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Cover Letter

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Dr Nandita Singh
Senior Science Editor, JoVE

Dear Dr Singh

Thank you for the comments and suggestions from the reviewers and the opportunity to revise our manuscript.

Please find here the revised manuscript titled “Preclinical Platform for Cardiac Electrophysiology Assessment by Dual Voltage and Calcium Optical Mapping of Human Organotypic Cardiac Slices” for publication in Journal of Visualized Experiments. Edits are redlined and a point by point response to the reviewer’s comments are included in the Response to Reviewers document.

Yours sincerely,

TITLE:

Preclinical Cardiac Electrophysiology Assessment by Dual Voltage and Calcium Optical Mapping of Human Organotypic Cardiac Slices

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KEYWORDS:

human heart, organotypic culture, cardiac slices, optical mapping, electrophysiology, voltage, calcium, drug testing

SUMMARY:

This protocol describes the procedure for sectioning and culturing human cardiac slices for preclinical drug testing and details the use of optical mapping for recording transmembrane voltage and intracellular calcium signals simultaneously from these slices.

ABSTRACT:

Human cardiac slice preparations have recently been developed as a platform for human physiology studies and therapy testing to bridge the gap between animal and clinical trials. Numerous animal and cell models have been used to examine the effects of drugs, yet these responses often differ in humans. Human cardiac slices offer an advantage for drug testing in that they are directly derived from viable human hearts. In addition to having preserved multicellular structures, cell-cell coupling, and extracellular matrix environments, human cardiac tissue slices can be used to directly test the effect of innumerable drugs on adult human cardiac physiology. What distinguishes this model from other heart preparations, such as whole hearts or wedges, is that slices can be subjected to longer-term culture. As such, cardiac slices allow for studying the acute as well as chronic effects of drugs. Furthermore, the ability to collect several hundred to a thousand slices from a single heart makes this a high-throughput model to test several drugs at varying concentrations and combinations with other drugs at the same time. Slices can be prepared from any given region of the heart. In this protocol, we describe the preparation of left ventricular slices by isolating tissue cubes from the left ventricular free wall and sectioning them into slices using a high precision vibrating microtome. These slices can then either be subjected

to acute experiments to measure baseline cardiac electrophysiological function or cultured for chronic drug studies. This protocol also describes dual optical mapping of cardiac slices for simultaneous recordings of transmembrane potentials and intracellular calcium dynamics to determine the effects of the drugs being investigated.

INTRODUCTION:

Animal models have been a valuable tool used for understanding the underlying mechanisms of human physiology and pathophysiology, as well as a platform for preliminary testing of therapies to treat various diseases[1]. Great strides have been taken in the field of biomedical research based on these animal studies[2]. However, significant interspecies differences exist between human and animal physiologies, including mice, rats, guinea pigs, rabbits, sheep, pigs, and dogs[3,4]. As a result, there have been numerous drug, gene, and cell therapies that showed promise during the animal testing stage but failed to live up to the results in clinical trials[5]. To bridge this gap, isolated cardiac myocytes and human induced pluripotent stem cells (iPSCs) were developed as models to test the response of human physiology to various drugs and diseases[6]. Stem cell-derived cardiomyocytes have been widely used in organ-on-a-chip systems as a surrogate of the heart[6–8]. However, the usefulness of iPSC-derived cardiomyocytes (iPSC-CMs) is impeded by their relatively immature phenotype and the lack of representation of the cardiomyocyte subpopulation; the mature myocardium is a complex structure comprised of several coexisting cell types such as fibroblasts, neurons, macrophages, and endothelial cells. On the other hand, isolated human cardiomyocytes are electrically mature, and different cardiomyocyte subpopulations can be obtained by altering culturing parameters[9]. Still, these myocytes generally exhibit altered action potential morphologies due to the lack of cell-cell coupling, rapid de-differentiation, and occurrence of proarrhythmic behavior in vitro[10,11]. Some of the limitations were addressed by 3D cell culture models of iPSC-CMs and cardiac myocytes. These models, which include spheroids, hydrogel scaffold encapsulated 3D cultures, engineered heart tissues (EHTs), and heart-on-a-chip systems, use multiple cardiac cell populations such as cardiomyocytes, fibroblasts, and endothelial cells. They either self-assemble or assemble along a scaffold to form 3D structures, and some even reproduce the complex anisotropic nature of the myocardium. These models have been reported to have cells of mature phenotypes, contractile properties, and molecular profiles similar to cardiac tissue. The heart-on-a-chip system also allows the study of systemic effects in drug testing and disease models. However, in vitro cell-based models lack the native extracellular matrix and therefore cannot accurately mimic organ level electrophysiology. Human cardiac slices, by contrast, have an intact extracellular matrix and native cell-to-cell contacts, making them useful for more accurately examining arrhythmogenic properties of the human myocardium.

Researchers have developed human cardiac organotypic slices as a physiological preclinical platform for acute and chronic drug testing and to study cardiac electrophysiology and cardiac disease progression[12–19]. When compared with iPSC-derived cardiomyocytes, human cardiac slices more faithfully replicate adult human cardiac electrophysiology with a mature cardiomyocyte phenotype. When compared with isolated human cardiomyocytes, cardiac slices exhibit physiological action potential durations because of the well-preserved cell-cell coupling and the intrinsic existence of their native intra- and extracellular environments.

This protocol describes the process of generating human cardiac slices from whole donor hearts, performing acute (i.e., hours-long) and chronic (i.e., days-long) studies to test cardiac electrophysiology parameters via optical mapping. While this protocol describes only the use of the left ventricular (LV) tissue, it has been successfully applied to other regions of the heart as well as other species such as mice, rats, guinea pigs, and pigs[14,20–22]. Our laboratory uses whole human donor hearts that have been rejected for transplantation for the last 5 years, but it is feasible for these same procedures to be carried out on any donor heart sample tissues obtained by alternative means (e.g., left ventricular assist device [LVAD] implantations, biopsies, myectomies) as long as the tissues have the ability to be sectioned into cubes. Optical mapping is employed for analysis in this study due to its capacity to simultaneously map optical action potentials and calcium transients with high spatial (100 x 100 pixels) and temporal (>1,000 frames/s) resolution. Alternative methods can also be used, such as multielectrode arrays (MEAs) or microelectrodes, but these techniques are limited by their relatively low spatial resolutions. Additionally, MEAs were designed for use with cell cultures, and sharp microelectrodes are more easily managed for use with whole hearts or large tissue wedges.

The goal of the article is to enable more researchers to use human cardiac tissues for cardiac electrophysiology studies. It should be noted that the technology described in this article is relatively simple and beneficial for short-term studies (on the order of several hours to days). More physiological biomimetic culture for longer-term studies (on the order of weeks) has been discussed and described by a number of other studies[12,18,23]. Electrical stimulation, mechanical loading, and tissue stretching are advantageous conditioning mechanisms that can help limit the onset of in vitro tissue remodelling[12,18,23].

PROTOCOL:

All methods described have been performed in compliance with all institutional, national, and international guidelines for human welfare. Research was approved by the Institution Review Board (IRB) at The George Washington University.

NOTE: Donor human hearts were acquired from Washington Regional Transplant Community as deidentified discarded tissue with approval from the George Washington University IRB. Explanted hearts are cardioplegically arrested by flushing the heart with a solution of ice-cold cardioplegia (the blood was cleared from the heart in this process) and transferred to the lab under standard organ transplant conditions.

1. Preparation of solutions

1.1. For each heart, make 4 L of cardioplegic solution (110 mM NaCl, 16 mM KCl, 16 mM $MgCl_2$, 10 mM $NaHCO_3$, 1.2 mM $CaCl_2$; pH = 7.4). Store 3 L at 4 °C and the remaining 1 L at -20 °C.

NOTE: This solution can be made up to several days in advance.

1.2. Freshly prepare 1 L each of Tyrode's slicing solution (140 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, 10 mM 2,3-butanedione monoxime [BDM]; pH = 7.4) and Tyrode's recovery solution (140 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, 10 mM BDM; pH = 7.4).

NOTE: Both the slicing and recovery solutions should be made the day of the experiment and can be stored at 4 °C.

1.3. Prepare stock solutions of the fluorescent dyes. Reconstitute the voltage-sensitive dye RH237 at 1.25 mg/mL in dimethyl sulfoxide (DMSO) and store it in 30 µL aliquots at 4 °C. Reconstitute the calcium indicator Rhod-2AM at 1 mg/mL in DMSO. Store in 30 µL aliquots at -20 °C.

NOTE: Di-4-ANEPPS (stock solution at 1.25 mg/mL in DMSO) can be used in experiments for single camera imaging of the transmembrane potential alone.

1.3.1. Before the start of the experiment, sonicate the dyes using an ultrasound sonicator for at least 10 min and dilute each aliquot of fluorescent dyes in 1 mL of the recovery solution. Add nonionic, surfactant polyol (**Table of Materials**) to Rhod-2AM at a 1:1 ratio before dilution in recovery solution.

1.4. Prepare a stock solution of the excitation-contraction uncoupler blebbistatin at 2 mg/mL solution in DMSO. Store in aliquots at -20 °C. During optical mapping experiments, dilute the stock solution of blebbistatin to a working concentration of 5−10 µM in the Tyrode's recovery solution.

1.5. Make fresh culture medium by supplementing medium 199 with 2% penicillin-streptomycin, 1x insulin-transferrin-selenium (ITS) liquid media supplement, and 10 mM BDM. Filter the medium using a 0.2 µm sterile filter.

NOTE: For pharmacological perturbation studies, drugs can be added directly to the culture medium. The culture medium can be stored at 37 °C.

1.6. Make a 4% agarose gel for tissue mounting by dissolving low-melting point agarose in distilled water and heating the mixture in a microwave until fully dissolved. Cure the agarose in a Petri dish at a thickness of 5 mm and store at 4 °C.

2. Equipment setup

2.1. Vibrating microtome setup

2.1.1. Calibrate the vibrating microtome prior to each experiment.

2.1.1.1. Using a high precision vibrating microtome (**Table of Materials**), load a ceramic cutting

blade into the holder and attach the calibrating device provided with the vibrating microtome. Choose the blade adjustment option from the menu and select ceramic for type of blade.

2.1.1.2. Select the vibrate option check for Z axis value. If this value is <1 µm, exit the calibration menu. If not, finely adjust the calibration screw attached to the top of the vibrating head and select the vibrate option. Repeat as many times as needed to set the Z axis to <1 µm.

2.1.2. Set the vibratome settings to 400 µm cutting thickness, 0.02 mm/s advance speed for atrial tissue and 0.04 mm/s advance speed for ventricular tissue, 2 mm horizontal vibration amplitude, and 80 Hz vibration frequency.

NOTE: While 400 µm is the recommended thickness to compensate for cell damage on the cut surfaces of the slice, thinner slices can also be prepared. Given that the oxygen diffusion limit is around 150 µm, slices around 300 µm are often used[14,17,18,24].

2.1.3. Fill the bath of the vibrating microtome with slicing solution at 4 °C and maintain the temperature by surrounding the outside of the bath with ice, replenishing as needed throughout the slicing protocol. Continuously oxygenate the slicing solution in the bath by bubbling with 100% oxygen during slicing.

