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# Engineering Studies Towards Simultaneous Imaging of Membrane Potential and Calcium Transients in Beating Hearts

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# ENGINEERING STUDIES TOWARDS SIMULTANEOUS IMAGING OF MEMBRANE POTENTIAL AND CALCIUM TRANSIENTS IN BEATING HEARTS

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BIOMEDICAL ENGINEERING

## ABSTRACT

Optical mapping uses fluorescent reporters to image propagation of signals like transmembrane potential (Vm) and/or calcium transients (CaT) in the heart with high spatiotemporal resolution. Simultaneous optical mapping of transmembrane potential and intracellular free calcium has been described in motionless *ex vivo* heart preparations, but simultaneous optical mapping of voltage and calcium in beating hearts is complicated by motion artifact and has yet to be reported. Studying these two parameters in beating hearts could produce invaluable information about the interactions between them under normal and pathophysiologic conditions. This thesis presents hardware development and design studies to demonstrate the feasibility of simultaneous Vm and CaT mapping in beating hearts. Our overall design is to load reporters for both voltage and calcium. Dyes are excited with two bands on alternating camera frames and two emission bands are recorded from each cardiac site. This produces four signals every two frames, and, with judicious filter choice, those signals include Vm + motion, CaT + motion, and motion only. The motion only signal can be used to cancel motion artifact in the other two. An optical splitter is needed to relay fluorescence emission to two cameras that record different wavelengths. In addition, color mixing is critical to avoid artifacts from spatial heterogeneity of excitation light. Two optical splitters were developed and optimized to record the above signals and a color mixing device was built that enabled independent filtering of two different LED wavelengths. A series of experiments in organotypic slices

of ventricular tissue and *ex vivo* swine hearts suggest two promising designs: (1) Di-4-ANEQ(F)PTEA (Vm dye)/Rhod-4 (CaT dye); green (525nm) and amber (590nm) excitation light; 540-560nm and 700nm longpass emission bands. (2) Di-4-ANBDQBS (Vm dye)/Rhod-2 (CaT dye); green and amber excitation; 550-580nm and 700nm longpass emission bands. With the optimized optical splitters, the promising spectral designs, and the color mixing device, simultaneous imaging of Vm and CaT in beating hearts should soon be realized.

Keywords: cardiac, dual mapping, optical splitter

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# INTRODUCTION

Optical mapping of cardiac electromechanical activity employs fluorescent signals recorded with high spatiotemporal resolution. It has assisted in the understanding of normal conditions, pathophysiologic conditions such as arrhythmias, and electrically induced events like pacing and defibrillations.[1-3] Electrical and mechanical function in the heart are bidirectionally coupled. This means electrical signals affect the mechanics of the heart and the motion of the heart can affect the electrical signals.[4] Due to this, measurement of both transmembrane electrical potential (Vm) and intracellular calcium concentration simultaneously is of great interest; this is possible when the heart is stained with both a calcium-sensitive fluorescent probe and a voltage-sensitive fluorescent probe.[5] Emissions from fluorescent markers are linearly related to signals of interest like transmembrane potential or intracellular free calcium and thus provide a method to produce high spatiotemporal maps when recorded with optical mapping equipment.[6] In addition to fluorescent probes, the basic elements of an optical mapping setup include excitation lights, lenses, filters, and image sensors.[7]

Simultaneous recordings of voltage and calcium have been reported in motionless *ex vivo* and *in vivo* heart preparations.[8-12] It is important, however, to be able to observe both voltage and calcium signals in beating hearts to allow the study of complex interactions between them such as mechano-electrical feedback[13] which has been implicated in arrhythmogenesis.[14-16] Motion-artifact has long been a hinderance to beating heart preparations[17], but recent advances have made beating-heart optical

mapping of Vm possible. Ratiometry is the first of these advances and works by taking the ratio of two images that have shared motion artifact/dye loading but different voltage or calcium sensitivity leaving only the desired voltage and/or calcium signals.[4, 18, 19] Excitation ratiometry was used in this study to limit the number of cameras necessary in the setup. Ratiometry either requires two signals of opposite polarity that, when divided into one another, amplify each other and remove artifact or one reference signal (a signal containing only artifact) that, when divided into a desired signal, removes artifact. The second of these advances is marker tracking. Small markers are placed on the epicardium of the heart to estimate and remove tissue deformation during recordings. These two methods enabled mapping of beating hearts.[17, 18, 20, 21]

Previous studies that have accomplished simultaneous mapping of transmembrane potential and intracellular free calcium suffer from drawbacks such as low spatial resolution (using needles to record signal),[8] inability to perform ratiometry (only used one camera),[9] narrow depth-of-field (rear-facing collimating lens),[10] and inefficient optical path (no collimating lens in front of mirror/filters)[11] that have hampered their methods from being used in beating hearts.

The objective of this study is to perform design and feasibility studies toward the establishment of a novel method to simultaneously record both voltage and calcium signals from a beating heart. There are three main components to our method. First, an optical beam splitter (a piece of equipment that separates one incoming optical path into two or more optical paths with the use of a mirror) that overcomes the shortcomings of previous systems needed to be fabricated. Next, a spectral design (choice of excitation sources, excitation filters, emission filters, dyes) was created and optimized. Finally, a new color mixer was produced and tested. Each component was first tested using

organotypic cardiac slices to avoid potentially confounding variables such as motion and complications with whole heart dye loading. Once validated in the *in vitro* environment, the spectral design and the splitter were assessed in the *ex vivo* environment using Langendorff-perfused pig hearts.

# METHODS

## Design Overview

Fluorescent Vm and CaT reporters will be loaded. Two excitation bands will be used to excite the dyes on alternating camera frames and two emission bands will be collected. This produces up to four signal collection opportunities every two camera frames. With careful excitation and emission filter choice, a Vm + motion, a CaT + motion, and a motion only signal can be collected from each site. The motion only signal can be collected at the isosbestic point of the Vm dye and used to cancel motion artifact in of the other two. To collect three signals of interest in two frames, two signals must be collected in one frame. An optical splitter is ideal for this situation as it takes emission from Vm and CaT reporters and relays them to two cameras that record different wavelengths. Spatial heterogeneities in excitation lighting are detrimental to optical mapping, thus a color mixing device for the excitation source is necessary for effective optical mapping. Studies in organotypic cardiac slices and *ex vivo* hearts will help optimize optical splitter design, dye/filter choice, and color mixer design.

## Optical Splitter Design

Previous works have accomplished simultaneous imaging of transmembrane potential and intracellular free calcium; however, each of the designs/methods implemented have suffered from a disadvantage (low spatial resolution, inability to perform ratiometry, narrow depth-of-field, or inefficient optical path)[8-11] that hindered

their ability to be used in beating heart preparations. To solve these issues, two different splitter designs were evaluated.

First, a relay lens beam splitter was built consisting of a front-facing objective lens, a rear-facing collimating lens, a half-silvered or dichroic mirror, two refocusing relay lenses, and two cameras. All lenses were full-frame format SLR camera lenses so lens aberrations were ignored.[24] SOLIDWORKS was used to aid in the design of the relay lens optical splitter. Once designed in SOLIDWORKS, the splitter was built. The mirror's housing was fabricated in house. Stock aluminum was purchased from McMasterCarr and milled to size. The housing (roughly 3"x3"x3") was made slightly larger than the diameter of the lenses that attach to it to limit excess weight and to keep the design small. Three circular holes were cut into the sides of the mirror housing and epoxy was used to secure ring adapters in them. Three 50mm f/1.4 Nikon lenses were attached to the mirror housing by screwing the lens filter threads to these ring adapters. Another circular hole was cut into the top of the mirror housing for the mirror/mirror-holder. The inside of the mirror housing was painted black, and any opening was covered with black tape. 15mm aluminum rods were attached underneath the mirror housing, lenses, and cameras to keep parts aligned and stabilize the system; the rods were attached to the splitter using rod clamps. A quick release plate was installed beneath the rods so the splitter could easily attach to our pre-existing camera mounts. The mirror-holder was also fabricated in house using 3D-printed parts and heat-set inserts/screws purchased from McMasterCarr. The holder is highly adjustable allowing mirror rotation, mirror tilt, and mirror movement left, right, up, or down. This level of adjustability was achieved by placing the mirror in a 3D-printed U-channel that attaches to a 3D-printed lid *via* a thumbscrew. Small aluminum bars were milled and attached to the top of the splitter

housing to hold the lid on and to lock the rotational adjustment of the mirror. The relay lens optical splitter design that we employed was composed of four lenses. The two optical paths shared their first two lenses (objective lens and collimating lens), diverged at the beam-splitting mirror, and had their own third lens (the refocusing lens); therefore, each optical path consisted of three lenses. As lenses are combined in series, light is lost. To determine the lens combination that maintained the highest light efficiency, an LED held at constant temperature was placed behind a target (~5x5cm) fashioned to represent the mapping area of a pig heart. Different focal length lenses were used in the beam splitter design over distances varying from 10-80cm. Short videos (3 frames) were recorded. The intensity of light emanating from the target was averaged spatially and temporally for each lens combination. Once the lens combination with the highest light efficiency was selected, the splitter was evaluated in cardiac slices and *ex vivo* hearts.

