical activity in the heart. *Science (New York, N.Y.)*. **191** (4226), 485–487 (1976).
5. Hortigon-Vinafre, M. et al. The use of ratiometric fluorescence measurements of the voltage sensitive dye Di-4-ANEPPS to examine action potential characteristics and drug effects on human induced pluripotent stem cell-derived cardiomyocytes. *Toxicological Sciences*. **154** (2), 320–331 (2016).
6. Blinova, K. et al. International multisite study of human-induced pluripotent stem cell-derived cardiomyocytes for drug proarrhythmic potential assessment. *Cell Reports*. **24** (13), 3582–3592 (2018).

7. Miller, E. W. et al. Optically monitoring voltage in neurons by photo-induced electron transfer through molecular wires. *Proceedings of the National Academy of Sciences*. **109** (6), 2114–2119 (2012).
8. Bedut, S. et al. High-throughput drug profiling with voltage- and calcium-sensitive fluorescent probes in human iPSC-derived cardiomyocytes. *American Journal of Physiology-Heart and Circulatory Physiology*. **311** (1), H44–H53 (2016).
9. McKeithan, W. L. et al. An automated platform for assessment of congenital and drug-induced arrhythmia with hiPSC-derived cardiomyocytes. *Frontiers in Physiology*. **8**, 766 (2017).
10. Duncan, G. et al. Drug-mediated shortening of action potentials in LQTS2 human induced pluripotent stem cell-derived cardiomyocytes. *Stem Cells and Development*. **26** (23), 1695–1705 (2017).
11. Asakura, K., Hayashi, S., Ojima, A., Taniguchi, T., Miyamoto, N. Improvement of acquisition and analysis methods in multi-electrode array experiments with iPS cell-derived cardiomyocytes. *Journal of Pharmacological and Toxicological Methods*. **75**, 17–26 (2015).
12. Lian, X. et al. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions. *Nature Protocols*. **8** (1), 162–175 (2013).
13. Burridge, P. W. et al. Chemically defined generation of human cardiomyocytes. *Nature methods*. **11** (8), 855–860 (2014).
14. Kleinsorge, M., Kleinsorge, M., Cyganek, L. Subtype-directed differentiation of human iPSCs into atrial and ventricular cardiomyocytes *STAR Protocols*. 100026 (2020).
15. Knollmann, B. C., Katchman, A. N., Franz, M. R. Monophasic action potential recordings from intact mouse heart: validation, regional heterogeneity, and relation to refractoriness. *Journal of Cardiovascular Electrophysiology*. **12** (11), 1286–1294 (2001).
16. Leopold, J. A., Loscalzo, J. Emerging role of precision medicine in cardiovascular disease. *Circulation Research*. **122** (9), 1302–1315 (2018).
17. Voigt, N., Zhou, X.-B., Dobrev, D. Isolation of human atrial myocytes for simultaneous measurements of Ca^{2+} transients and membrane currents. *Journal of Visualized Experiments* (77), e50235 (2013).
18. Voigt, N. et al. Enhanced sarcoplasmic reticulum Ca^{2+} Leak and increased Na^{+}-Ca^{2+} exchanger function underlie delayed afterdepolarizations in patients with chronic atrial fibrillation. *Circulation*. **125** (17), 2059–2070 (2012).
19. Voigt, N. et al. Cellular and molecular mechanisms of atrial arrhythmogenesis in patients with paroxysmal atrial fibrillation. *Circulation*. **129** (2), 145–156 (2014).
20. Fakuade, F. E. et al. Altered atrial cytosolic calcium handling contributes to the development of postoperative atrial fibrillation. *Cardiovascular Research*. cvaa162 (2020).
21. Gross, E., Bedlack Jr, R. S., Loew, L. M. Dual-wavelength ratiometric fluorescence measurement of the membrane dipole potential. *Biophysical Journal*. **67** (1), 208–216 (1994).
22. Matiukas, A. et al. Near-infrared voltage-sensitive fluorescent dyes optimized for optical mapping in blood-perfused myocardium. *Heart Rhythm*. **4** (11), 1441–1451 (2007).
23. Mutoh, H. et al. Spectrally-resolved response properties of the three most advanced fret based fluorescent protein voltage probes. *PLoS One*. **4** (2), e4555 (2009).
24. Hochbaum, D. R. et al. All-optical electrophysiology in mammalian neurons using engineered microbial rhodopsins. *Nature Methods*. **11** (8), 825–833 (2014).
25. Huang, Y.-L., Walker, A. S., Miller, E. W. A photostable silicon rhodamine platform for optical voltage sensing. *Journal of the American Chemical Society*. **137** (33), 10767–10776 (2015).
26. Deal, P. E., Kulkarni, R. U., Al-Abdullatif, S. H., Miller, E. W. Isomerically pure