2.1.4. Set up a second dish with as many 100 µm nylon mesh cell strainers and meshed washers as needed (one cell strainer per slice). Fill this dish with recovery solution and oxygenate it by bubbling with 100% oxygen at room temperature (RT).

NOTE: This solution is maintained at RT during the experiment.

2.2. Optical mapping setup

NOTE: A more detailed description of the optical mapping system is provided in previous publications[16,25,26].

2.2.1. Attach a tissue bath with polydimethylsiloxane (PDMS) gel layer at the bottom (to pin the slices) to a perfusion system. Circulate 1 L of the recovery solution at 37 °C and oxygenate with 100% oxygen, through the perfusion system at a flow rate fast enough to maintain the temperature and clear accumulation of bath perfusate.

2.2.2. Adjust the focus and alignment of the two CMOS cameras (**Table of Materials**) using a target.

NOTE: More details on alignment can be found in previous studies[26].

2.2.3. Use a green LED light source with a wavelength of 520 ± 5 nm to excite the voltage-sensitive and calcium indicator dyes simultaneously.

NOTE: The excitation light source is attached to the excitation filter cube of the optical mapping system and is reflected off a 550 nm dichroic mirror for epicentric illumination. The emitted light is collected by a 1x lens and split by a second dichroic mirror at 630 nm into voltage and calcium components before it is filtered by 690 ± 50 nm and 590 ± 33 nm filters, respectively, and recorded by the two cameras.

3. Slicing protocol

3.1. When ready for tissue dissection and experimentation, prepare a bath of ice-cold cardioplegia by mixing frozen and liquid cardioplegia. Keep the heart submerged in the cardioplegia bath until tissue collection for slicing (**Figure 1A**).

3.2. Glue the premade agarose gel to the back of the metal tissue holders of the vibratome.

3.3. Identify the left ventricular free wall and cut 1 cm^3 cubes of tissue in cold cardioplegic solution. Then, quickly mount the tissue blocks onto the metal tissue holders with the endocardial surfaces facing up and attach them to the agarose gel using topical skin adhesive (**Figure 1B**).

NOTE: The selected tissue region should be away from large blood vessels to avoid holes in the slices. The plane of sectioning is approximately parallel to the fiber orientation in the endocardial layer (**Figure 1E**) and the plane of sectioning could be at a slight angle due to rotational anisotropy in a deeper layer of the LV wall. To increase throughput, up to two tissue blocks can be mounted side by side on the same holder.

3.4. Transfer the metal holders into the vibratome bath filled with the ice-cold oxygenated slicing solution. Ensure that the tissue cubes are completely submerged.

3.5. Move the blade to the front edge of the tissue and turn on the vibratome to begin slicing with the preset parameters. Discard the first several slices until the blade reaches beyond the trabeculae into the smooth endocardial tissue.

3.6. Once each slice is cut, carefully transfer the slice to an oxygenated (100% O_2) bath of Tyrode's recovery solution at RT. Gently place each slice in individual 100 µm nylon mesh cell strainers and cover with meshed washers to keep the tissue slices from curling (**Figure 1C**). Keep slices in the recovery solution for at least 20 min.

NOTE: Slices can be kept in the bath under these conditions for up to 3−4 h without detrimental electrophysiological effects.

4. Slice culturing under static conditions

NOTE: To minimize the chance of contamination, sterilize the forceps using a bead sterilizer before each transfer step.

4.1. When ready to culture, carefully transfer each slice to the individual wells of a 6 well plate filled with sterile phosphate-buffered saline (PBS). Gently rock the well plate to rinse the slices of recovery solution.

NOTE: From this point forward, the slices should only be exposed in a BSL2 laminar flow culture hood, and sterile forceps should be used to handle the tissue.

4.2. Transfer the slices to wells of fresh PBS to thoroughly rinse and sterilize the slices for culture. Perform this wash step 3x to ensure complete removal of recovery solution from the slices.

4.3. Transfer the slices to individual wells of a 6 well plate filled with 3 mL of prewarmed (37 °C) culture medium (with or without drugs, **Figure 1D**). Place plates onto an orbital shaker at 20 rpm in a humidified incubator at 37 °C with 30% O_2 and 5% CO_2. Aspirate and replace culture medium every 48 h.

5. Functional characterization—optical mapping

5.1. For optical mapping studies either immediately following slicing or after culturing, carefully transfer the slice of interest to the tissue bath of the perfusion system at 37 °C with 100% O_2 in Tyrode's recovery solution, and pin down the four corners to the PDMS gel layer while applying minimal stretch (i.e., just enough to keep the slice from easily moving under flow conditions). Allow the slices to rest in this bath with circulating recovery solution for approximately 10 min.

5.2. Add 0.3−0.5 µL of the stock blebbistatin to the reservoir containing the recovery solution. Let the slice incubate with blebbistatin for approximately 10 min.

5.3. Reconstitute 30 µL of the stock voltage-sensitive dye, RH237, in 1 mL of recovery solution at 37 °C. Turn off the pumps and slowly load 0.2−0.3 mL of working dye solution onto the surface of the slice over a period of 30 s. Allow the slice to incubate in the dye with the pumps off for a period up to 90 s, then turn the pumps on again to allow any excess dye to wash out.

NOTE: Care should be taken to apply the dye evenly all over the slice. Additionally, very slow dye application is crucial to prevent the dye from floating away from the slice.

5.4. Repeat step 5.3 to reconstitute the stock calcium indicator Rhod-2AM.

5.5. Focus the cameras onto the slice by adjusting the distance of the slice from the lens.

5.6. Position a bipolar platinum-iridium pacing electrode such that its tip is in contact with the middle of the slice and apply pacing stimuli of increasing amplitude to determine the minimum pacing threshold required to elicit an electrical response. Pace the slice at 1 Hz with a 2 ms pulse width duration at 1.5x the amplitude of the predetermined pacing threshold. Place a coverslip over the tissue slice.

5.7. Illuminate the slice using a 520 ± 5 nm LED excitation light source. Record the emitted voltage and calcium signals with the two CMOS cameras at 1,000 frames/s.

5.8. Check the recording for good signal quality and suppressed motion artifacts (**Figure 1F**). Add more blebbistatin to the reservoir in steps of 0.1 µL until the motion no longer produces artifacts in the optical signals. Add more dye, if needed, to improve the signal quality.

NOTE: Good signal quality refers to qualitatively assessed large signal amplitude and low background noise in the recorded optical signals.

6. Data processing with RHYTHM1.2

NOTE: RHYTHM1.2 is a MATLAB based user interface that is used to display, condition, and analyze optical mapping data acquired by single or dual camera optical mapping systems (**Figure 3**). It is used in conjunction with the imaging system.

6.1. Loading data files

6.1.1. Load the data files into RHYTHM1.2 by selecting any one of the four display windows on the screen and using the **Select Directory** and **Load** buttons.

6.1.2. The program will prompt the user to select Camera 1 or Camera 2 data for the dual voltage and calcium recordings. Select **Camera 1** for the voltage signal.

6.1.3. Repeat the process to load the calcium signal (Camera 2) to one of the adjacent display windows.

6.1.4. Select the **Dual Data Linking** checkbox between the two selected display windows to link the two display windows, so the two data sets are recorded from the same region on the slice.

NOTE: Alternatively, if the same field of view is maintained, this function can be used to link two images that were obtained at separate time points to see the time-response in electrophysiology of the drug being investigated. Double clicking any one display window allows for that one window to be maximized to allow for ease of analysis.

6.2. Signal conditioning

6.2.1. Select **Data Analysis** | **Condition Parameters** to condition the voltage and calcium optical mapping data prior to further analysis.

6.2.2. Use the "**Remove Background**" function to remove pixels that do not contain any signal based on a fluorescent intensity threshold (background [BG] threshold) and degree of pixel clustering (excitation [EX] threshold).

6.2.3. Perform spatial averaging of the optical mapping data using the "**Bin**" function to improve signal quality.

6.2.4. Filter the optical mapping data using a band pass filter with the "**Filter**" function.

6.2.5. Use the "**Drift**" function to remove the baseline drift in the optical mapping signal.

6.2.6. Using the "**Normalize**" function, normalize the optical mapping data from each pixel to have a maximal amplitude of 1.

6.2.7. Use the "**Inverse Signal**" function to invert the calcium traces for further analysis.

6.2.8. Click **Display Wave** to select the conditioned trace from any given spot in the display window and plot it in the Waveform window. Adjust the signal conditioning parameters to obtain optimal action potential and calcium transient traces.

6.3. Conduction velocity (CV) calculation

NOTE: The activation time is defined as the time of maximum derivative of the optical signal (dF/dt_{max}).

6.3.1. Select **Data Analysis** | **Activation Map** and enter a Start Time and End Time to encompass a single action potential in the trace. Press **Calculate** and select the region of interest (ROI) to display the activation map of the selected region (**Figure 1G**).

6.3.2. Select **Data Analysis** | **CV Map** and similarly, choose the Start Time and End Time. Enter the interpixel resolution values based on the setup.

NOTE: For 1x magnification systems, interpixel resolution is 0.1 mm in the X and Y direction.

6.3.3. Press the **Generate Vec. Map** button to select an ROI and display CV vectors within that region. The mean, median, standard deviation, and number of vectors included in the analysis as well as the average angle of propagation of the CV vectors in the selected region is calculated and displayed in the Statistics section.

6.3.4. Click the **Draw Line** button to draw a line along a given direction of propagation. All CV vectors in that direction will be selected. Click **Calculate CV** to use only those selected CV vectors to calculate the statistics that will be displayed in the Statistics section.

6.4. Action potential duration (APD) and calcium transient duration (CaTD) calculation

NOTE: The APD is another fundamental parameter of the cardiac electrophysiology. APD maps can be generated by determining the time difference between activation and a specified

percentage of repolarization of each optical action potential. APD heterogeneity or APD prolongation and shortening can be used to predict arrhythmia susceptibility.

6.4.1. To calculate APD in RHYTHM1.2, select **Data Analysis** | **APD/CaT Map**.

6.4.2. Select the Start Time and End Time to encompass one full action potential. Set a minimum and maximum value of APD/CaTD to remove any outliers (for example, 0 and 1,000 ms, respectively). Enter the percent repolarization that will determine the APD, for example, 0.8 for $APD_{80}/CaTD_{50}$ or 0.5 for $APD_{50}/CaTD_{50}$.

6.4.3. Click **Regional APD Calc** to select an ROI and generate the APD map. The mean, median, standard deviation, and number of pixels included in the analysis will be displayed in the Statistics section.

6.5. Rise time

NOTE: The rise time is another electrophysiological parameter that can be measured from optical signals of voltage and calcium transient traces. It provides an estimate for how long it takes for the depolarizing ion channels to trigger an action potential, or how long it takes for calcium to be released into the cytoplasm from the sarcoplasmic reticulum. Optical rise time is not a perfect substitute for rise times measured by microelectrodes and may not be as sensitive to changes in depolarization, because optical action potentials are an average of the transmembrane potential of many cells. Spatial and temporal resolution of the system can also affect optical rise time values. However, it can still be used to predict large changes in depolarization[27].