The second splitter was a variation of the relay lens optical splitter detailed above. This second splitter consisted only of the final lenses in the relay lens splitter (50mm f/1.4 Nikon lenses) and is referred to as the simplified splitter elsewhere in the paper. A 5x close-up lens was attached in front on the two 50mm lenses. All other splitter components (mirror-housing, stabilizing rods, etc.) were shared between splitters. This splitter was based on a splitter used in Lee et al. (2017). Because the second splitter only used one lens per optical path instead of three (as in the first splitter), it should retain more light. The lack of collimation in front of the mirror/filters could decrease reliability of those elements as they are designed to process parallel light beams. The splitters were compared for their light efficiency using the target method discussed at the bottom of the previous paragraph.

## Spectral Design

Excitation ratiometry was used in this work. Because we have two parameters of interest (voltage and calcium), our design is to collect one reference signal (artifact only signal) that can be used to cancel motion and dye loading artifacts in both voltage and calcium recordings. A reference signal can be obtained at an isosbestic point (a combination of excitation band and emission band at which a voltage-sensitive dye has no voltage sensitivity) of a ratiometric dye. Isosbestic wavelengths of our voltage dye were found by varying excitation and emission filters until the voltage dye showed little to no voltage sensitivity (see "Methods: Cardiac slices: Experimental Protocol" for further detail).

The first candidate voltage dye Di-4-ANEQ(F)PTEA was selected for testing based on its long-wavelength spectral properties as well as its use in previous works.[23, 25] Calcium dyes Rhod-2, Rhod-4, Fluo-3, Fluo-4, Cal-520, and Calbryte-520 were tested for their loading speed in cardiac slices. Dyes can be recirculated in an *ex vivo* environment to promote better staining[11], but a quick loading dye was preferred to decrease recirculation times reducing the likelihood of injuring the heart *via* lack of nutrients.

Two set of lights/filters were primarily utilized in this work (Figure 1). All lights and filters were selected to maximize signal while minimizing crosstalk. First, cyan (~505nm) and green (~525nm) LEDs (Luxeon C, Lumileds) were utilized as excitation sources. Four LEDs of each color were soldered in close proximity to the same circuit board. The physical proximity of the LED chips aids color mixing. Because these LEDs have a spectral half-width of ~30 nm, 530nm shortpass excitation filters were used to prevent cyan and green excitation light from interfering with emission signals. A single filter was used for all LEDs on each circuit board. Calcium emission was collected from a

band shorter than the voltage dye's emission band to avoid crosstalk with voltage emission (~540-560nm) and voltage emission was collected from wavelengths longer than the calcium's dye's emission band to avoid crosstalk with calcium emission (>700nm). These lights and filters were utilized in both cardiac slices and the Langendorff preparation before the new color mixer was designed. Green (~525nm) and amber (~590nm) Luxeon C LEDs were the excitation sources in the second set of lights/filters employed. Using these excitation sources, only voltage was recorded, so a 625nm shortpass excitation filter was used and voltage emission was collected with a 700nm longpass filter. To record both Vm and CaT with these excitation wavelengths, the two excitation wavelengths must be filtered independently. The color mixing device described in the next section was designed to achieve this.

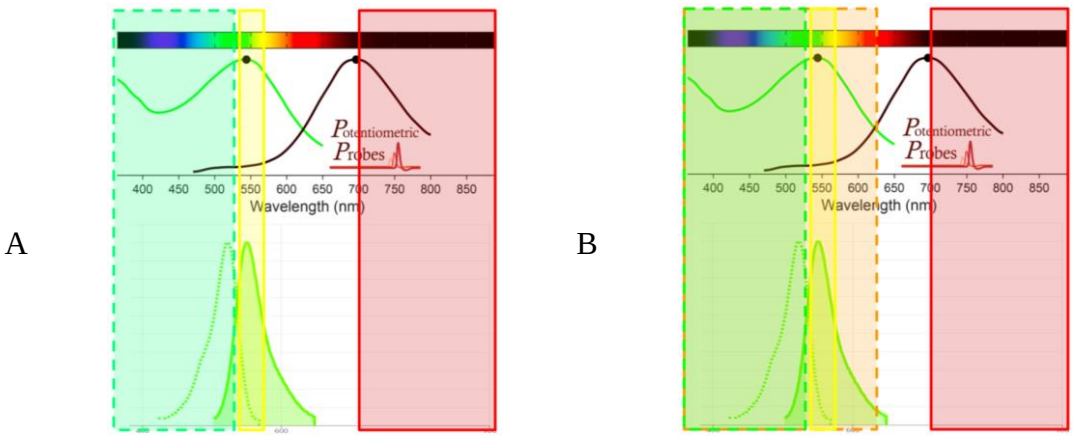



Figure 1. Spectral Designs. Both spectral designs used Di-4-ANEQ(F)PTEA (top spectra) and Rhod-4 (bottom spectra). Excitation bands are shown as dashed rectangles and emission bands are shown as solid rectangles. (A) The first spectral design using cyan and green excitation. A single 530nm shortpass excitation filter is used. Emissions are recorded between 540-560nm (CaT) and 700nm longpass (Vm). This spectral design was employed in organotypic ventricular slices and in an *ex vivo* heart. (B) The second spectral design using green and amber excitation. Two excitation filters are used: 530nm shortpass for green excitation and 625nm shortpass for amber excitation. Emissions would be recorded between 540-560nm (CaT) and 700 longpass (Vm). This spectral

design was employed in an *ex vivo* heart. In the heart, we only stained with Vm dye, so the 530nm shortpass excitation filter and the 540-560nm emission filter were omitted.

## Color Mixing Device Design

With the previous iteration of our excitation light design, two different color LEDs were placed on one printed circuit board. Only one excitation filter could be used for both colors of LEDs because of their physical proximity. When using a common shortpass excitation filter, both excitation sources must be a lower wavelength than the emission band of the calcium dye lest we saturate the calcium emission band. However, using LEDs that are farther apart in wavelength than cyan and green may produce better results for recording voltage because the signals elicited by the two excitation wavelengths would have larger differences in voltage sensitivity. When one of the excitation bands is within the emission band of the calcium dye, it becomes necessary to filter the two excitation bands independently. One approach for this is to use a filter with multiple pass bands. However, we were unable to source such a filter. An alternative approach is to build a device that mixes light emitted from separate circuit boards with independent filters.

A hollow square pyramid structure was constructed out of black acrylic sheet. Acrylic was cut using a laser cutter and parts were attached using hot glue. The inside of the pyramid was painted matte white to diffusely reflect light. Each side of the square pyramid held an LED board, a small color-mixing chamber, and a water cooling jacket; the LEDs shined into the middle of the pyramid. The LED boards on opposite sides of the pyramid were the same color. The middle of the pyramid was used as a second color mixing chamber and light came out of a 1.5” circle cut into the bottom of the pyramid. The 1.5” circle cut-out in the bottom of the pyramid is referred to as the “aperture” and

the aperture size was varied to see how it affected color-mixing. Varying lengths of PVC pipe were also attached to the color mixer at the aperture to see how they affected color mixing and are referred to as “hoods”. Each LED board in the color mixer could use a separate shortpass excitation filter. The color mixing design was then tested to determine its color mixing efficiency. Color exiting the light source should show homogeneity between the two colors at the object distance for ratiometry to be effective. A 35mm f/1.4 lens attached to a BrainVision MiCAM03 N256 camera was mounted ~40cm away from a piece of paper; the paper was used to visualize the pattern that the different color lights in the light source created. The light source was placed behind the piece of paper and directed at paper at distances varying from 2-20cm. Short videos (3 frames) were recorded; three videos were recorded at each distance for each color. Videos were averaged temporally to create an image, images of different colors at the same distance and trial were subtracted to create a difference image, and then the variance of the difference image was determined and plotted. The design of the color mixer with the lowest variance was selected for ratiometry tests in cardiac slices.