tetramethylrhodamine voltage reporters. *Journal of the American Chemical Society*. **138** (29), 9085–9088 (2016).
27. Fluhler, E., Burnham, V. G., Loew, L. M. Spectra, membrane binding, and potentiometric responses of new charge shift probes. *Biochemistry*. **24** (21), 5749–5755 (1985).
28. Fromherz, P., Muller, C. O. Voltage-sensitive fluorescence of amphiphilic hemicyanine dyes in neuron membrane. *Biochimica et Biophysica Acta*. **1150** (2), 111–122 (1993).
29. Salama, G. et al. Properties of new, long-wavelength, voltage-sensitive dyes in the heart. *Journal of Membrane Biology*. **208** (2), 125–140 (2005).
30. Jin, L. et al. Single action potentials and subthreshold electrical events imaged in neurons with a fluorescent protein voltage probe. *Neuron*. **75** (5), 779–785 (2012).
31. Kralj, J. M., Douglass, A. D., Hochbaum, D. R., MacLaurin, D., Cohen, A. E. Optical recording of action potentials in mammalian neurons using a microbial rhodopsin. *Nature Methods*. **9** (1), 90–95 (2012).
32. Tsutsui, H., Karasawa, S., Okamura, Y., Miyawaki, A. Improving membrane voltage measurements using FRET with new fluorescent proteins. *Nature Methods*. **5** (8), 683–685 (2008).
33. Lundby, A., Mutoh, H., Dimitrov, D., Akemann, W., Knöpfel, T. Engineering of a genetically encodable fluorescent voltage sensor exploiting fast Ci-VSP voltage-sensing movements. *PLoS One*. **3** (6), e2514 (2008).
34. Bradley, J., Luo, R., Otis, T. S., DiGregorio, D. A. Submillisecond optical reporting of membrane potential in situ using a neuronal tracer dye. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. **29** (29), 9197–9209 (2009).
35. Herron, T. J., Lee, P., Jalife, J. Optical imaging of voltage and calcium in cardiac cells & tissues. *Circulation Research*. **110** (4), 609–623 (2012).
36. Kappadan, V. et al. High-resolution optical measurement of cardiac restitution, contraction, and fibrillation dynamics in beating vs. blebbistatin-uncoupled isolated rabbit hearts. *Frontiers in Physiology* . **11**, 464 (2020).
37. Kettlewell, S., Walker, N. L., Cobbe, S. M., Burton, F. L., Smith, G. L. The electrophysiological and mechanical effects of 2,3-butane-dione monoxime and cytochalasin-D in the Langendorff perfused rabbit heart. *Experimental Physiology*. **89** (2), 163–172 (2004).
38. Képiró, M. et al. para-Nitroblebbistatin, the non-cytotoxic and photostable Myosin II inhibitor. *Angewandte Chemie International Edition*. **53** (31), 8211–8215 (2014).