6.5.1. To determine the rise time, select **Data Analysis** | **Rise Time**.

6.5.2. Select **Start Time** and **End Time** to select the upstroke of one single action potential or calcium transient. Enter the values of Start% and End% (typically 10–90% is recommended for non-noisy signals), which allows the user to select just the portions of the signal without including noise in the case of noisy signals.

6.5.3. Click **Calculate** to select the ROI and determine the mean, median, standard deviation, and number of pixels included in the analysis of rise time.

6.6. Calcium decay

NOTE: For calcium traces, the time constant of the decay of the calcium transient can be determined. This allows for the analysis of changes in the reuptake of calcium ions from the cytoplasm back into the SR.

6.6.1. Select **Data Analysis** | **Calcium Decay** and enter the start time and end time to encompass the entire decay portion of a single calcium transient signal.

6.6.2. Click **Calculate Tau** to select the ROI and determine the mean, median, standard deviation, and number of pixels included in the analysis of calcium decay time constant.

REPRESENTATIVE RESULTS:

Human organotypic slices were collected from the left ventricle of a donor human heart according to the protocol detailed above and illustrated in **Figure 1**. A dual camera optical mapping system like that in **Figure 2** was used in the upright imaging configuration to perform simultaneous optical mapping of voltage and calcium about 1 h after the completion of the slicing protocol. Data were analyzed using RHYTHM1.2 (**Figure 3**), an open source optical mapping data analysis tool previously published by our laboratory and freely available on Github (https://github.com/optocardiography/Rhythm-1.2). The electrophysiological parameters measured are illustrated in **Figure 4**. The action potential and calcium transient traces were signal conditioned and representative traces used in further analysis are illustrated in **Figure 4A**. Activation times were determined for each pixel and isochronal maps of activation times determined from voltage and calcium traces are illustrated in **Figure 4B**,**C**. Note that activation in the calcium isochrone lags behind that of the voltage as expected. CV vectors plotted in **Figure 4D** were calculated using the activation times and the known interpixel resolution. The average CV in the transverse direction was determined to be 21.2 cm/s in this slice. This CV value is comparable to previously reported ventricular transverse CV measured from explanted whole human hearts (24 ± 4 and 28 ± 7 cm/s in hearts with diffuse and patchy fibrosis)[28]. The calcium transient decay constant was measured by fitting a polynomial to the decaying portion of the calcium traces and average decay constant was determined to be 105.3 ms in this slice (map in **Figure 4E**). Next, APD and CaTD were measured as the time duration between activation time and a specified percent of repolarization/calcium removal form the cytoplasm. Average APD_{80} and $CaTD_{80}$ were determined to be 343.1 ms and 442.6 ms, respectively (**Figure 4F**,**G**). Previous in vivo human studies report the activation-recovery interval (ARI) measured from unipolar electrograms recorded from in vivo human hearts during steady state pacing at 1 Hz as a surrogate for APD. ARI values in these studies range from 250–450 ms[29,30]. The APD values from human cardiac slices reported here are comparable to the previous ARI values. Regions with motion artifacts were removed from APD and CaTD calculations. Finally, the rise times (i.e., the duration of the upstroke) of the voltage and calcium traces were measured and mapped. These are shown in **Figure 4H** and **Figure 4I**, respectively. The average values were determined to be 10.2 ms and 13.3 ms, respectively. The artifacts in the maps to the right of the point of pacing are due to the pacing wire within the field of view. Finally, the use of this slice model in acute drug testing was demonstrated when slices were cultured for 24 h with doxorubicin (DOX), a chemotherapeutic agent known to have cardiotoxic effects. Treatment of slices with 50 µM DOX resulted in a reduction of transverse conduction velocity from 19.4 ± 3.4 cm/s to 9.6 ± 3.2 cm/s, as illustrated by the activation maps in **Figure 5A**, CV vector maps in **Figure 5B**, and summary data in **Figure 5C**. The smaller sample size in the DOX group was due to a higher number of nonviable slices after DOX treatment. Increased motion artifacts in DOX-treated slices prevented the calculation of accurate APD values. Another important parameter that can be measured by dual voltage and calcium imaging is V_m-Ca delay, to determine robust excitation-contraction coupling in the tissue[31]. Delays or negative values of this parameter could be detrimental.

FIGURE LEGENDS:

Figure 1: Human cardiac organotypic slice preparation. (**A**) Human hearts were stored in an ice-cold cardioplegic bath (mixture of cardioplegia solution and cardioplegia ice) upon retrieval. (**B**) The 1 cm^3 cubes of left ventricular tissue were cut and mounted onto a metal tissue holder with 4% agarose gel glued to the back wall of the holder and transferred to an ice-cold bath of oxygenated slicing solution. (**C**) Once cut, slices were transferred to oxygenated recovery solution at RT in individual 100 µm nylon mesh cell strainers. Meshed washers covered the tissues to keep the slices from curling. (**D**) For long term culture, slices were washed in PBS and cultured in 6 well plates with 3 mL of tissue medium at 37 °C. (**E**) Mason's trichrome stained slice section showing fiber orientation. (**F**) Representative optical action potential recorded from a slice. (**G**) Representative activation map of a slice paced at the center (blue).

Figure 2: Dual camera optical mapping system. Dual camera optical mapping system in the upright imaging configuration. System parts include: 1) master and slave cameras for dual mapping; 2) tissue bath with PDMS gel; 3) filter cubes that house the excitation and emission filters and dichroic mirrors; 4) lens holders and lenses; and 5) excitation light source (520 nm green LED). Inset on right details filter and dichroic mirror combinations for dual optical mapping of V_m and calcium using RH237 and Rhod-2-AM, respectively.

Figure 3: RHYTHM1.2 graphical user interface (GUI) of the open source optical mapping data analysis tool for single parameter and multiparameter analysis. The data file loading options and file list are displayed in the red box. Data analysis options are listed in the data analysis dropdown menu indicated in green. Display windows for displaying data maps are indicated in dark blue. Checkboxes to link dual mapping data files for simultaneous analysis of dual camera data are indicated in purple. The waveform window to plot action potential and calcium transient traces is displayed in yellow.

Figure 4: Transmembrane potential and calcium transients mapping from a human organotypic cardiac slice preparation. (**A**) Representative optical action potential (black) and calcium transient (burgundy). (**B**,**C**) Activation maps obtained from V_m and calcium recordings, respectively. (**D**) Conduction velocity (CV) vector map. (**E**) Calcium transient decay constant (Tau) map. (**F**,**G**) Action potential duration (APD_{80}) and calcium transient duration ($CaTD_{80}$) map, respectively. (**H**,**I**) Maps of the rise time of the upstrokes of action potential and the calcium transient, respectively.

Figure 5. Doxorubicin treatment slows transverse conduction velocity. (**A**) Activation maps from slices cultured for 24 hours without (control) and with doxorubicin at 50 µM (DOX). (**B**) Conduction velocity vector maps from control and DOX slices. (**C**) Average transverse conduction velocity calculated from control and DOX-treated slices. *$P < 0.05$.

DISCUSSION:

Here, we present step-by-step methods to obtain viable cardiac slices from cardioplegically arrested human hearts and to functionally characterize the slices using dual optical mapping of transmembrane potential and intracellular calcium. With preserved extracellular environment

and native cell-cell coupling, human cardiac slices can be used as an accurate model of the human heart for fundamental scientific discovery and for efficacy and cardiotoxicity testing of pharmacological agents and gene therapies. The technology also allows for structural-functional mapping of specific regions of the heart, such as the sinoatrial node, atrioventricular node, and Purkinje fibers. The protocol described here lists basic steps for cardiac slice generation, imaging, and analyses that are best for short-term studies. Static culture of tissue slices have been shown to induce changes in cardiac tissue over prolonged periods of time, so for longer-term studies, we encourage the reader to incorporate more physiological culture conditions, such as electromechanical stimulation[12–14].

Care should be taken for a number of steps to maintain tissue health. For example, the time between heart harvesting and tissue slicing should be minimized. As shown previously[21], extended length of cardioplegic arrest can lead to altered electrophysiology. Also, to preserve the integrity of the extracellular matrix and cell-cell coupling, excessive handling and stretching of the slices should be avoided during slice collection, culturing, and functional studies. Excessive stretching of the slices could result in conduction blocks. Additionally, all solutions described in this protocol should be kept at a pH of 7.4. The Tyrode's solution described here utilizes HEPES to maintain the proper pH using 100% O_2. Sodium bicarbonate may be used to control pH instead, but this solution should be bubbled with 95% O_2 and 5% CO_2. Finally, during transportation and slicing of the heart, one should ensure that during transportation and slicing of the heart, the cardioplegia and slice cutting solution are kept as close to freezing temperature as possible to preserve tissue viability.

ACKNOWLEDGMENTS:

Funding by NIH (grants R21 EB023106, R44 HL139248, and R01 HL126802), by Leducq foundation (project RHYTHM) and an American Heart Association Postdoctoral Fellowship (19POST34370122) are gratefully acknowledged.

DISCLOSURES:

The authors have nothing to disclose.