## Organotypic Cardiac Slices

### *Slice Preparation*

Organotypic slices of swine ventricular myocardium were prepared in a similar fashion to previous works.[26] 4L of HEPES buffered physiological solution (140 mM NaCl, 3 mM KCl, 2.6 mM CaCl2, 0.6 mM MgCl2, 10 mM HEPES, 12 mM glucose, 20 mM BDM; pH = 7.4) was prepared the day of the experiment and was stored in an ice bath. The animal was anesthetized and chest opened as previously described.[21] 500 IU/kg heparin was given intravenously. The heart was rapidly excised and placed into a bucket containing 3L of ice cold 0.9% saline. The aortic root was cannulated and the coronary

arteries were flushed with 1L of ice cold HEPES. The ventricles of the heart were then sliced into ~1$cm^3$ chunks and placed back into the bucket of saline. The epicardial surface of the 1$cm^3$ heart chunks were glued to a tissue holder. The tissue holder was transferred into the bath chamber of a vibratome. A large initial slice was cut to create a flat, rectangular surface.

*Instrumentation*

The vibratome was calibrated until the z-axis value was less than 1 um. The vibratome settings were adjusted to 380-400 um cutting thickness, 0.03 mm/sec advance speed, and 80 Hz vibration frequency. The bath of the vibratome was filled with HEPES buffered physiological solution at ~4 ºC. Ice was continually placed around the bath to maintain the slicing solution's temperature and 100% oxygen was continuously bubbled into the slicing bath throughout the experiment. Anti-foam was occasionally utilized to decrease surface bubbles.

For determining slice viability, a Canon 85mm f/1.4 lens was fitted with a 5x close-up lens and attached to a BrainVision MiCAM03 N256 camera with 256x256 pixels on a 17.6x17.6mm CMOS sensor. The camera was mounted above the mapping chamber containing HEPES buffered physiological solution and the cardiac slices. 10uL of Di-4-ANEPPS (0.520uM) was used to stain the slice. The slices were backlit with cyan (~505nm) LEDs cooled with recirculating water and 600 nm longpass emission filter was utilized.

*Experimental Protocols*

Once slices were obtained, they were moved to a mapping chamber. The mapping chamber consisted of a petri dish containing HEPES solution at ~37 ºC and stimulation

electrodes. The electrodes were used for bipolar field pacing at 1 Hz and anywhere from 10-100mV in magnitude.

Four different experiments were run. First, the isosbestic point of Di-4-ANEQ(F)PTEA was determined. Cyan (~505nm) and green (~525nm) LEDs were used. Slices were loaded with 10 uL of Di-4-ANEQ(F)PTEA (0.027uM) by dispersing it over the slice with a micropipette. Imaging was performed immediately after loading. The cyan excitation source was used to determine if the slice was viable. Green excitation was used to see if the slice showed any voltage sensitivity (green was the expected isosbestic point). A 625nm shortpass excitation filter and a 700nm longpass emission filter were used. The excitation was switched back to cyan to again verify slice viability.

Next, select calcium dye was loaded into a petri dish containing a cardiac slice. For Rhod-2 (8.746uM) and Rhod-4 (9.745uM), 20uL of dye was used to stain the slice. For Fluo-3 (8.763uM), Fluo-4 (9.026uM), Cal-520 (4.511uM), and Calbryte-520 (4.561uM), 20uL of dye was used along with 200uL of Probenecid (2.278mM) and 10uL of Pluronic to stain the slice. Imaging was performed using cyan excitation at 0-, 15-, 30-, and 60-minutes post dye loading to determine dye loading speed. When not being imaged slices were kept in an incubator at ~37 ºC with 5% $CO_2$.

Dual dye loading/imaging was attempted with both splitters. Rhod-4 was loaded as stated above and voltage dye was loaded by adding 20uL Di-4-ANEQ(F)PTEA (13.570uM) to the imaging solution. A 675nm longpass filter was used for the Vm emission instead of the 700nm longpass filter mentioned in the spectral design.

The color mixer with 1” and 4” hoods was tested to verify that the color mixing was effective enough for ratiometry. 10 uL of Di-4-ANEPPS (0.520uM) was loaded into a petri dish containing a cardiac slice. Di-4-ANEPPS was used because the color mixer

contained blue and cyan LEDs and Di-4-ANEPPS has a shorter wavelength spectrum than Di-4-ANEQ(F)PTEA. Recordings were made using a 600nm longpass emission filter. Motion was induced by bumping the mapping chamber. The blue and cyan LEDs in the color mixer alternated each frame (blue = odd frames, cyan = even frames) to perform excitation ratiometry.

*Data Processing*

A frame from a recording was selected, normalized, a mask was created using MATLAB's imbinarize function with a 0.25 threshold, and then Vm visualization was done in similar fashion to our previous works.[21] MATLAB's movmean function was first used with a large window to remove baseline drift and then with a small window to remove noise. The root mean square of the Vm signal with baseline drift and noise removed was taken and divided by the root mean square of the noise signal. The result was squared to get the signal to noise ratios.

## Langendorff-Perfused Pig Hearts

Two *ex vivo* experiments were performed. Instrumentation was varied between the two experiments.

*Heart Preparation*

The Langendorff perfusion heart preparation were done in a similar fashion to previous works.[21] 6L of modified Tyrode's (123 mM NaCl, 11 mM glucose, 0.98 mM MgCl2, 4.5 mM KCl, 1.01 mM NaH2PO4, 1.8 mM CaCl2, 20mM NaHCO3, 0.04 g/L albumin, 20 mM BDM; pH = 7.4) was prepared the day of the experiment. The solution was stirred for 5 minutes to remove excess $CO_2$, bubbled with 95% $O_2$, 5% $CO_2$ for ten minutes, and was then stored in an ice bath. BDM was prepared separately and added to the perfusate after recordings were made in beating hearts. The animal was anesthetized and the chest

opened.[21] 500 IU/kg heparin was given intravenously. The heart was rapidly excised and placed into a bucket containing 3L of ice cold 0.9% saline. The aortic root was cannulated, and the coronary arteries were flushed with 2L of ice cold Tyrode's. Approximately 20 black circular markers (~5mm spacing) were glued to the anterior left ventricular epicardium with tissue glue. The heart was then suspended in the mapping system and by the aortic cannula and perfused at 200 ml/min with 37°C oxygenated modified Tyrode's solution. Bipolar pacing electrodes were placed in the subepicardium. Paddle electrodes were used to reinitiate organized activity.

*Instrumentation*

Both beam splitters were utilized for signal collection. The relay lens splitter consisted of a 24mm f/1.4 Canon front-facing objective lens, a 50mm f/1.4 Nikon rear-facing collimating lens, two 50mm f/1.4 Nikon refocusing relay lenses, and two BrainVision MiCAM03 N256 cameras with 256x256 pixels on 17.6x17.6mm CMOS sensors. The simplified splitter consisted only of two 50mm f/1.4 Nikon refocusing relay lenses and two BrainVision MiCAM03 N256 cameras. The mirror was omitted from both setups as we only stained for voltage and thus did not need to see through both cameras. 675nm and 700nm longpass emission filters were used.

In the first *ex vivo* heart, the relay lens splitter was mounted about 15cm away from the heart. The first set of lights/filters was used for this *ex vivo* experiment. The heart was lit with either cyan LEDs (~505nm), green LEDs (~525nm), or both (in alternating fashion) cooled with recirculating water. The heart was stained with 250 uL of Di-4-ANEQ(F)PTEA (9.076uM). The dye was dissolved in ~100 ml of perfusate and recirculated through the coronary circulation for ~5 minutes. The heart was then

alternated between large volume perfusion and small volume staining perfusion at roughly 5 minute intervals until dye loading had plateaued (visualized with splitter).

In the second *ex vivo* heart, both splitters were used with the second set of lights/filters. Splitters were mounted 15-30cm away from the heart. The heart was lit with either green LEDs (~525nm), amber LEDs (~590nm), or both (in alternating fashion) cooled with recirculating water. The heart was stained with 250 uL of Di-4-ANEQ(F)PTEA (9.076uM). The dye was dissolved in ~100 ml of perfusate and recirculated through the coronary circulation for ~5 minutes.

*Experimental Protocols*

Imaging runs lasted about 10 seconds and the frame rate was varied from 300 Hz (to increase light) to 750 Hz (to test the splitters at commonly used cardiac imaging frame rates[21, 23]). While the heart was beating, LED excitation color alternated with the frames for excitation ratiometry (ex. odd frames = cyan, even frames = green). BDM was added to the perfusion solution and more imaging runs were conducted. Frame rates were again varied from 300-750 Hz. In some videos, only one color LED was turned on (cyan only or green only). In others, LED excitation color was again allowed to alternate with the frames.

*Data Processing*

Tissue motion tracking and Vm visualization was done in similar fashion to our previous works.[21] MATLAB's movmean function was first used with a large window to remove baseline drift and then with a small window to remove noise. The root mean square of the Vm signal with baseline drift and noise removed was taken and divided by the root mean square of the noise signal. The result was squared to get the signal to noise ratios.

# RESULTS

## Optical Splitter

### *Design and Components*

Both splitters were constructed in-house. The design and photos of both optical splitters are shown in Figure 2.