Figure 1

Click here to access/download;Figure;Figure 1(revised)_Seibertz_JoVE_2020.tif



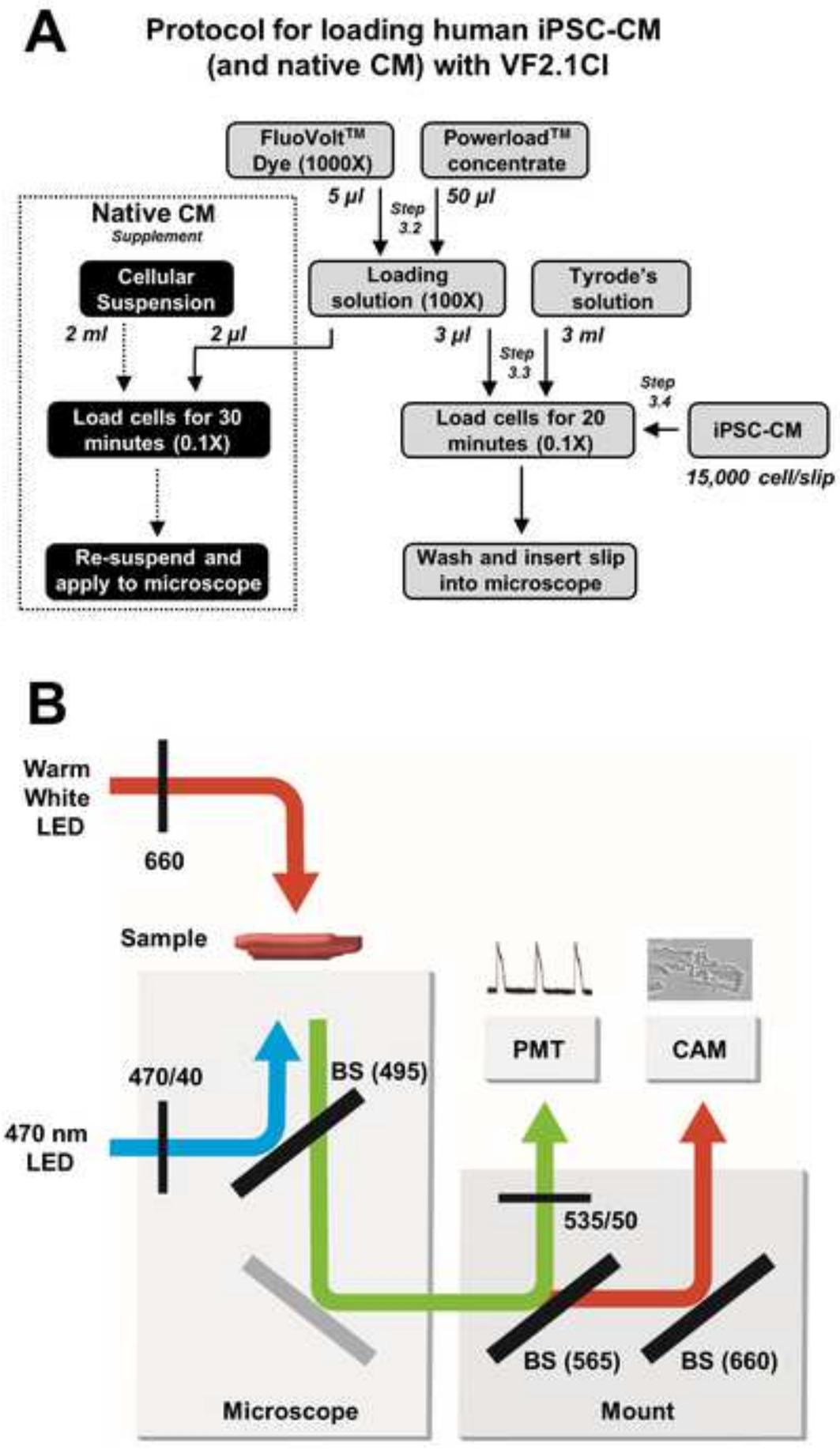


Figure 3

Click here to access/download;Figure;Figure 3(revised)_Seibertz_JoVE_2020.tif

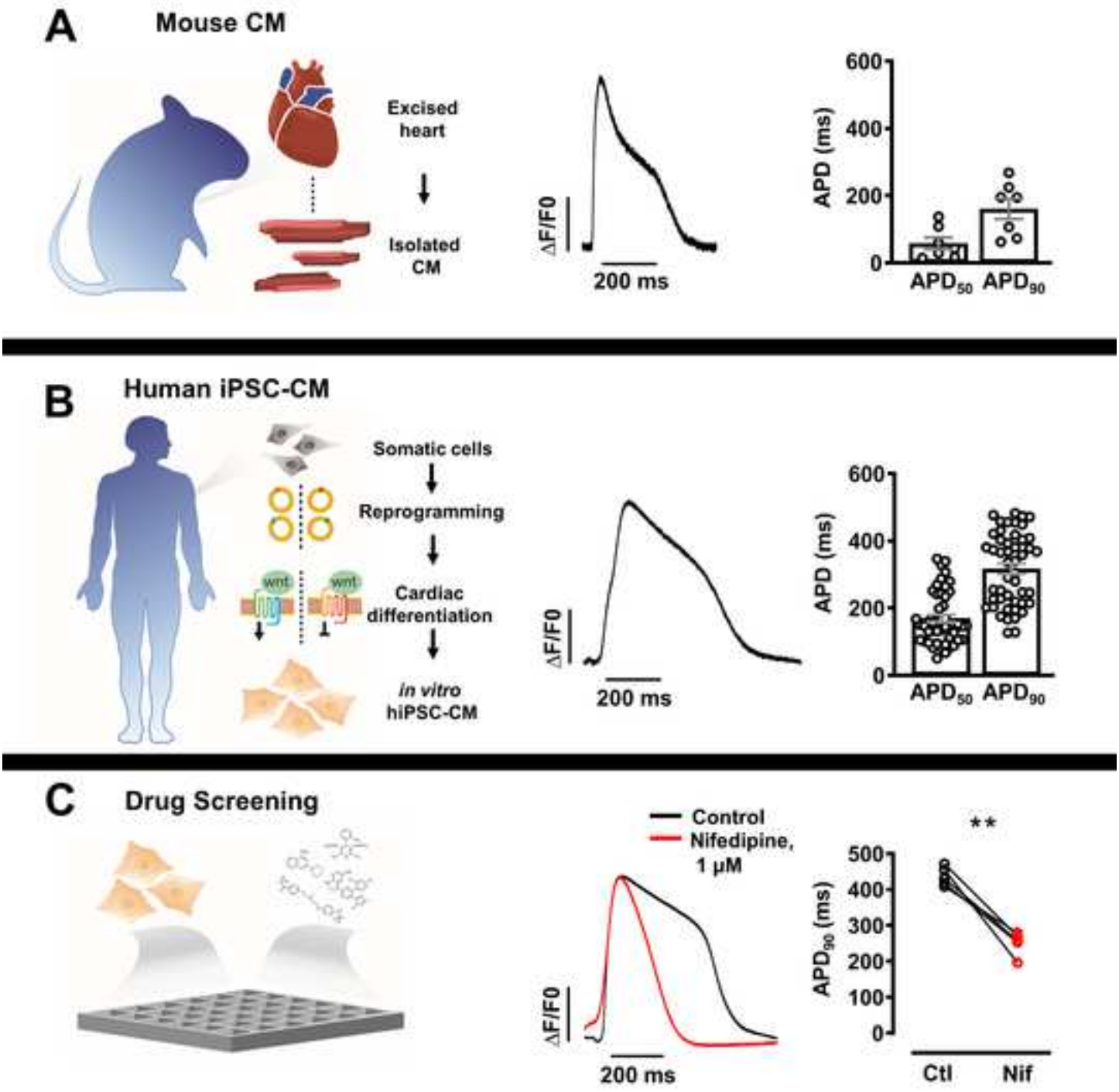


Table 1

Click here to access/download;Table;Table 1(revised)_Seibertz_JoVE_2020.xlsx

	Sensitivity (% ΔF/F per 100mV)	**Speed** (ms)
PeT-based dyes		
VF2.1Cl[7]	27	<1
BeRST1[25]	24	<1
RhoVR1[26]	47	<1
Hemicyanine dyes		
Di-8-ANEPPS[21]	10	<1
Di-4-ANEPPS[27]	8	<1
Di-4-ANBDQBS[22]	10–20	<1
RH237[28]	11	<1
PGH1[29]	17.5	<1
GEVIs		
ArcLight[30]	32	123[24]
Arch D95N[31]	40	41[24]
Mermaid[32]	40	17.4
VSFP 2.3[33]	13.3	25[23]
QuasAr2[24]	90	11
FRET		
Dio/DPA[34]	56	2

Table of Materials

Click here to access/download;Table of Materials;Materials(revised)_Seibertz_JoVE_2020.xlsx

Name of Material/ Equipment	Company	Catalog Number
Reagents		
0.25 Trypsin EDTA	Gibco	25200056
B27 Supplement	Gibco	17504044
CaCl2	Carl Roth	HN04.2
D(+)-Glucose anhydrous *BioChemica*	ITW Reagents	A1422
Fetal Bovine Serum	Gibco	10270-106
FluoVolt Membrane Potential Kit	Invitrogen	F10488
HEPES	Carl Roth	HN77.4
KCl	Sigma-Aldrich	6781.1