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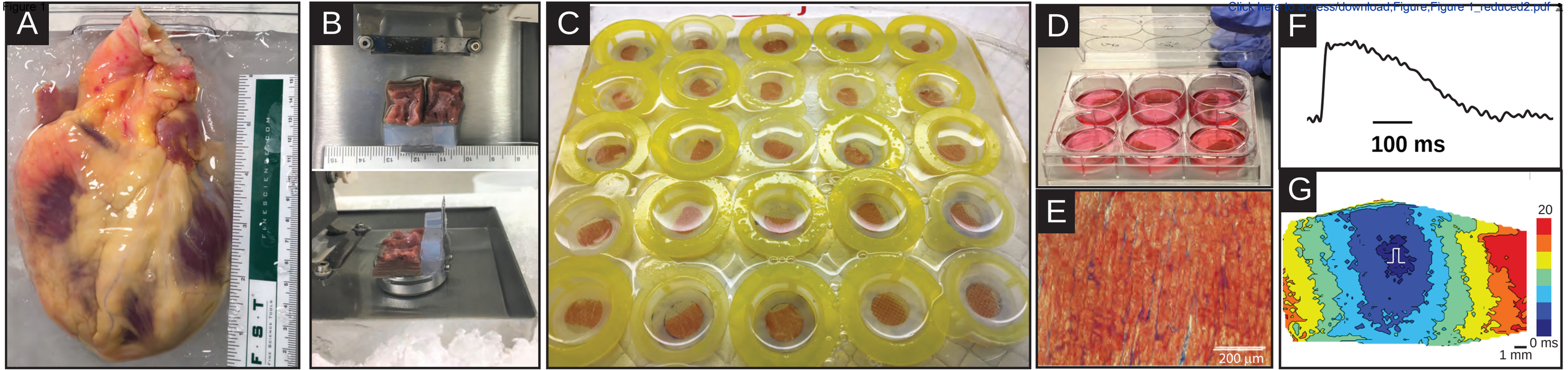
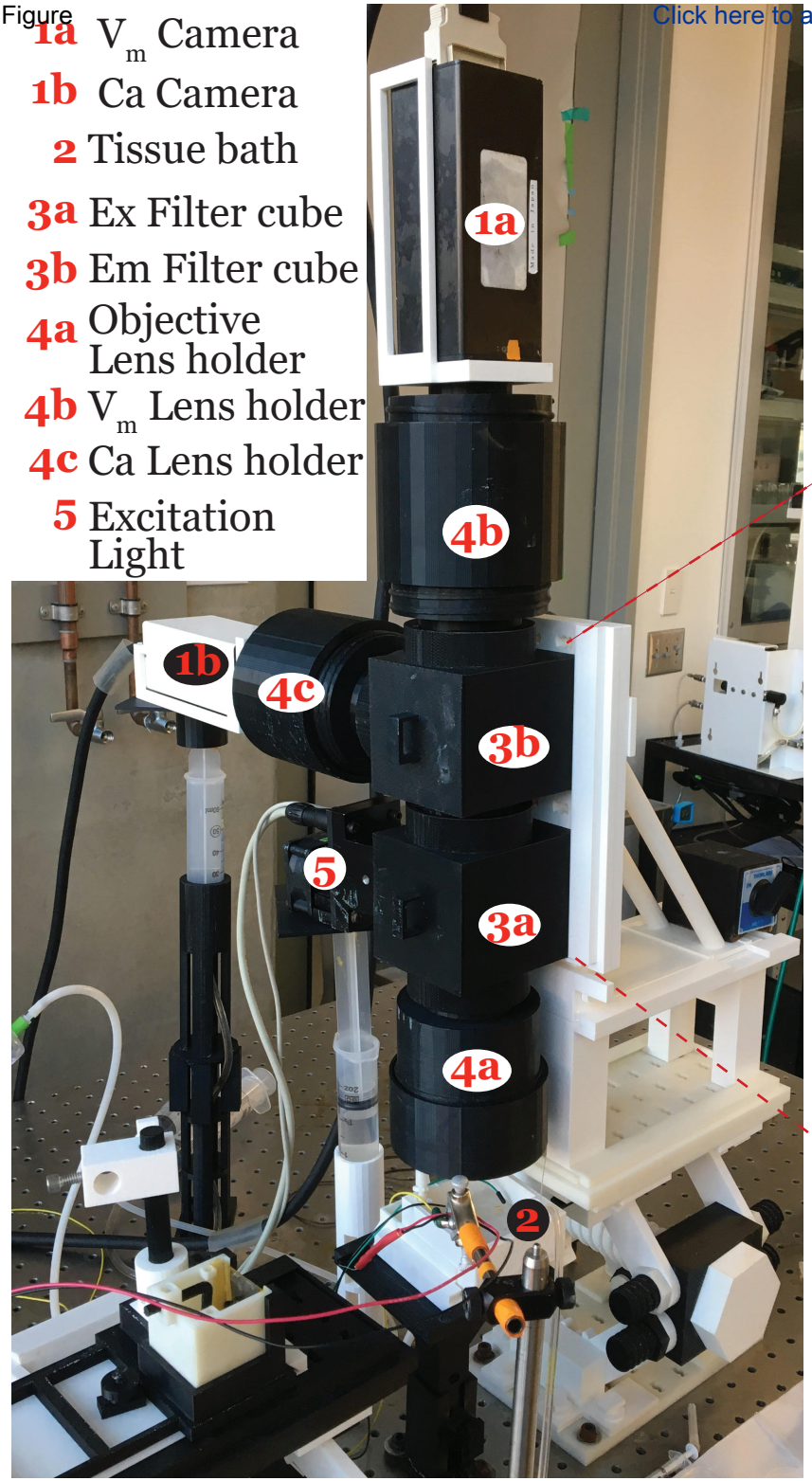
Figure 1

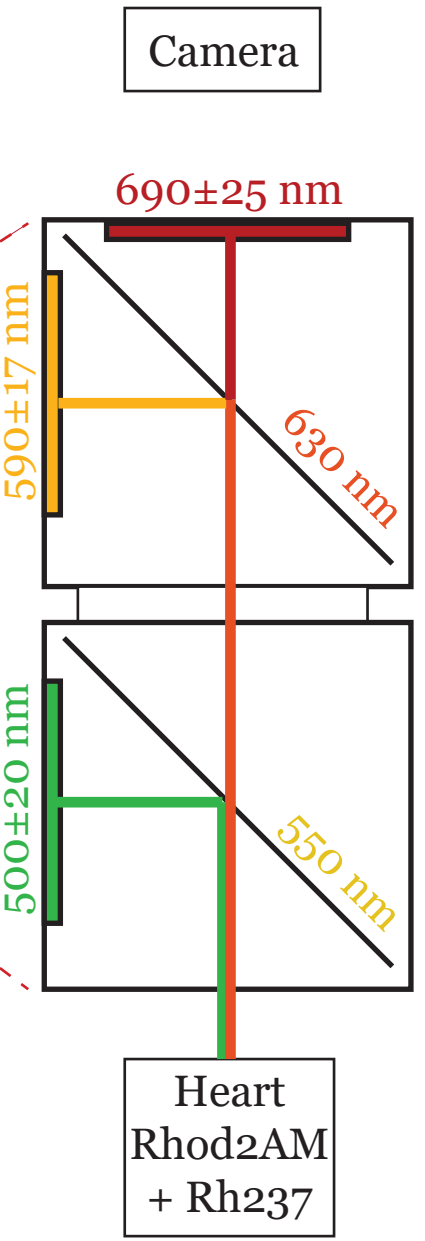


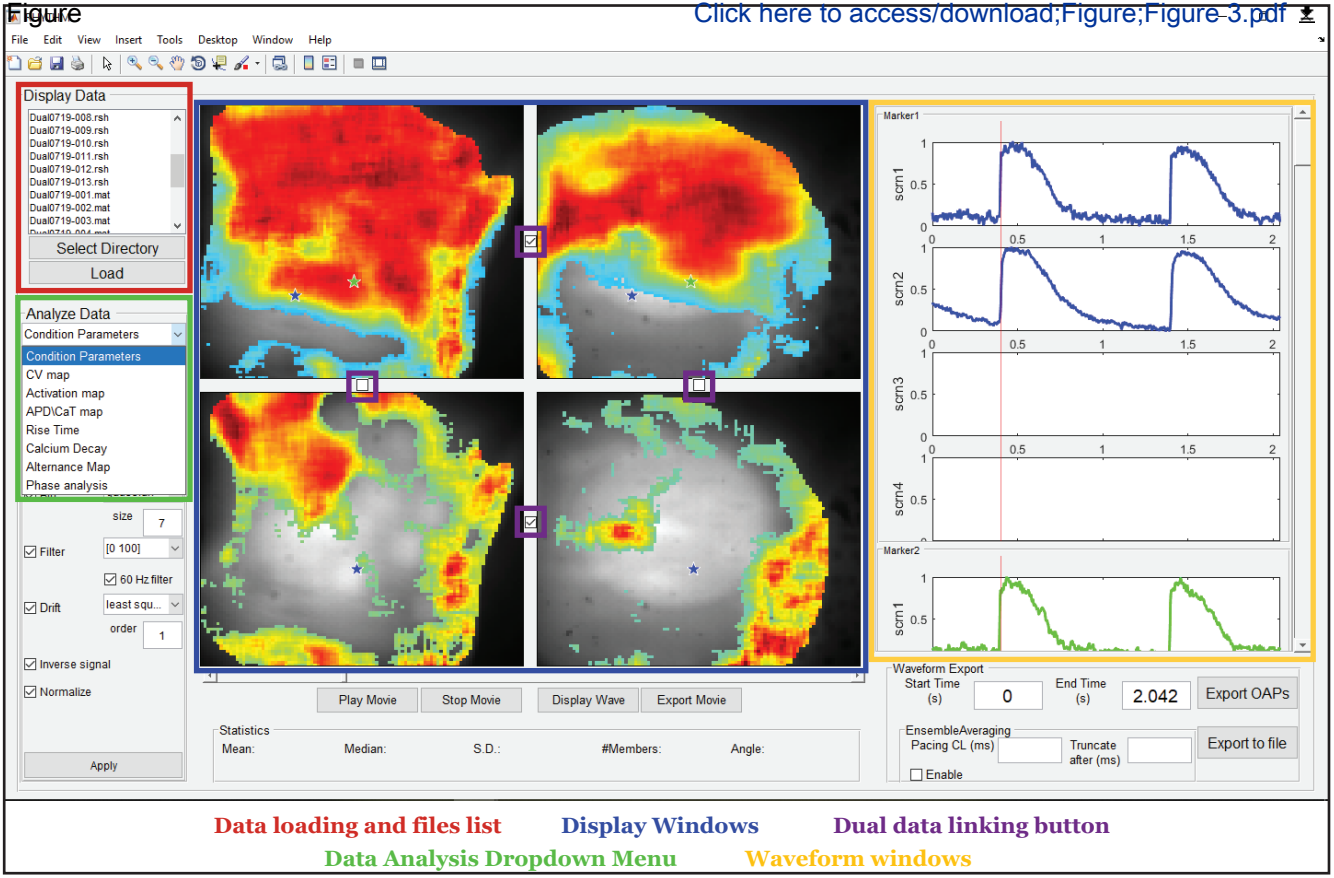
Figure

1a V_m Camera
1b Ca Camera
2 Tissue bath
3a Ex Filter cube
3b Em Filter cube
4a Objective Lens holder
4b V_m Lens holder
4c Ca Lens holder
5 Excitation Light



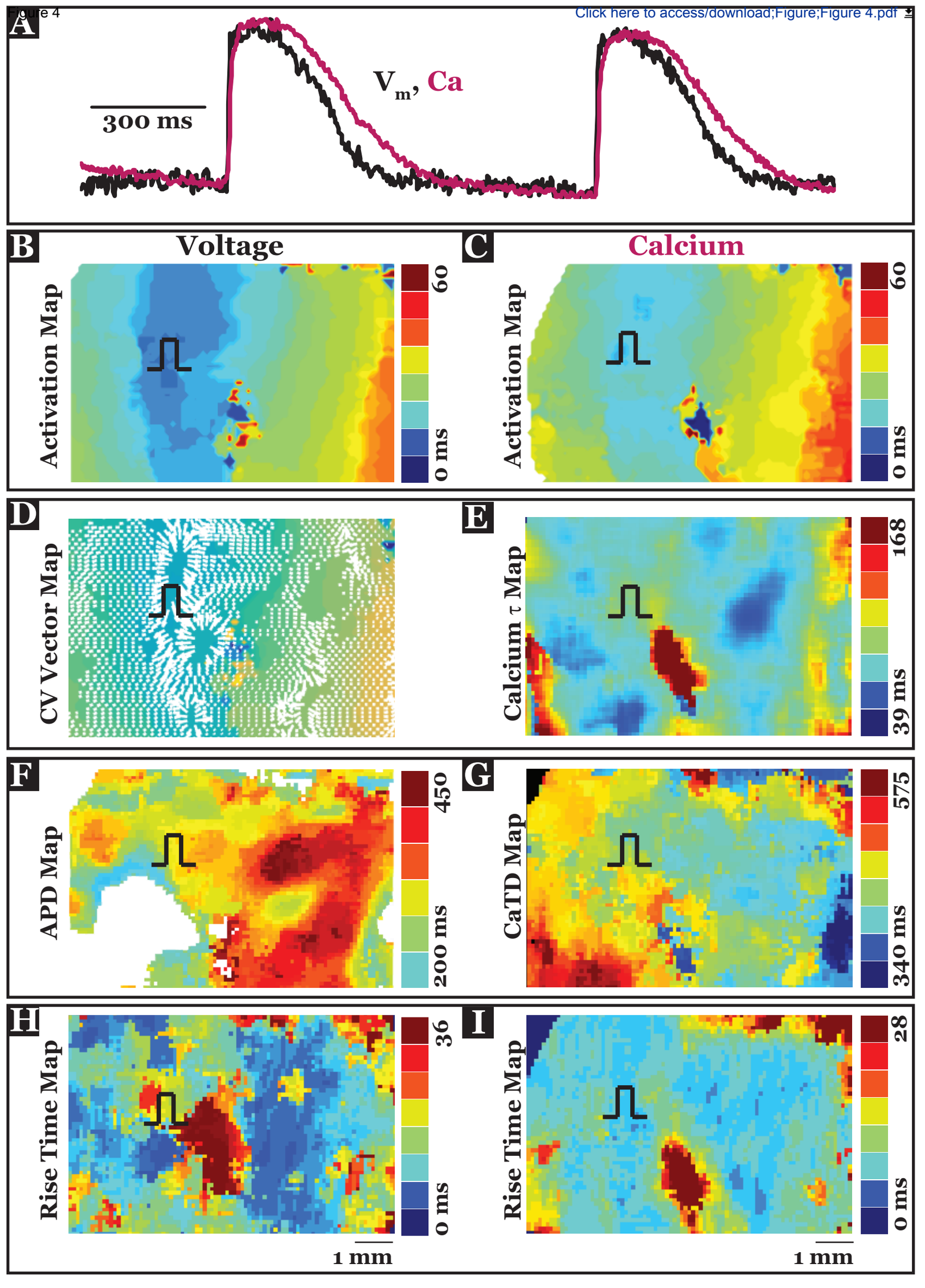
Optics for Rhod-2-AM /RH237 dual optical mapping