A B C

Figure 2. Optical Splitters. (A) SOLIDWORKS design of the splitter housing with its mechanical supports. Lenses and cameras were not included in SOLIDWORKS. (B) The relay lens optical splitter modeled after the SOLIDWORKS rendering including lenses, cameras, and extension tubing attachments. (C) The simplified splitter including lenses, cameras, and 5x close-up lens.

### *Lens combination tests*

For the relay lens splitter, tests were performed to determine which combination of lenses would retain the most light at varying distances. Lens combinations are denoted as “focal length of front objective lens-focal length of collimating lens-focal length of refocusing lens”. First, the focal length of the front lens was varied while keeping the focal lengths of the back two lenses constant (shown below in Figure 3). As the focal length of the objective lens increased, the distance between the objective lens and the collimating lens

had to increase for the splitter to focus. The objective lens and collimating lens are connected by extension tubing and there was a limited amount of extension tubing on hand; therefore, at longer focal lengths, the splitter was unable to focus at shorter object distances (thus why data points at shorter object distances are not shown for longer focal length objective lenses). N = 9 for each lens combination at each object distance. In n = 3 of the 24-50-50 lens combination, intensities across all distances were lower likely due to an aperture being partially closed.

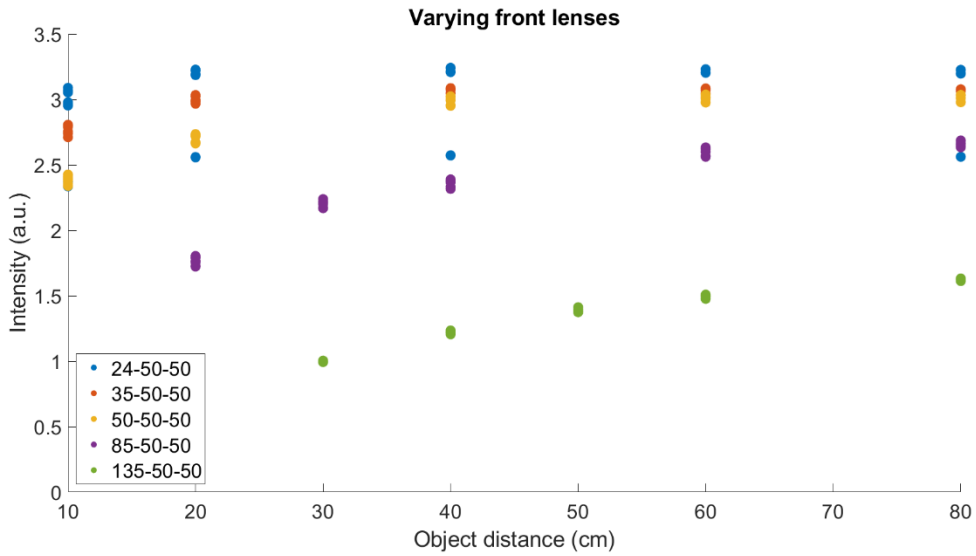



Figure 3. Splitter: Front Lens Variations. Intensities recorded by the relay lens splitter while varying the focal length of the front lens and increasing object distance. Lens combinations are denoted as "objective lens focal length-collimating lens focal length-refocusing lens focal length".

As the focal length of the objective lens increased, the intensity of light collected over the target region decreased. The lens combination that recorded the highest intensity of light over the target region was 24-50-50; with this combination, intensity of light recorded did not vary much over distance.

After varying the focal length of the objective lens, the focal lengths of the collimating lens and the refocusing lens were varied (seen below in Figure 4). Again, lens

combinations are denoted as “focal length of front objective lens-focal length of collimating lens-focal length of refocusing lens”.

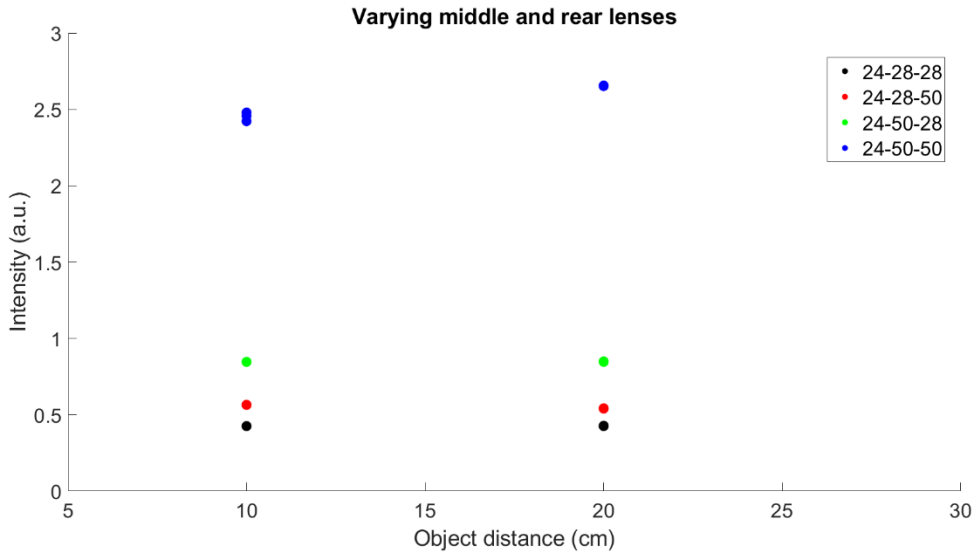



Figure 4. Splitter: Rear Lenses Variations. Intensities recorded by the relay lens splitter while varying the focal lengths of the collimating lens and the refocusing lens while holding the objective lens focal length constant. Lens combinations are denoted as “objective lens focal length-collimating lens focal length-refocusing lens focal length”.

N = 3 for each lens combination at each object distance. The intensity recorded over the target region for the 24-50-50 lens combination clearly surpassed competing combinations. With the 24mm focal length lens recording the highest light intensity when varying the objective lenses and the 50mm-50mm focal length combination recording the highest light intensity when varying the collimating and refocusing lenses, the combination of 24mm f/1.4 objective lens, 50mm f/1.4 collimating lens, and two 50mm f/1.4 refocusing lenses were selected for use in the relay lens splitter.

*Splitter comparison*

The light efficiency between the relay lens beam splitter and the simplified beam splitter (based on Lee et al. (2017)) were compared. The results of the comparison are shown below in Figure 5.