Lamanin	Sigma-Aldrich	114956-81-9
Matrigel	BD	354230
NaCl	Sigma-Aldrich	9265.2
Nifedipine	Sigma-Aldrich	21829-25-4
Penicillin/Streptomycin	Invitrogen	15140
ROCK Inhibitor Y27632	Stemolecule	04-0012-10
RPMI 1640 Medium	Gibco	61870010
Versene EDTA	Gibco	15040033
Equipment		
495LP Dichroic Beamsplitter	Olympus	
Axopatch 200B Amplifier	Molecular Devices	
Circle Coverslips, Thickness 0	Thermo Scientific	CB00100RA020MNT0
Digidata 1550B	Molecular Devices	
Dual OptoLED Power Supply	Cairn Research	
ET470/40x Excitation Filter	Chroma	
ET535/50m	Chroma	
Etched Neubauer Hemacytometer	Hausser Scientific	

Filter Cubes	Cairn Research
IX73 Inverted Microscope	Olympus
MonoLED	Cairn Research
Multiport Adaptors	Cairn Research
Myopacer Cell Stimulator	IonOptix
Optomask Shutter	Cairn Research
Optoscan	Cairn Research
PH-1 Temperature Controlled Platform	Warner Instruments
PMT Amplifier Insert	Cairn Research
PMT Supply Insert	Cairn Research
RC-26G Open Bath Chamber	Warner Instruments
SA-OLY/2AL Stage Adaptor	Olympus
T565lpxr Dichroic Beamsplitter	Chroma
T660lpxr Dichroic Beamsplitter	Chroma
TC-20 Dual Channel Temperature Controller	npi Electronic
UPLFLN 40X Objective	Olympus
USB 3.0 Colour Camera	Imaging Source

Software

Clampex 11.1	Molecular Devices
Clampfit 11.1	Molecular Devices
IC Capture 2.4	Imaging Source
Prism 8	Graphpad

Rebuttal letter

JoVE61890R1 - "Single-cell optical action potential measurement in human induced pluripotent stem cell-derived cardiomyocytes"

Editorial Reviewer Comments:

We thank the editors for giving such precise instructions for shaping the manuscript into a text that is appropriate for the *Journal of Visualized Experiments*. A point-by-point response is detailed below.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. Please use American English throughout.

The text has been thoroughly checked by all authors and all 'british-isms' changed to American English.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

Formatting has been followed as instructed.

3. As one of the authors is from UK please agree to UK ALA in the additional details page of the submission site.

This has been agreed to on the submission site.

4.Please provide at least 6 keywords or phrases.
5. We cannot have commercial terms in the keyword. Please remove FluoVolt and use generic term instead.