Data loading and files list Display Windows Dual data linking button

Data Analysis Dropdown Menu Waveform windows



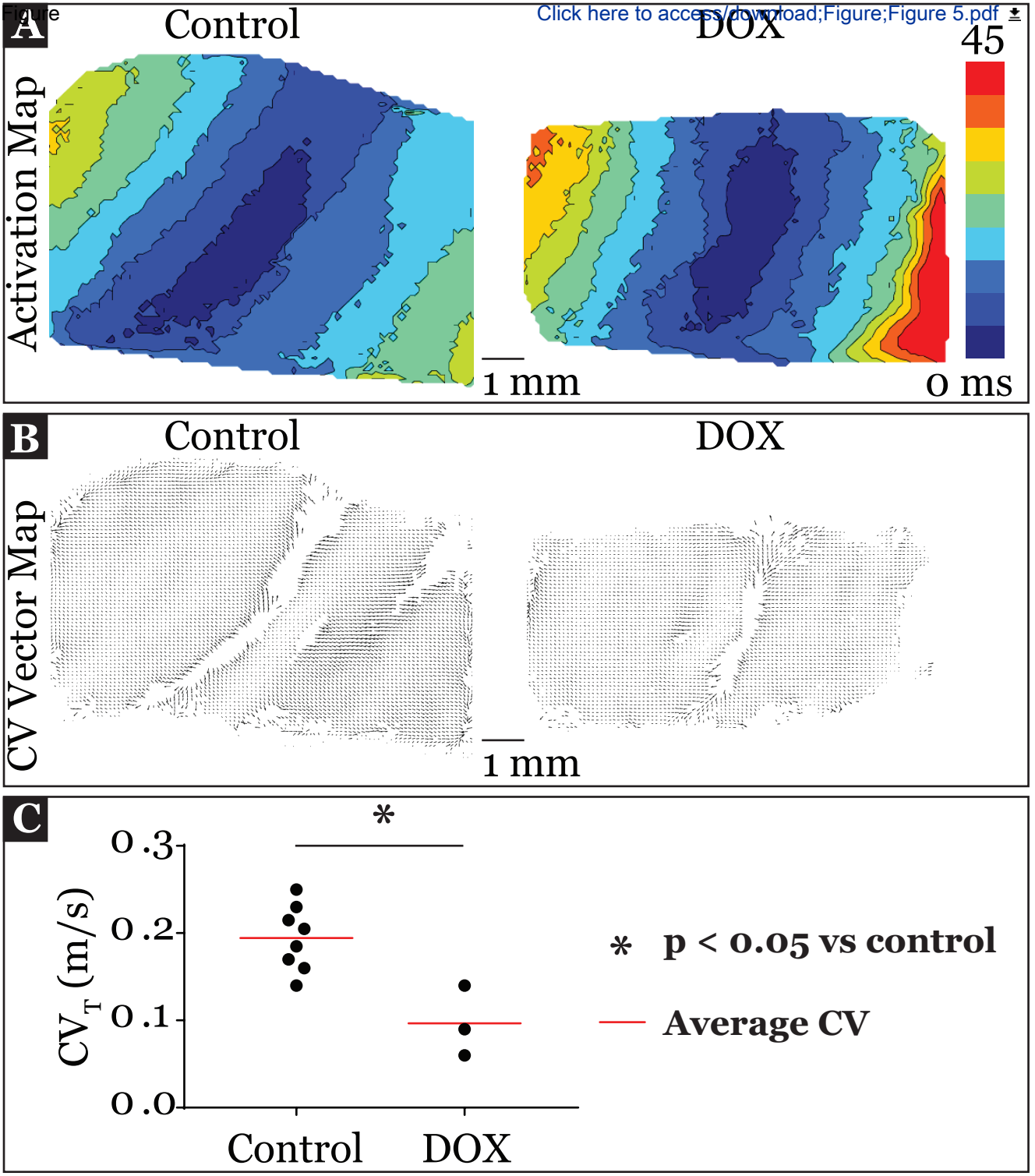


Table of Materials

Click here to access/download;Table of Materials;60781_TableofMaterials.xls

Name of Material/Equipment	Company	Catalog Number	Comments
1mL BD Syringe	Thomas Scientific	309597	
2,3-butanedione monoxime	Sigma-Aldrich	B0753	
6 well culture plates	Corning	3516	
Biosafety cabinet	ThermoFisher Scientific	1377	
Blebbistatin	Cayman	13186	
Bubble Trap	Radnoti	130149	
Calcium chloride	Sigma-Aldrich	C1016	
Corning Cell Strainers	Fisher Scientific	07-201-432	
Di-4-ANEPPS	Biotium		stock solution at 1.25 mg/mL in DMSO
DMSO	Sigma-Aldrich	D2650	
Dumont #3c Forceps	Fine Science Tools	11231-20	
Emission dichroic mirror	Chroma	T630LPXR-UF1	
Emission filter (RH237)	Chroma	ET690/50m	
Emission Filter (Rhod2AM)	Chroma	ET590/33m	
Excitation dichroic mirror	Chroma	T550LPXR-UF1	
Excitation Filter	Chroma	ET500/40x	
Falcon 50mL Conical Centrifuge Tubes	Fisher Scientific	14-959-49A	
Glucose	Sigma-Aldrich	G8270	
Heat exchanger	Radnoti	158821	
HEPES	Sigma-Aldrich	H3375	
Incubator	ThermoFisher Scientific	50145502	
Insulin Transferrin Selenium (ITS)	Sigma-Aldrich	I3146	
LED excitation light source	Prizmatix	UHP-Mic-LED-520	
Magnessium chloride hexahydrate	Sigma-Aldrich	M9272	
Medium 199	ThermoFisher Scientific	11150059	
Micam Ultima L type CMOS camera	Scimedia	N/A	
Minutien Pins	Fine Science Tools	26002-10	
Pennicillin-Streptomycin	Sigma-Aldrich	P4333	
Peristaltic Pump	Cole Parmer	EW-07522-20	

Platinum pacing wire	Alfa Aesar	43275	
Pluronic F127	ThermoFisher Scientific	P6867	nonionic, surfactant polyol
Potassium chloride	Sigma-Aldrich	P3911	
Powerlab data acquisition and stimulator	AD Instruments	Powerlab 4/26	
RH237	Biotium	61018	
Rhod2AM	ThermoFisher Scientific	R1245MP	
Rhod-2AM	Invitrogen, Carlsbad, CA		
Sodium bicarbonate	Sigma-Aldrich	S6014	
Sodium chloride	Sigma-Aldrich	S9625	
Sterilizer, dry bead	Sigma-Aldrich	Z378550	
Stone Oxygen Diffuser	Waterwood	B00O0NUVM0	
TissueSeal - Histoacryl Topical Skin Adhesive	gobiomed	AESCULAP	
UltraPure Low Melting Point Agarose	Thermo Fisher Scientific	16520100	
Ultrasound sonicator		Branson 1800	
Vibratome	Campden Instruments	7000 smz	

We thank the reviewers and the editor for thorough and very helpful reviews and for the opportunity to revise the manuscript. Please see below point-by-point responses as to how we addressed the reviewers concerns in this version of the manuscript. To facilitate review process we used black font to restate original questions from the critiques, and blue font for our responses.

Editorial comments:

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.
2. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.
3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Sigma, Invitrogen, Biotium, Branson, Pluronic, Tocris Bioscience, Sigma Aldrich, Campden Instruments Ltd., Fisher Scientific, MiCAM Ultima-L, SciMedia, etc.
4. Please revise the Protocol to contain only action items that direct the reader to do something (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." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.
5. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. For actions involving software usage, please provide all specific details (e.g., button clicks, software commands, any user inputs, etc.) needed to execute the actions. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Thank you! Necessary edits have been made.

6. Section 1: Please list an approximate volume of solutions to prepare.

The volumes of the solution to be prepared are listed in Section 1.

7. 2.1.1: Please describe how calibration is done.

The calibration step has been further explained.

8. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.
9. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.
10. 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.

The necessary steps have been highlighted.

11. Discussion: Please discuss any limitations of the technique.

The limitation that this protocol is for short-term culturing has been included in the Discussion section.

12. Figure 1G: Please include a space between the numbers and their units (1 mm, 0 ms).

The figure has been edited accordingly.

13. Please reference each panel of the figures in the text.

Each Figure panel is now referenced in the text.

14. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate journal titles. See the example below:
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

Thank you or pointing this out. The references have been converted to the JoVE format. The Mendeley citation style listed on the JoVE website has been used.

15. Table of Materials: Please remove any ™/®/© symbols. Please sort the materials alphabetically by material name.

The Table of Materials has been edited accordingly.

Reviewer #1:
Minor Concerns:

Just to be comprehensive in citation for the previous work, the authors need to cite 2 recent papers in addition to references 12-16: These 2 manuscripts just published this year in Nature communication (Fischer et al: https://www.nature.com/articles/s41467-018-08003-1, and Watson et al.,https://www.nature.com/articles/s41467-019-10175-3)

We thank the reviewer for this note. We have now cited both these recent studies in our manuscript.