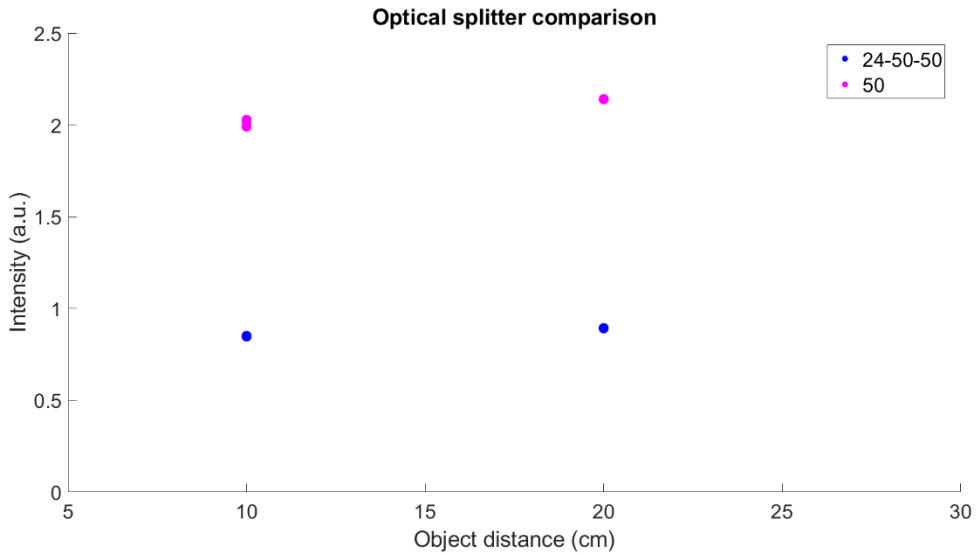


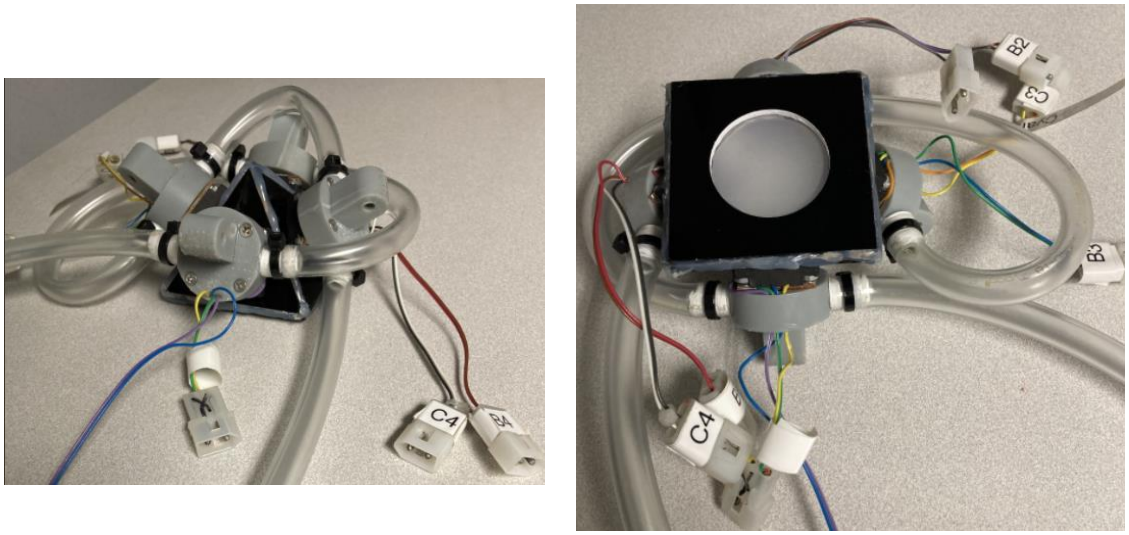

Figure 5. Splitter Comparison. A comparison between the 24-50-50 relay lens optical splitter and the simplified splitter. Lens combinations are denoted as "objective lens focal length-collimating lens focal length-refocusing lens focal length" or simply "objective lens focal length" for the simplified splitter design.

The splitter based on the splitter used in Lee et al. (2017) captured ~2.5 times more light than the relay lens optical splitter utilized in our cardiac slice and *ex vivo* experiments. For signal magnitude, this simplified splitter design is the clear choice; however, as mentioned before, as it does not include a collimating element before the dichroic mirror and emission filters, the splitter would need to be assessed for crosstalk between dyes in cardiac slices and then in *ex vivo* hearts.

## Color Mixer

### *Design and components*

The color mixer was devised and constructed in-house. Photos of it are shown in Figure 6.




A B

Figure 6. Color Mixer. (A) A picture of the 1.5” aperture, 0in hood color mixer (aperture face down). (B) A picture of the 1.5” aperture, 0in hood color mixer (aperture face up).

*Color mixing tests*

Tests were performed to determine the color-mixing efficacy of the new light source design. A variance under 1% is considered acceptably homogenous. N = 3 for each light source variation at each distance. Variations on the light source design are denoted as “aperture size (in), hood length (in).” Aperture size did not decrease variance as desired. Hood length (the length of PVC pipe placed over the aperture) did largely decrease variance with all hood lengths recording variances near zero (see Figure 7). The 4” hood length did have the lowest overall variance. When the new light source was used in cardiac slices, it, along with a 1.5” aperture, was used.

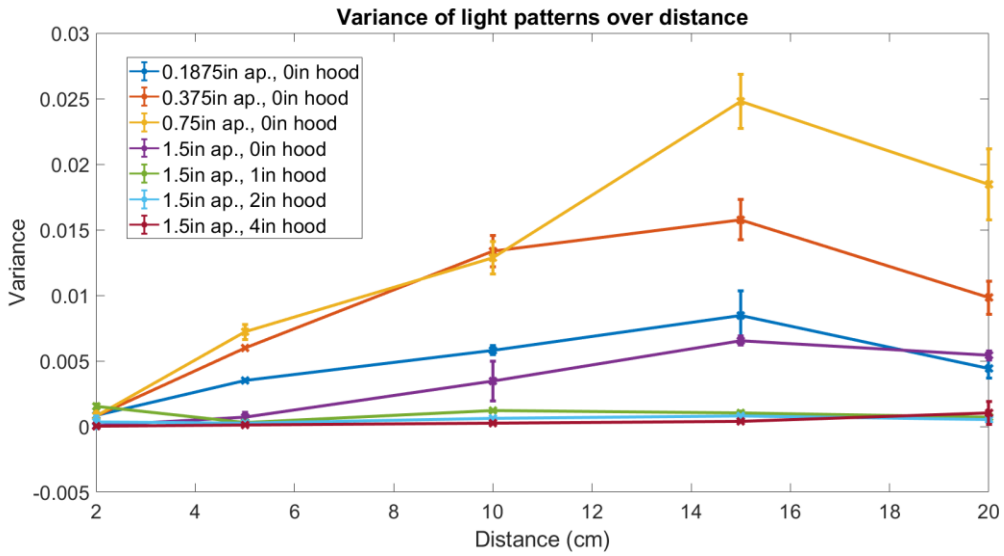



Figure 7. Variances using color mixers. Variances between cyan and blue lights over distance in variations of the color mixer.

## Cardiac Slices

### *Isosbestic point of Di-4-ANEQ(F)PTEA*

The isosbestic point of a potentiometric dye is a combination of excitation and emission wavelengths that do not produce voltage sensitivity. While attempting to determine the isosbestic point of Di-4-ANEQ(F)PTEA, many combinations of excitation sources, excitation filters, and emission filters tested. Figure 8 shows the single pixel signal from a cardiac slice stained with Di-4-ANEQ(F)PTEA, excited with green LEDs passed through a 625nm shortpass filter and collected through a 700nm longpass filter. No Vm signal is present.

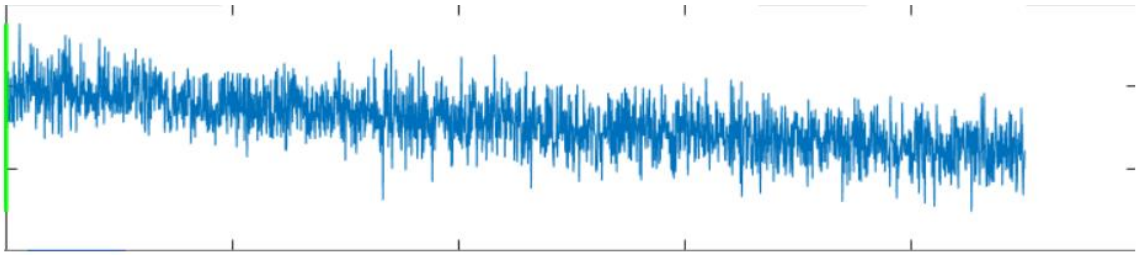


Figure 8. Isosbestic Point of Di-4-ANEQ(F)PTEA. An isosbestic point of Di-4-ANEQ(F)PTEA. No Vm signal is visible using green excitation with a 625nm shortpass filter and a 700nm longpass emission filter.

*Calcium dye loading*

Table 1. Calcium dye incubation times to produce signal.

| Dye | Loading time (minutes) |
|---|---|
| Rhod-2 | 5 |
| Rhod-4 | 20 |
| Fluo-3 | 90 |
| Fluo-4 | N/A |
| Cal-520 | 90 |
| Calbryte-520 | 90 |

Several calcium probes were assessed for their possible use (Table 1). A short loading time was desirable to allow for recirculating staining of *ex vivo* heart preparations. Two dyes presented as viable options: Rhod-2 produced good signal quality after ~5 minutes of incubation and Rhod-4 produced good signals after ~20 minutes of incubation. To help minimize crosstalk, the slightly shorter wavelength Rhod-4 calcium dye was selected.

*Dual imaging with splitter*

A few slices were dual stained with both Di-4-ANEQ(F)PTEA and Rhod-4. The relay lens splitter was used first with the cyan/green spectral design (see Methods: Spectral

Design). The resulting recordings had no discernable voltage or calcium signals were visible. Next, the simplified splitter was used with the green/amber spectral design (see Methods: Spectral Design); however, a 675nm longpass emission filter was used instead of the 700nm longpass filter mentioned in the spectral design. Figure 9 shows signals resulting from the imaging.