Keyword number has been updated to 6 and no longer contains commercial language.

6. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: FluoVolt, Invitrogen, TrypLE, Accutase, falcon, Axon Instruments Digidata, Eppendorf, PowerLoad, etc.
7. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end we ask you remove the term FluoVolt and use generic term instead.

Commercial language in the protocol has been removed. FluoVolt has been replaced with the generic term VF2.1Cl (VoltageFluor2.1Cl).

8. Please ensure the Introduction include all of the following with citation:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

We have made major revisions to the introduction which now covers each of these points.

9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.

Text has been changed to the imperative tense throughout the protocol. Notes have been used where appropriate.

10. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.
11. We cannot have non numbered paragraph as well. Please convert these to notes instead.

Each step now contains a maximum of three actions and non-numbered paragraphs have been removed.

12. The Protocol should contain only action items that direct the reader to do something.
13. Please ensure you answer the "how" question, i.e., how is the step performed?

We have taken care to ensure that the protocol uses direct language and details how each step is to be performed.

14. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Appropriate and filmable protocol content has now been highlighted. It is well within a 3 page limit.

16. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

As our figure is entirely self-made and every graphical element is completely original, we have removed this citation.

17. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations: a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have made major revisions to the discussion it now satisfactorily covers all of these points.

18. Please sort the materials table in alphabetical order.

The materials have been sorted into alphabetical order in their respective groups.

19. Please check if the attached supplementary file is correct. If this needs filming please include this with the actual protocol and highlight accordingly.

The supplemental file is correct and does not require filming. We feel it is still necessary to include in order to make clear to the readers that many cellular and animal constructs can be assessed with this multi-purpose protocol.

Reviewer 1 Comments:

This manuscript provides a detailed protocol to acquire optical recordings of action potentials in hiPSC-derived cardiomyocytes using a commercially available voltage-sensitive dye, FluoVolt. This is a method that is gaining interest for researchers studying hiPSC-cardiomyocytes due to its high throughput. The method is also technically less challenging than the traditional method of measurement using the patch-clamp technique. Hence, the publication is timely and of value to the readers. There are no major concerns.

We thank this reviewer for his/her positive evaluation of our manuscript.

Minor Concerns: The reason for the addition of probenecid should be included in the text.

We are incredibly grateful to this reviewer for pointing out this sloppy mistake. Probenecid is used in other protocols we employ for imaging of cytosolic calcium and ensuring the indicator is not extruded from the cell. This has been removed from the manuscript. The Tyrode's solution constituents have been double checked and now are listed correctly.

Reviewer 2 Comments:

We thank this reviewer for his/her detailed evaluation of our protocol and their incredibly helpful comments which have guided us to substantially improve our revised manuscript. We have made major adjustments, particularly to the discussion, based on this reviewers suggestions. A point-by-point response is detailed below.

The authors describe a well built system for optical mapping of single cells. The VSD chosen has been in the market for a few years and has all the qualities described in the paper. The use of the fluovolt has been well described and results documented in a fairly large number of publications. The main contribution of this manuscript is the description of how to assemble a PMT-based optical mapping system. Although Cairn contributed financially and provided materials for assembly of the system, a PMT-based optical mapping system can theoretically be assembled with parts from other brands. It would be desirable to have that clearly stated in the manuscript, otherwise this would be a simple assembly manual for a Cairn system (which by the way manufactures excellent and high quality equipment).

Firstly, we agree and wholly support Cairn Products! Secondly we thank the reviewer for highlighting this point. To our knowledge, Cairn is only distributor that provides a modular system which does not directly integrate the PMT into their acquisition hardware. Respectively, this allows for simultaneous measurements of multiple parameters and gives the operator more control over analogue signal processing prior to digitization. We have incorporated these points into our discussion and reduced our commercial plugs for Cairn equipment.