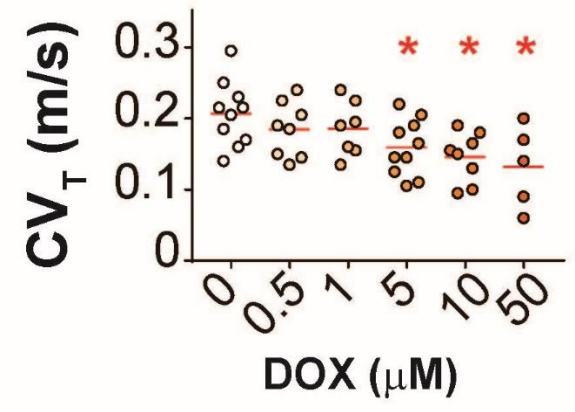
Reviewer #2:
Manuscript Summary:

Major Concerns:
The major concerns are the short introduction and discussion which should be expanded. The present manuscript also proposes the transwell methodology as a reliable long term culture method which can be utilised for drug testing. This is debatable. Although being simple and inexpensive, this methodology induces important changes in cardiac tissue (mostly due to the unphysiological conditions) which are clearly described in several papers PMID:31092830, PMID:30671746 and PMID:21972180). And these changes result in unreliable/unphysiological readouts. In 2019 three papers have been published and they all describe biomimetic culture systems able to limit in vitro tissue remodelling and therefore provide more reliable data. The authors (in the introduction and/or in the discussion) should mention them and ideally encourage the use of these systems over the transwell methodology.

We thank the reviewer for bringing this point to our attention. We have added these references to our manuscript in both the introduction and discussion, indicating that the purpose of our protocol is for studying tissues in short-term culture rather than long-term culture. Indeed, long-term culture (more than 1 month) will require further technology development by tissue slice culture community, which is fortunately growing.

Furthermore, pharmacological testing is supported with an exemplary experiment in which Doxorubicin (DOX) is shown to have profound effects on tissue function. DOX is used at a concentration of 50uM which is associated with a lower survival rate of myocardial slices (discussion). This concentration, however, is significantly higher than the concentration reported in the literature to induce cardiotoxicity in vitro (PMID:28967302, PMID:28300219). To support the assertion that myocardial slices can be used for drug testing, a concentration of DOX similar to in vivo treatments (or at least at the correct in vitro concentration) should be used.

We appreciate this comment. However, for the purposes of this protocol-focused manuscript, we were interested in simply highlighting just one condition that would show clear changes in tissue function when compared to control culture conditions. We have performed more comprehensive studies on varying concentrations of DOX, and these results will be reported in a separate, more mechanistic paper. Below is a graph of summary data included in a poster we presented at the 2019 Heart Rhythm Society annual meeting that demonstrates the DOX dose-dependent decrease in CV.



Minor Concerns:
1 - Line 54-55. It stated that myocardial slices are prepared with living donor human hearts rejected for transplantation. However, this is only one of the supply of human specimens. Other sources can be used such as LVAD implantation, myectomies etc. The text should be altered to clarify this point.

We have updated the text to suggest applying this protocol to myocardial tissues procured by alternative means. However, in our studies we use only donor hearts rejected for transplantation.

2 - Line 91-93 this sentence is misleading. Although cell isolation is time-consuming and labour-intensive, there are now machines for high throughput calcium and contractility acquisition and data analysis or automated patch clamp systems which allow a data acquisition superior to what is currently achievable with myocardial slices.

This is a good point. We have removed this sentence from the manuscript.

3 - Line 93 Myocardial slices have been used for decades, therefore it is not appropriate to consider them a "novel preclinical platform".

The word “novel” has been removed from this manuscript.

4 - Line 96 The bibliography can also be updated. In the past couple of years some major papers were published on this methodology, but they were completely ignored. A Nature Protocol Paper is available online on the same matter (PMID:29189769) and only this year two manuscripts describing the preparation and in vitro culture of myocardial slices were published in Nature Communications (PMID:31092830 and PMID:30631059).

These references have been added. Thank you.

5 - Line 158 myocardial slices of 400um thickness are recommended. It should be specified however that thinner slices can also be prepared (300um is the most common thickness used in other publications).

This is a good point. We have updated the text with this comment and included references.

6 - line 259 the meaning of "while applying minimal stretch" should be better explained. How can minimal stretch be standardized? As mentioned in the discussion, stretch has a profound effect on function, structure and excessive stretch can result in tissue damage.

We have clarified the meaning of minimal stretch that we use for our optical mapping procedures. Further explanation on how stretch can be standardized is included in the discussion.

7 - it should be clearly stated that the loading protocol of loading and calcium indicator has been specifically designed for human samples. If this method is used for other large animal models the loading will likely require re-optimization.

We thank the review for this important comment. However, our lab actually uses the same dye loading protocols for mouse, rat, dog and human slices, with equal success. But in this manuscript, we only focus on the procedure for human heart slices.

8 - the application of Blebbistatin is often not sufficient to immobilize cardiac tissue. Other laboratories use a mixture of BDM and Blebbistatin. This should briefly be mentioned/discussed.

While it is true that blebbistatin might not always completely immobilize cardiac tissue, we have found that to be the case only in whole hearts – not slices. One of the reasons for insufficient immobilization by blebbistatin is its sensitivity to light. We take precautions to minimize light exposure and blebbistatin works just fine. Thus, our lab does not to mix the two excitation-contraction uncouplers of BDM and blebbistatin because of the various ways these two agents can impact cardiac physiology. BDM has been shown to detrimentally affect calcium cycling, ion channel kinetics, and action potential morphology. While blebbistatin brings its own concern, it is still a less toxic and a more specific inhibitor of contraction than other electromechanical uncouplers (PMCID: PMC3586237).

9 - the camera acquisition speed (frames/second) is not mentioned.

This has been added to the manuscript. Thank you.

Reviewer #3:

Major Concerns:

Most importantly, the authors also need to summarize and discuss all the advantages and (specifically) disadvantages of the proposed platform in comparison with alternative platforms especially iPSC-CM based multicellular (cardiomyocytes enhanced with cardiac fibroblasts and epithelial cells) monolayers and 3D constructs.

We thank the reviewer for pointing out the lack of comparison between 3D cell culture models and the human slice platform described here. We have now included a discussion on this topic in the Introduction Section.

Second, the authors need to show an example of optical mapping from a cultured slide as they claim this application in the abstract. They also need to demonstrate its vitality over a period of culturing. This is the most critical aspect for the application of this platform for chronic drug and genetic testing.

The reviewer accurately points out that culturing the cardiac slices is one of the aims of this manuscript and in Figure 5 we illustrate slices that were cultured for 24 hours in the absence (Control) or presence of Doxorubicin (DOX). Using the method described in this manuscript, our group has previously demonstrated that electrophysiology was preserved in slices after 24 hours of culturing (PMID: 27356882) whereas additional controls were required for prolonged culture periods (PMID: 29960680). Therefore, in this manuscript we focused on the 24 hour slice culturing protocol.

I assume the authors tried to select an LV region with more parallel to surface orientation of the fibers. Otherwise, the tissue integrity would be affected. It would be thus beneficial to show histological section and demonstrate fiber orientation in slides used. It was also shown that in the LV free wall, fibers change their orientation from epicardium to endocardium. How was this acknowledged in the study?

The reviewer is correct in that the tissue cubes were oriented on the vibratome holders with fibers roughly parallel to surface, endocardial side facing upwards. This way, the plane of sectioning was approximately parallel to the fibers, which minimized cell damage. Thus, the slices collected were not transmural in nature. An image of a Mason Trichrome stained slice is now included in Figure 1E to demonstrate fiber orientation.

Even so, the LV wall has rotational anisotropy as the reviewer points out and hence the plane of sectioning is not perfectly parallel but roughly parallel to the fiber orientation in the tissue. This has been explained as a note in Section 3.2.1.

Minor Concerns:
Line 132: What temperature of recovery solution was used to dilute the dyes? I was wondering if fluorescent dyes precipitate when they are dissolved at room (or colder) temperatures as it was shown for blebbistatin (Swift, L. M. et al. Pflugers Arch. 464 (5), 503-512, 2012)? The same question is for the following section 1.4.

The dyes were reconstituted in recovery solution at 37°C and this is now indicated in the manuscript, Sections 5.1.3 and 5.1.4.

Line 177: Please, introduce 'PDMS' abbreviation.
Thank you for pointing this out, we have now included the abbreviation for PDMS at first mention in section 2.2.1.

Line 199: Please, specify if blood was washed out with ice-cold cardioplegic solution via coronary perfusion during heart harvesting procedure.

Yes, blood was washed out from the heart during ice-cold cardioplegia perfusion. This is now explained in Section 3.1.1.

Line 208: Specify what glue was used.

Thank you for this question. Histoacryl topical skin adhesive was used to attach the tissue cubes and the agarose gel to the vibratome tissue holder. This is listed in Section 3.2.1 and in Table 1.

Line 209: Please, provide more details on how the regions of interest was selected. What were the criteria used for selection of a region of interest to cut a cube? Should this region be supplied by coronary arteries? Contain Purkinje fibers? Papillary muscles? How the fibers should be aligned within this cube? It is better (in terms of fiber orientation) to use a tissue from apex or base of the ventricle?

The major criterion for selected the region of interest was to avoid areas of large coronary vessels as this will create holes in the slices. This is now explained in Section 3.2.1. The slices with trabeculae are also discarded as explained in Section 3.2.4. But, the choice of regions with Purkinje fibers, papillary muscles, LVbase/apex is dependent on the users interest. These regions can have different characteristics that can confound the results of the study. The fiber orientation within the cube is roughly parallel to the plane of sectioning as described in Section 3.2.1.

Line 228: 'Up to 3-4' what? Hours/days?

We thank the reviewer for pointing out this lack of information. We have now corrected the sentence to state that slices can be kept in the bath for 3-4 hours.

Line 250: What is the remaining 65% (30% O2 and 5% CO2)?

The Heracell Vios 160i incubator has HEPA filtered air in the chamber and allows to set the percentage of oxygen and carbon dioxide at specific levels. In our experiments, O_2 is set at 30% and CO_2 at 5% and the remaining is composed of various components of atmospheric air.

Line 262: What was the final concentration of blebbistatin used in the study? The same question is for fluorescent probes as well.

The final concentration of blebbistatin was ~5-10 μM as indicated in Section 1.4. This range is due to the fact that slices from some hearts may require more blebbistatin to prevent motion. Blebbistatin concentration was kept consistent between slices from the same heart.

How do you control a homogeneous staining of the preparation via a surface application of the fluorescent probes?

It is true that depending on how the dye is applied over the slice during dye incubation, homogenous staining of the tissue may not be achieved. However, after repeated trials, we found that uniformly applying the dye over the slice in a very slow manner so as to not cause dye to float away from the slice in the bath and by using uniform illumination during optical mapping, we were able to get signals of comparable amplitude from all regions of the slice. The

only exception is the edges of the slice where dead/damaged tissue could affect dye retention and/or signal quality. Slice edges were always excluded from the analysis.

278: 'it's - 'its'?

Thank you for catching this typo. It has been corrected.

Line 278: Specify what 'good' signal quality means quantitatively?