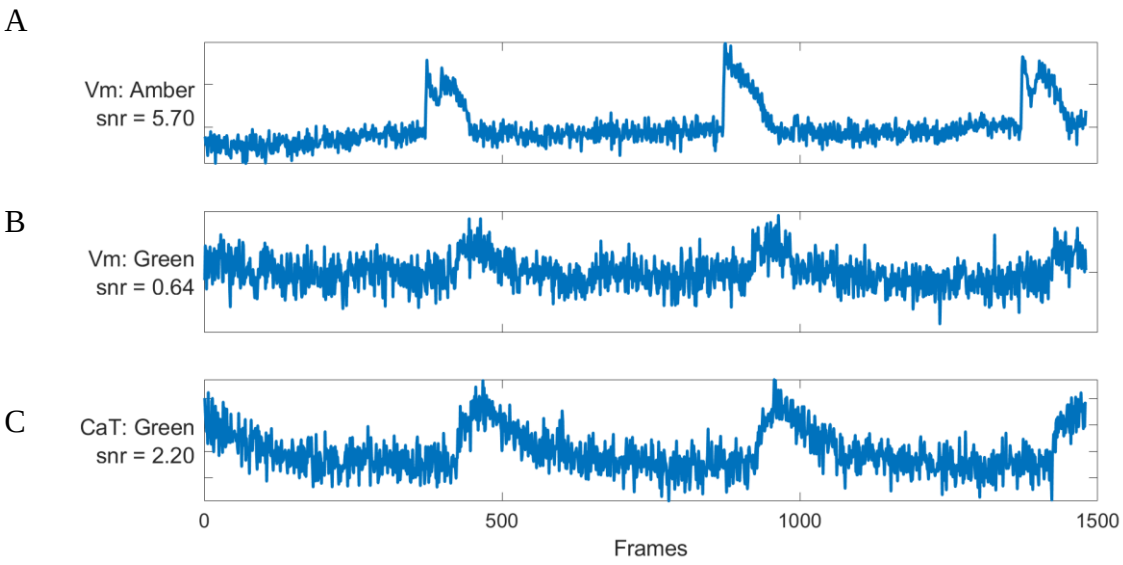



Figure 9. Dual-Imaged Slices. Dual stained slice signals imaged with the simplified splitter and green/cyan excitation. (A) Vm signal with amber excitation. (B) Vm isosbestic signal with green excitation. (C) CaT signal with green excitation.

SNRs for Figure 9A-C are 5.70, 0.64, and 2.20. Two other slices were dual stained and imaged producing similar results. The use of a 675nm longpass filter instead of the 700nm longpass filter likely caused a shift away from the isosbestic point of the Vm dye leading to the slight signals seen in Figure 9B.

*Color Mixing Device*

The color mixer with 4” hood was used to record Vm signals from cardiac slices with induced motion. The ratio of odd (blue) and even (cyan) frames were taken and data was

baseline and noise corrected as mentioned in the Methods section. Resulting signals are shown in Figures 10.

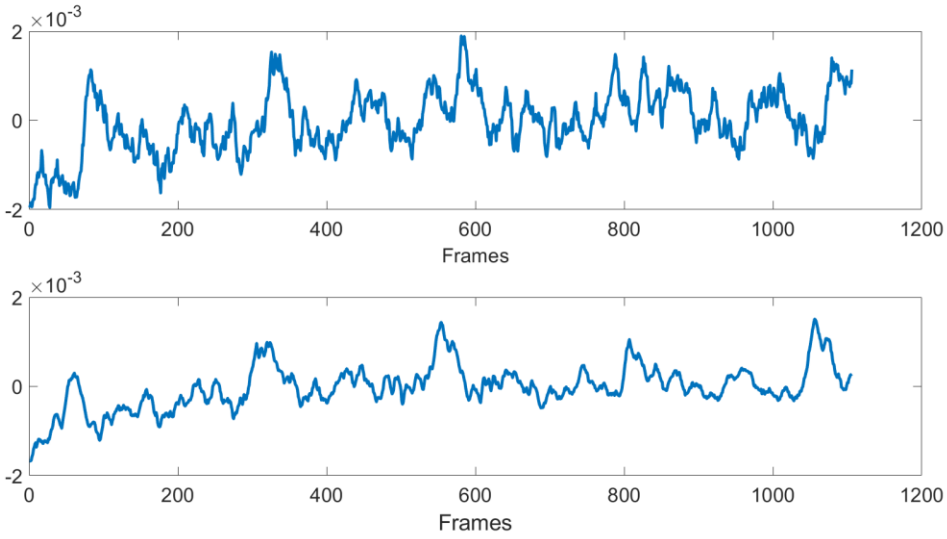



Figure 10. Induced Motion Vm with 4” Hood Color Mixer. Two ratiometric Vm signals from recorded from an induced motion cardiac slice illuminated by the color mixer with 4” hood.

The SNR of the top signal in Figure 10 is 0.42 and the SNR of the bottom signal in Figure 10 is 2.16. Discernable ratiometric signals were only visible on a small portion of the slice.

The color mixer with the 1” hood was also used to record Vm signals from cardiac slices with induced motion. The ratio of odd (blue) and even (cyan) frames was taken and data was baseline and noise corrected as mentioned in the Methods section. Resulting signals are shown in Figures 11.

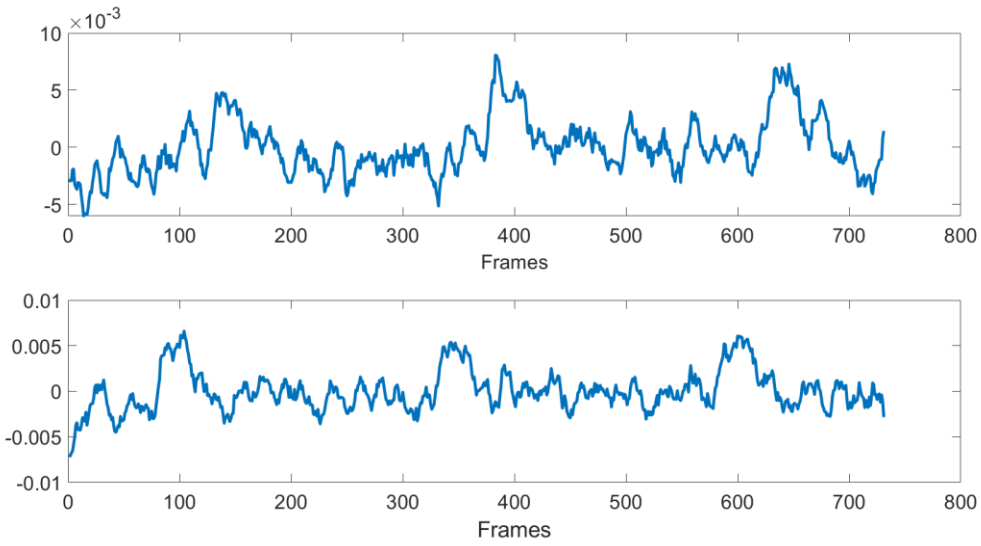



Figure 11. Induced Motion Vm with 1" Hood Color Mixer. Two ratiometric Vm signals from recorded from an induced motion cardiac slice illuminated by the color mixer with 1" hood.

The SNR of the top signal in Figure 11 was 0.33 and the SNR of the bottom signal in Figure 11 was 0.30. Discernable ratiometric signals were visible over almost the entire slice.

## Langendorff-Perfused Pig Hearts

In the first heart, recordings taken with heart motion and recordings taken at fast frame rates (750 Hz) produced poor results. Signals from the voltage dye were small and thus were swamped by motion or too small to see when fast frame rates let in less light. After BDM was added to the perfusion solution and heart motion was stopped, signals remained poor but became discernable using a spatial filter (Figure 12).