In addition we have added this sentence to our discussion to make this clear for the readers:

"Many optics-based setups are commercially available and can be optimized for a wide range of signal recording requirements"

Furthermore, it is imperative to indicate the limitations of this system in regards to the loss of spatial resolution. It is particularly important because the CiPA and JICSA efforts to validate the use of hiPSC-CMs for cardiotoxicity screening was performed on syncytia of hiPSC-CMs. Being direct to the point, please discuss the inability to assess conduction velocity (an important contributor to the establishment of arrhythmia) with a PMT.

We agree that this is a valuable point. We have indeed directly included this limitation into the discussion and devoted a full paragraph to discussing the advantages and disadvantages of alternative imaging systems and their implications.

We have included the following paragraph into our discussion:

"Whilst the temporal resolution of our system brings many advantages, it should be noted that parameters requiring analysis of the spatial distribution of fluorescence signals, such as excitation patterns or conduction velocity, are not suitable for investigation by the photometry techniques that we used here and are in the realms of optical mapping. The hardware described here can be readily converted to an optical mapping configuration by exchange of the photomultiplier for a suitable specialist camera with high frame rates and large well capacity. Our described configuration and protocol can offer extremely detailed temporal information at a fraction (up to 1/10th) of the commercial price of advanced large area cameras with equivalent capabilities. Confocal line scan techniques are also more spatially detailed than our method, however limitations in z restrict fluorescence acquisition from multiple transverse planes at once. This is not ideal for imaging membrane bound reporters held by iPSC-CMs with typically heterogeneous morphologies. Photometry measurements using a standard epifluorescence microscope avoid this issue by accessing the entire cellular surface instantaneously, again at a much lower cost per data point."

In addition, we have discussed this method in the context of proarrhythmia screening throughout the manuscript. This is best highlighted in the introduction:

"A recent study has demonstrated the suitability of ratiometric voltage sensitive probe photometry to accurately quantify the cardiac action potential (Hortigon-Vinagre et al., 2016). Furthermore the ability to readily scale up optical photometry approaches lends this technique to large scale cardiotoxicity screens critical in therapeutic drug development (e.g. CiPA). Development of standardized cardiotoxicity protocols in a blinded multi-site study using microelectrode array and voltage-sensing optical techniques has demonstrated the key value of this approach (Blinova et al. 2018)."

Additionally, it would increase the strength of this manuscript to indicate that the over-killing acquisition rate might not be required for obtaining precise measurements of upstroke velocity, but would be incredibly useful for measuring APD in other excitable cells that have incredibly short APDs, like neurons.

We thank the reviewer for this useful comment which prompted us to fully investigate and review our digitization procedure. We agree that we are oversampling. Reliable analysis of upstroke gradient will require 5 or more data points. On the assumption that a typical cardiac AP rise time is 10 ms, an analogue signal cut off at 500 Hz is appropriate. We double this to 1 kHz, both to ensure a detailed recording, and because our PMT amplifier only allows for filtering at exponents of 10 (1, 10, 100 etc..). To satisfy Nyquist requirements and prevent aliasing we would therefore only need a digitization rate of 2 – 3 kHz.

In the protocol itself, we have directed attention to these steps, and we have added the following sentence to the discussion:

"High sampling rates are required to ensure reliable measurements of action potential upstroke in cardiomyocytes and can be critical in other excitable constructs (i.e. neurons) with extremely fast excitation kinetics"

It would be interesting to discuss that the system described may be suitable for measuring duration of intra-cellular calcium release and perhaps amplitude or relative amplitude.

We completely agree with this comment. We have now added a section directly discussing this systems ability to provide readouts of intracellular calcium. This is helpful on multiple levels, because describing the multi-functionality of such a system of course increases its desirability.

The following sentences have been added to the discussion:

"Addition of a second photomultiplier channel, with appropriate filter sets to avoid spectral overlap, will enable true simultaneous measurement of the fluorescent intracellular calcium indicator CAL-590 alongside VF2.1Cl. It is also important to note that this multipurpose system can be used alternatively for deep assessment of intracellular calcium handling as previously described (Voigt et al., 2013, Voigt et al., 2012, Voigt et al., 2014, Fakuade et al., 2020)."