At this stage of the protocol, “good signal quality” refers to qualitatively 1) large amplitude and low noise and background values as indicated in BV Analysis software and 2) lack of motion artifacts. This is specified in Section 5.1.8. Signal quality is further quantitatively assessed in post-acquisition analysis by a number of steps. Low signal to noise ratio and background noise intensity thresholds are typically applied to exclude pixels from analysis in our studies. For example, SNR of a ‘good’ optical signal from a single pixel from the voltage recording illustrated in Figure 4 is 30.43 whereas for background noise this value was 0.98. A suitable threshold is then applied to remove noisy/background signals from analysis.

Line 384: As it was shown previously, rise time for optical voltage traces is much smaller than that measured by patch clamp or sharp microelectrodes. It highly depends on the sampling rate, and 1,000 fps might be too slow to fully capture 2-ms action potential upstroke phase. Therefore, optical rise time for a voltage signal should be used with precautions. The authors should acknowledge this and compare their rise time values with those measured by microelectrodes.

The reviewer is right that optical rise times are not as accurate as rise times measured from single cells by microelectrodes. A note has been added in Section 6.5.1 to indicate some factors that can affect optical rise time and to caution the readers. However, it has previously been reported to still be able to detect changes in depolarization induced by conditions such as metabolic ischemia (PMID: 30707595).

Another valuable parameter that can be calculated from a dual voltage-calcium imaging, is AP-CaT delay which indicates an excitation-contraction time (Lou Q et al. Circulation. 2011; 123(17):1881-90).

We agree with the reviewer that this AP-CaT delay is another important parameter that can be measured as we reported in our 2011 paper. We have indicated this in the manuscript, and will be incorporating this additional parameter in future versions of our open source optical data analysis program Rhythm1.2

Line 426: What was the longitudinal conduction and conduction anisotropy in the slide? How comparable are they with those measured in a native human ventricle?
It would be highly beneficial for readers to provide comparisons of electrophysiological parameters measured in slides (CV, APD, CaTD etc) with those from native human hearts to validate the platform used.

Longitudinal conduction velocity is not reported in this manuscript because only a few CV vectors were measurable in the longitudinal direction were available for analysis in all slices (as

evidenced by vector maps in Figures 4 and 5). This is because, faster conduction in the longitudinal direction very quickly propagated over the small area of the 1x1 cm^2 slice.

We appreciate this suggestion from the reviewer and have now included text in the representative results section to compare APD and CV values from cardiac slices that we obtained to Activation Recovery intervals (ARI) and CV values measured from electrograms from in vivo or explanted whole human hearts. The values we report here in the manuscript are comparable to those previously reported from in vivo/explanted whole human hearts.

Figure 4F: Since dark blue areas were excluded from the analysis, the time scale should be adjusted for a real range of APDs shown in the map. It might be reasonable to show the excluded areas in white color to avoid a possible confusion with short APDs/CaTDs.

We thank you for this suggestion. The edits have been made to Figure 4 and related texts.

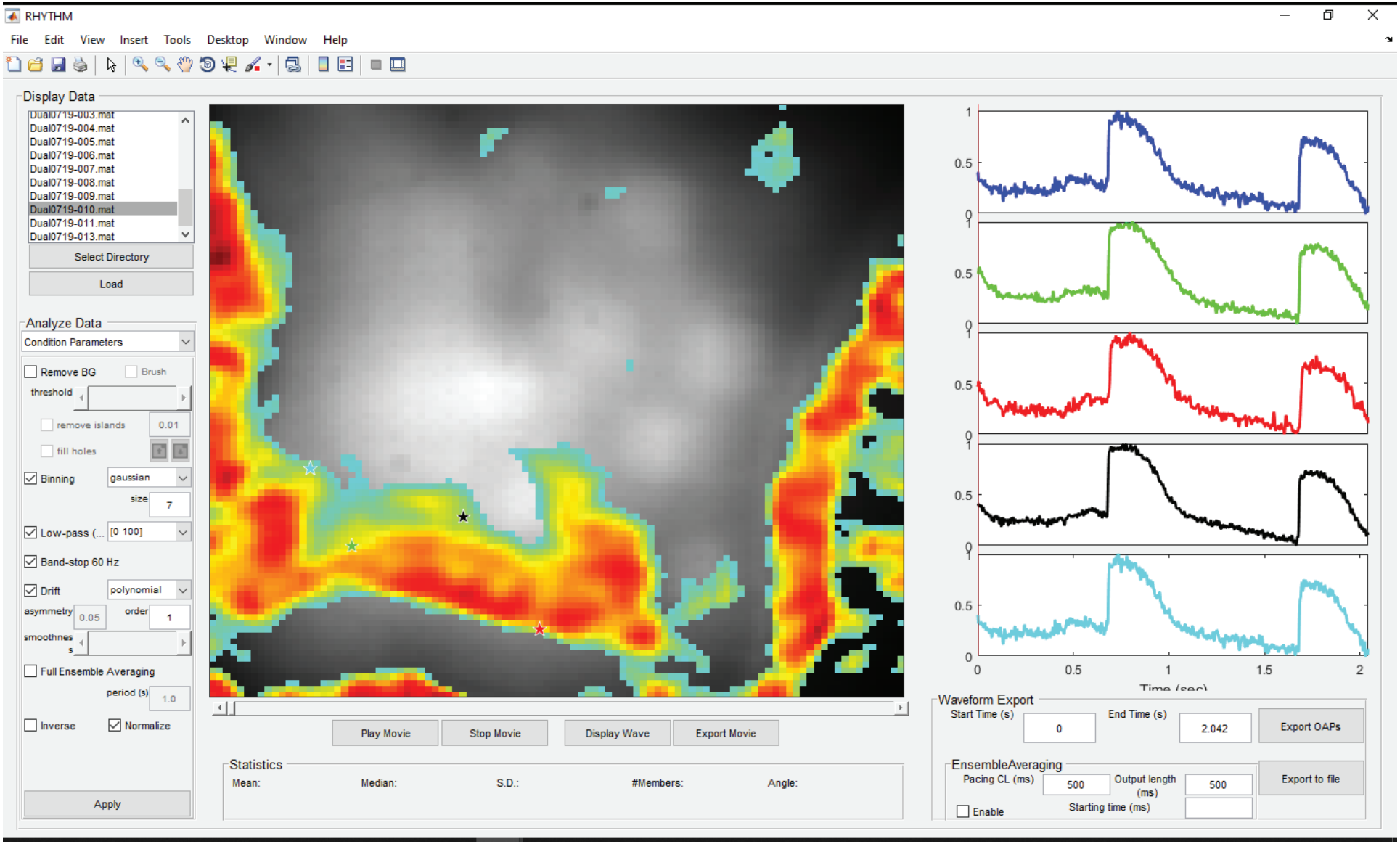
Figure 5C: Did DOX affect longitudinal CV?

Due to very few CV vectors in the longitudinal direction, predominantly close to the edge of the slice which was excluded from analysis, CV in the longitudinal direction was not robust and reproducible in all slices. As such, we did not report longitudinal CV or anisotropy from slices in this study.