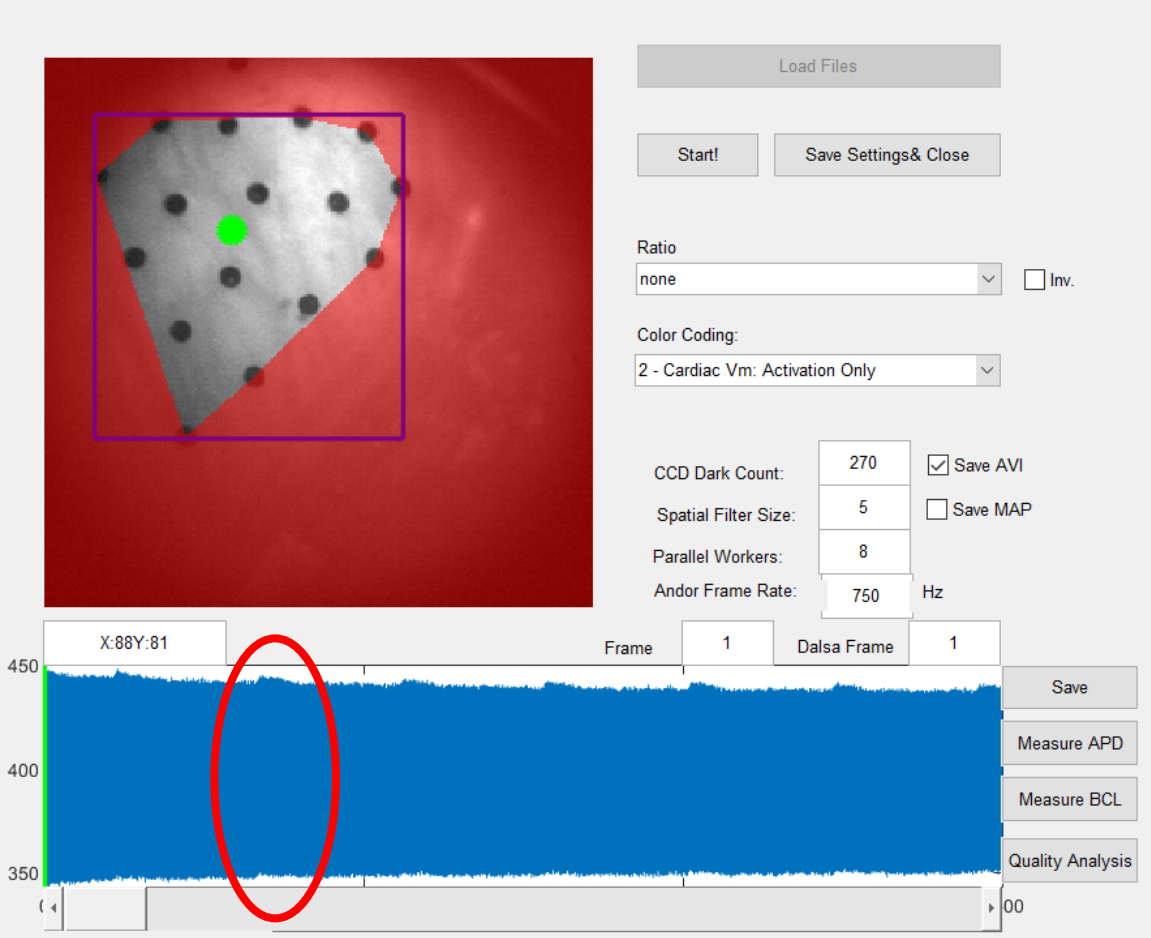
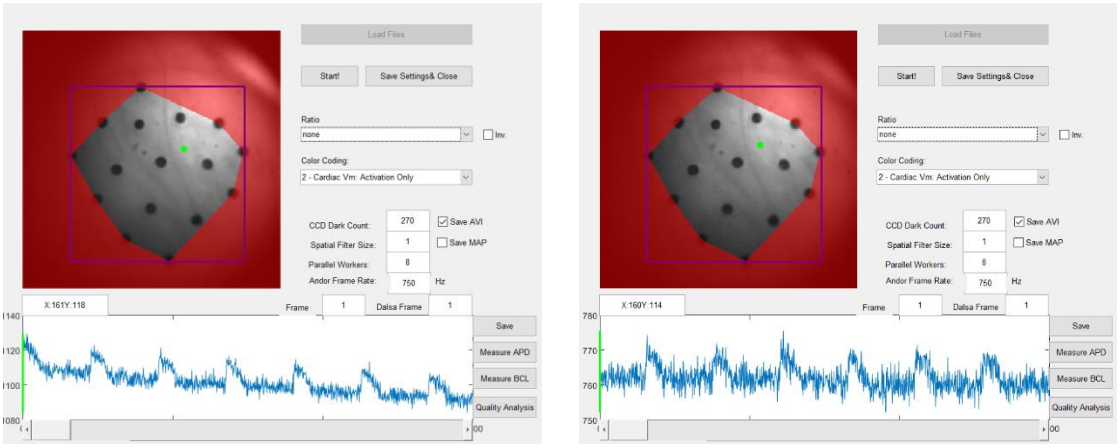



Figure 12. *Ex Vivo* Cyan/Green Vm. This recording, taken at 300 Hz and after BDM was added to the perfusion solution, shows small positive action potentials using both cyan and green excitation light. A spatial filter with a 5 pixels radius was applied to the above recording.

Green excitation was intended to be located at the isosbestic point of Di-4-ANEQ(F)PTEA. Because the green excitation also produced positive action potentials instead of no voltage sensitivity, ratiometry was largely ineffective as dividing the positive signal from cyan by the positive signal from green cancelled out the action potential signal.

When a 675nm emission filter was used, both cyan and green produced larger, cleaner signals, but this filter choice shifted the green excitation further away from the isosbestic point of the dye (Figure 13).




A B

Figure 13. *Ex Vivo* Single Excitation Vm. These recordings were taken at 300 Hz, after BDM was added to the perfusion solution, and with a 675 emission filter instead of the 700nm emission filter. (A) Recorded using only cyan excitation light show positive action potentials with a spatial filter with a 1 pixel radius. (B) Recorded using only green excitation light show positive action potentials with a spatial filter with a 1 pixel radius.

The use of the shorter emission filter showed that poor staining was not the primary culprit behind the poor signal production. Although the shorter emission filter produced better results, it's problematic because calcium signal was detected using a 675nm longpass emission filter after generous spatial filtering was applied in some of our earlier cardiac slice experiments. So, to avoid dye crosstalk, using a 675nm longpass emission filter is unlikely a good long-term solution. The spectral design or the splitter (or both) need to be optimized.

In the second heart, in which excitation was green and amber, recordings taken while the heart was beating produced poor results. The 675nm longpass filter was again used instead of the 700nm longpass filter mentioned in the spectral design. Either the

excitation light used was not color mixing well enough or the signals recorded by the relay lens splitter were simply too small for the motion correction techniques to overcome. Signals acquired shortly after BDM was added to the perfusate produced Vm signals (see Figure 14A). Vm signals were also visible with induced motion after the addition of BDM (Figure 14B).

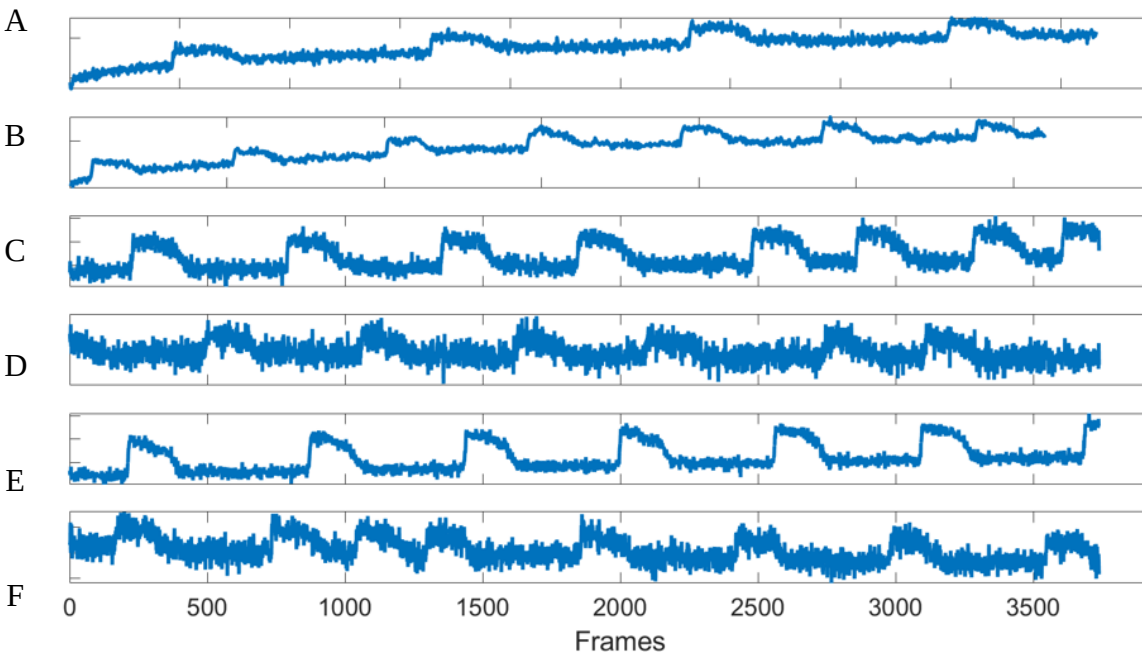



Figure 14. Second *Ex Vivo* Heart. (A) Signal taken with the relay lens splitter at 750Hz after BDM was added to the solution. (B) . Signal taken with the relay lens splitter at 500Hz after BDM was added to the solution with induced motion. (C) Signal recorded with the relay lens splitter with amber excitation at 750Hz. (D) Signal recorded with the relay lens splitter with green excitation at 750Hz. (E) Signal recorded with the simplified splitter with amber excitation at 750Hz. (F) Signal recorded with the simplified splitter with green excitation at 750Hz

Single color recordings were then taken with the relay lens splitter and the simplified splitter. These are shown in Figures 14C – 14F. The SNRs for Figures 14C – 14F are 5.50, 0.81, 12.13, and 1.58 respectively. This shows that the signals produced by the simplified splitter are about 2-2.5 times cleaner than those produced by the relay lens splitter. SNRs for green excitation are much smaller than SNRs for amber excitation as

green excitation is supposed to provide the isosbestic point of our voltage dye. If the longer wavelength emission filter was used (700nm longpass instead of 675nm longpass), Vm signals from green excitation would be smaller as the 700nm longpass emission filter in combination with green excitation is closer to the isosbestic point of Di-4-ANEQ(F)PTEA than the 675nm longpass emission filter.