Mapping is yet far from being able to measure specific currents. Nevertheless, it still can significantly reduce the amount of patch clamp work if it is used for assessment of APD morphology in order to do educated decisions on the ionic currents that should be assessed in following experiments.

We would like to clarify that we are only comparing our method to traditional current-clamp protocols. We thoroughly agree with the reviewer that our method will never replace voltage-clamp based assessment of individual ionic currents.

To address this, we have clarified this comparison with current-clamp in the introduction and included this brief reminder in the discussion:

"This method allows for rapid and precise quantification of repolarization mechanics, which can provide valuable insights into cellular ionic abnormalities."

DZHK
DEUTSCHES ZENTRUM FÜR
HERZ-KREISLAUF-FORSCHUNG E.V.

SFB 1002

UNIVERSITÄTSMEDIZIN GÖTTINGEN : UMG

**Institut für
Pharmakologie und Toxikologie**

Universitätsmedizin Göttingen, 37099 Göttingen

Prof. Dr. med. Niels Voigt
Professor für Molekulare Pharmakologie
Robert-Koch-Straße 40,
37075 Göttingen
Tel: +49 551 / 39-65174
Fax: +49 551 / 39-65169
niels.voigt@med.uni-goettingen.de
www.molecular-pharmacology.de

September 18, 2020

Sebastian Clauß, MD
Guest Editor
&
Vineeta Bajaj
Review Editor

Journal of Visualized Experiments

Re: JoVE61890R1 - Single-cell optical action potential measurement in human induced pluripotent stem cell-derived cardiomyocytes

Dear Dr. Clauß and Dr. Bajaj,

I respectfully submit the revised version of our above-indicated manuscript, which has been edited in response to your editorial correspondence and the reviewer comments of 4.09.2020. We have addressed all comments and modified the introduction and discussion significantly in response to the suggestions from the reviewers.

These changes will provide more valuable and accessible information to the potential reader about the advantages and limitations of this optical technique. We have also provided more context regarding the relevance of this protocol today in the "age" of pluripotent stem cell derived constructs.

We thank the reviewers for their suggestions which have helped us to improve the protocol, and we also thank you for detailed editorial guidance. We hope that you and the reviewers find the revised paper acceptable for publication in the *Journal of Visualized Experiments.*

Sincerely,

Niels Voigt, MD

Single-cell optical action potential measurement in human induced pluripotent stem cell-derived cardiomyocytes

Seibertz F, Reynolds M, Voigt N

Supplemental material

Native murine cardiomyocyte preparation for voltage imaging

Protocols for murine cardiomyocyte optical voltage investigation are largely identical to those outlined in the main text for human iPSC-CMs. Differences of note include the entirety of the cellular preparations outlined in section 1. Murine cardiomyocytes are not cultured, instead they are mechanically and enzymatically isolated from excised hearts as previously described[1]. VF2.1Cl loading concentration remains the same as listed, however, loading protocol 3.3–7.7 proceeds under alternative directions. A visual guide is displayed in **Figure 2A.**

Mouse cardiomyocyte isolation yields a pellet suspended in 0.2 mM Ca^{2+} storage solution in a 15 ml falcon tube. Decant this solution to 2 ml and add 2 µl 0.1X VF2.1Cl loading solution (Contents provided step 3.2). Loading proceeds in low light conditions for 30 minutes at room temperature. After, aspirate all supernatant and resuspend the cardiomyocytes with 2 ml fresh, warm Tyrode's solution (Contents provided in step 3.1). An identical bath chamber (Setup detailed in step 3.5) is utilized however the rectangular coverslip is coated with 2 µl of 1 mg/mL laminin prior to the direct application of 500 µl cell suspension. This is to limit cardiomyocyte movement during perfusion.

References:

1. Voigt, N., Zhou, X.-B., Dobrev, D. Isolation of human atrial myocytes for simultaneous measurements of Ca^{2+} transients and membrane currents. *Journal of Visualized experiments*. (77), e50235 (2013).