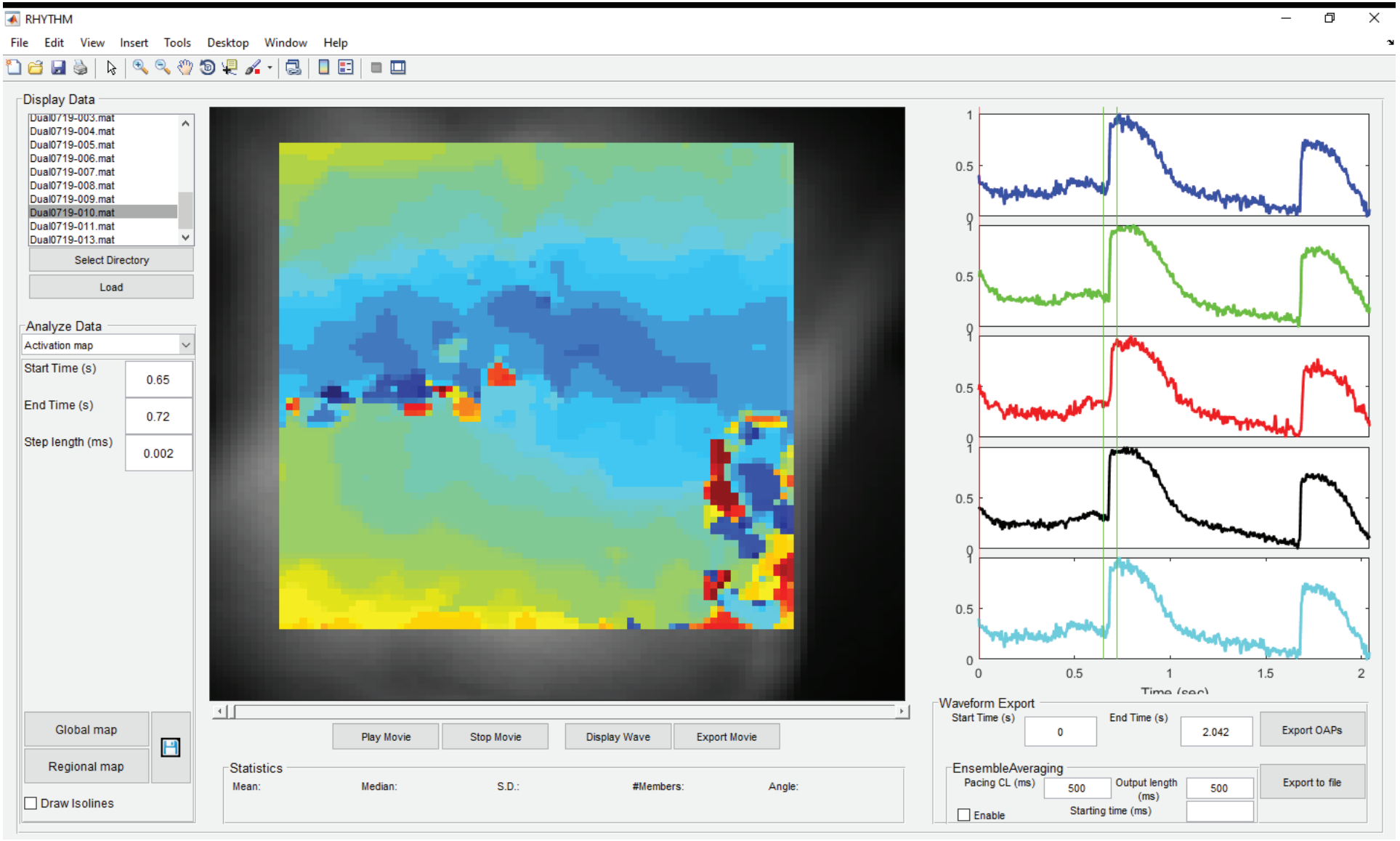
Screenfiles

Screenshot 1: Signal Conditioning

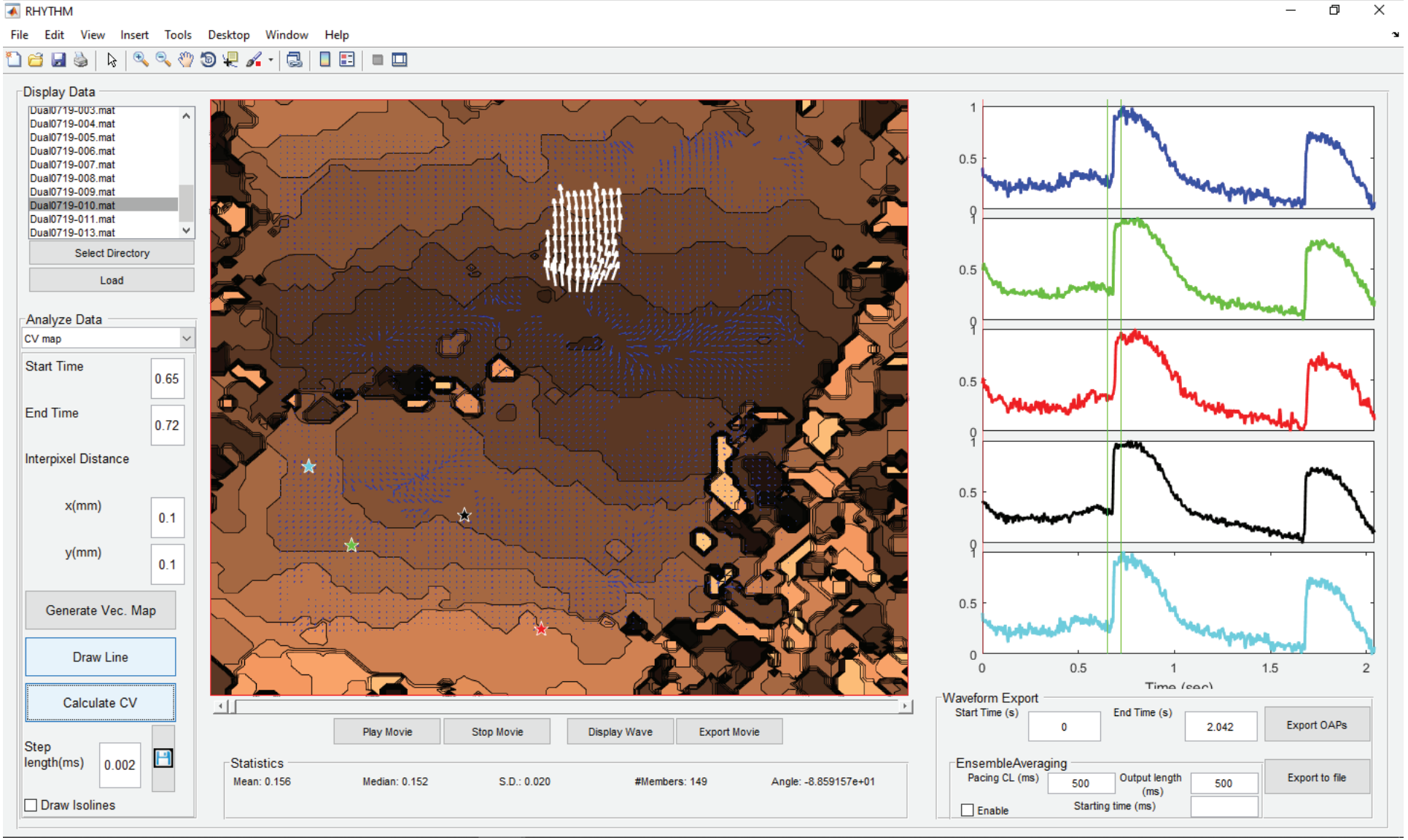
Click here to access/download;Supplemental File (Figures, Permissions, etc.);60781_Software screenshots.pdf



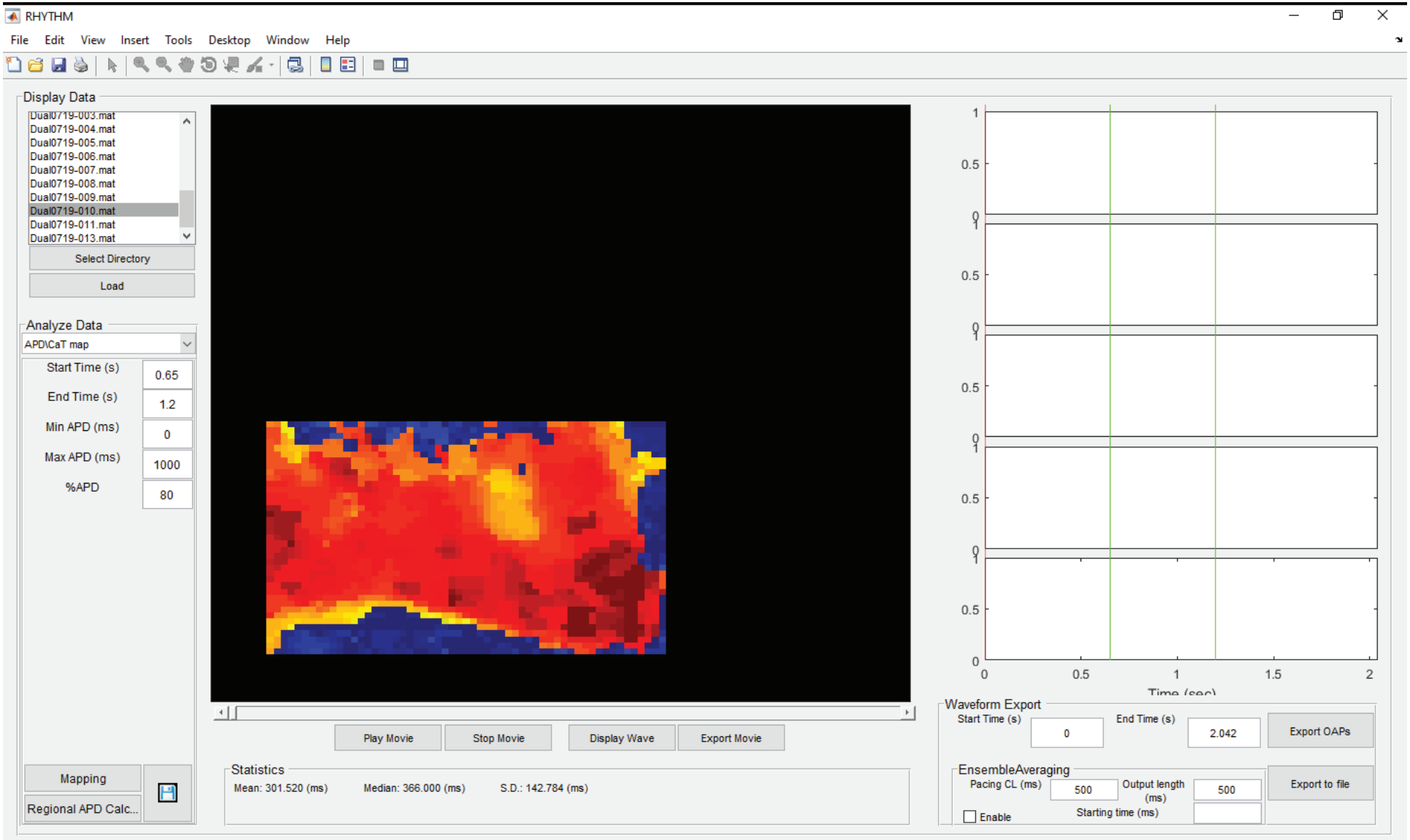
Screenshot 2: Conduction Velocity Calculation - Activation Map



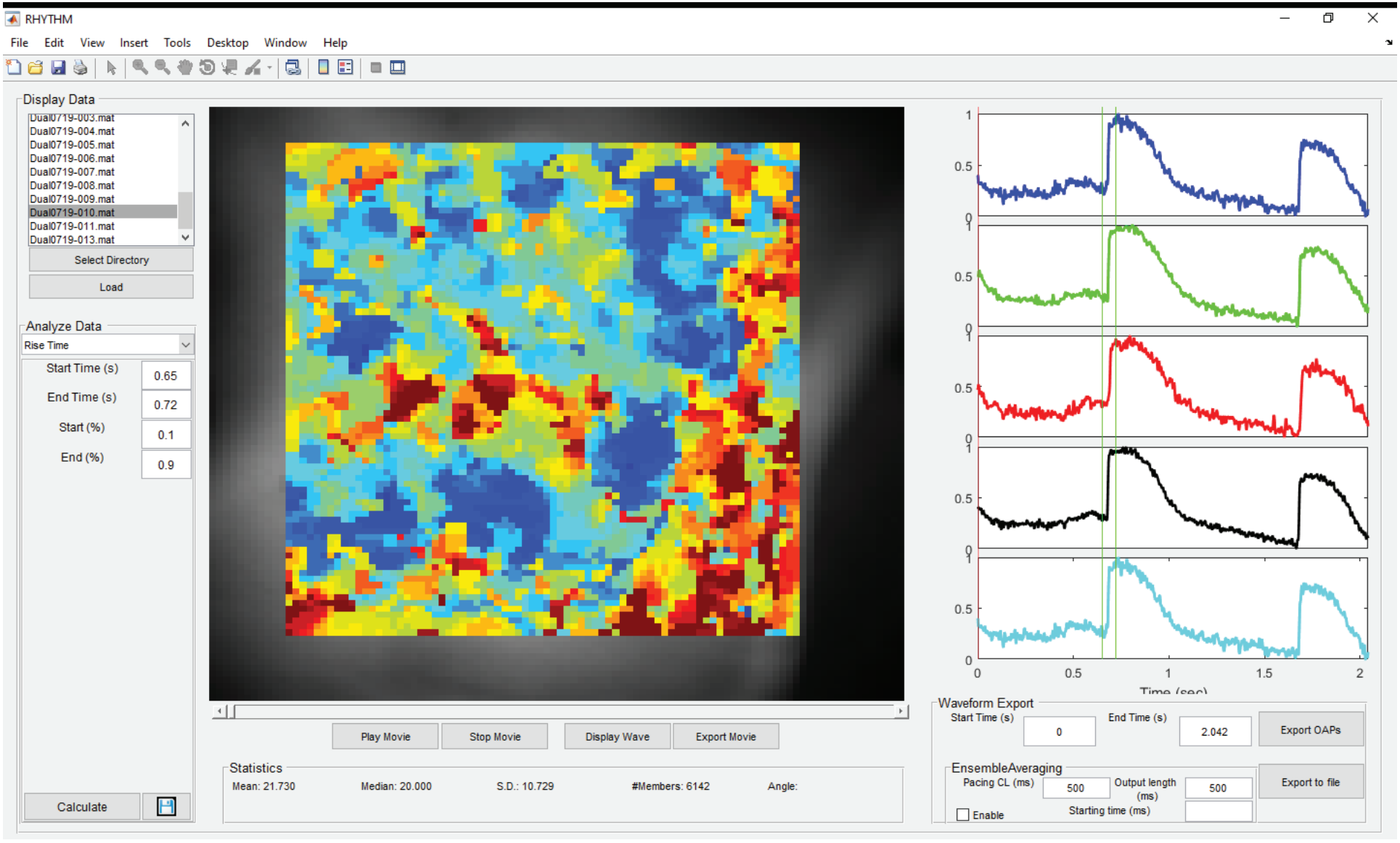
Screenshot 3: Conduction Velocity Calculation - CV Vector Map with transverse CV vectors selected in one direction



Screenshot 4: APD Map



Screenshot 5: Rise Time Map



Screenshot 6: Calcium Decay Constant Map