# DISCUSSION

Several tasks towards simultaneous imaging of Vm and CaT in beating hearts were accomplished in this work. Two designs of optical splitters were constructed, optimized, and tested: the relay lens splitter and the simplified splitter. Two spectral designs were ideated and show promise: (1) Di-4-ANEQ(F)PTEA (Vm dye)/Rhod-4 (CaT dye); green (525nm) and amber (590nm) excitation light; 540-560nm and 700nm longpass emission bands. (2) Di-4-ANBDQBS (Vm dye)/Rhod-2 (CaT dye); green and amber excitation; 550-580nm and 700nm longpass emission bands. A prototype of a new color mixing device that independently filters LEDs was developed that could be useful for the above spectral designs. Work that remains to be done is discussed in limitations.

## Optical Splitter Design

Two optical splitters were used throughout this project. The relay lens beam splitter was designed to overcome shortcomings of previous dual-mapping instrumentation. The simplified splitter was created to overcome a shortcoming of the relay lens splitter. The relay lens splitter has effective spatial resolution and depth-of-field, it collimates light before it hits the mirror, and it can perform ratiometry. The three lenses per optical path lose large amounts of light resulting is small, noisy signals. The simplified splitter has effective spatial resolution and depth-of-field, it can perform ratiometry, and it retains more light than the relay lens splitter. This splitter does not collimate light before it enters the mirror. This could create issues with dye crosstalk as dichroic mirrors are designed to work with collimated light rays. Crosstalk analysis with

this splitter needs to be performed to see if the lack of collimation compromises the mirror's effectiveness.

## Spectral Design

One reason we experienced small and noisy voltage signals in our first *ex vivo* experiment could be that our spectral design needs to be further optimized. Below, the spectral design used in our first *ex vivo* experiment is recapped (Table 2) and several options are presented that may create greater signal fidelity.

Table 2. Spectral design used in first *ex vivo* experiment

| Vm dye: Di-4-ANEQ(F)PTEA<br>Ca dye: Rhod-4 | | Emission filters | |
|---|---|---|---|
| | | 540-560nm | 700nm LP |
| Excitation Filter:<br>530nm SP | Cyan (~505nm) | Ca signal + motion | Vm signal + motion |
| | Green (~525nm) | Unused | Isosbestic (motion-only reference) |

With our initial excitation light design, we could only use one excitation filter for both colors of LEDs. When using a shortpass excitation filter, both our excitation sources had to be a lower wavelength than the emission band of Rhod-4 lest we saturate the calcium emission band. One way to use LEDs that are farther apart in wavelength from one another is to use a multi-bandpass excitation filter instead of a shortpass excitation filter (Table 3). Using LEDs that are farther apart in wavelength may produce better results during ratiometry as the slopes of the absorption spectrum at those wavelengths should vary more from one another, which would cause larger differences in voltage sensitivity.

Table 3. Spectral design Option 1

| Vm dye: Di-4-ANEQ(F)PTEA<br>Ca dye: Rhod-4 | | Emission filters | |
|---|---|---|---|
| | | 540-560nm | 700nm LP |
| Excitation Filter:<br>Multi-bandpass | Green (~525nm) | Ca signal + motion | Isosbestic (motion-only reference) |
| | Amber (~590nm) | Unused | Vm signal + motion |

A longer wavelength voltage dye should also produce better signals. In Table 4, Di-4-ANBDQBS replaces Di-4-ANEQ(F)PTEA. This dye's emission spectrum is about 20nm longer than Di-4-ANEQ(F)PTEA. The multi-bandpass idea is also retained in this design.

Table 4. Spectral design Option 2

| Vm dye: Di-4-ANBDQBS<br>Ca dye: Rhod-4 | | Emission filters | |
|---|---|---|---|
| | | 540-560nm | 700nm LP |
| Excitation Filter:<br>Multi-bandpass | Green (~525nm) | Ca signal + motion | Isosbestic (motion-only reference) |
| | Amber (~590nm) | Unused | Vm signal + motion |

With a longer wavelength voltage dye (as in the spectral design of Table 4), a longer wavelength calcium dye can also be used. Rhod-2 is used in Table 5 because of its faster loading time than Rhod-4 making it more optimal for loading *ex vivo*. Again, the multi-bandpass idea is retained in this spectral design.

Table 5. Spectral design Option 3

| Vm dye: Di-4-ANBDQBS<br>Ca dye: Rhod-2 | | Emission filters | |
|---|---|---|---|
| | | 550-580nm | 700nm LP |
| Excitation Filter:<br>Multi-bandpass | Green (~525nm) | Ca signal + motion | Isosbestic (motion-only reference) |
| | Amber (~590nm) | Unused | Vm signal + motion |

With the new excitation light design in mind, a different spectral design was employed in the second *ex vivo* experiment (Table 6). The heart was only stained for voltage and the new light design was not used, but if the heart was dual stained, the new light design that can filter excitation lights independently would be used.

Table 6. Spectral design used in second *ex vivo* experiment

| Vm dye: Di-4-ANEQ(F)PTEA<br>Ca dye: N/A | | Emission filters | |
|---|---|---|---|
| | | N/A | 700nm LP |
| Excitation Filter:<br>625nm SP | Green (~525nm) | N/A | Isosbestic (motion-only reference) |
| | Amber (~590nm) | N/A | Vm signal + motion |

## Color Mixing Device Design

The color mixing devices with PVC hoods were effective at color mixing. Signals recorded with the 1” hood and the 4” hood both produced ratiometric Vm signals while recording with induced motion. Discernable signals were only seen over a small area with the 4” hood while signal was discernable over almost the entire slice when the

1” hood was used. More experiments need to be run to determine the optimal hood length.

## Cardiac Slices

Dual Vm/CaT staining and imaging was accomplished using the simplified splitter and the green/amber spectral design. Imaging was not simultaneous as the appropriate light source was unavailable. To produce the traces in Figure 9, an amber LED was used to excite the slice, the LED was replaced with a green LED and the slice was imagined again. In order to retrieve CaT signals, the half-silvered mirror was employed; it remained in the splitter for every imaging session. This means that half of all signals were lost at the mirror.

## Langendorff-Perfused Pig Hearts

More experiments (specifically dual staining experiments) need to be carried out in *ex vivo* hearts. A dichroic mirror must be purchased once a final spectral design is settled upon. The half-silvered mirror the splitter currently operates with could work to visualize a dual stained heart, but 50% of any signal is lost at the mirror.

In the first *ex vivo* experiment, no beating recordings produced acceptable signals. Vm signals were only acquired at low frame rates after BDM was added to the perfusate. The spectral design was not optimized resulting in signals cancelling each other out during ratiometry.

In the second *ex vivo* experiment, recordings taken while the heart was beating again produced no acceptable signals. Either the relay lens splitter was not recording enough signal for the motion correction techniques to overcome or the excitation light used was not color mixing well enough to perform ratiometry. The effective color mixing distance of the light used in this experiment is currently unknown and needs to be

determined. The simplified splitter needs to be tested while the heart is beating to see if its increased light efficiency would allow for effective motion-reducing ratiometry.

With an optimized optical splitter, the new color mixer, and myriad of spectral designs, optical mapping of voltage and calcium in beating hearts should be realized soon.

## LIMITATIONS

Multiple factors limited the production of this project. The first limitation was lack of pig availability for experiments during the latter stages of the project. The lack of pigs and our decreased experimental timeline forced us to abandon dual voltage and calcium staining experiments in *ex vivo* hearts. Thus, we were unable to validate that 1) the splitter could be used to image both voltage and calcium signals in *ex vivo* hearts and that 2) the reference signal obtained from one dye (voltage) could be used as a reference for both dyes (voltage and calcium). Recordings should be taken of a dual stained heart while it is beating and employ the method of using the isosbestic voltage signal as a reference for both voltage and calcium. After a few recordings are taken, BDM (a commonly used electromechanical uncoupler)[7, 13, 27] should be added to the circulating Tyrode's solution. Once motion is eliminated in the heart, more recordings should be taken (recordings after BDM addition no longer require ratiometry). Signal morphology before and after BDM addition could be compared to see the effects of the ratiometry method.

The relay lens splitter utilizes relay lenses to create factors, such as a broader depth-of-field and collimation of light before hitting the mirror, necessary to imaging a beating *ex vivo* pig heart. It records much smaller signals than the traditional single lens/camera used in optical mapping due to the two three-lens optical paths. The simplified splitter has no collimating element in front of the mirror and thus may cause inefficiencies in a dichroic mirror.

BrainVision MiCAM03 N256 cameras have quantum efficiencies around 50-55% at 550-600nm. This is likely the highest quantum efficiency of the camera in the visible spectrum. When recording voltage signals with a 700nm longpass filter, it's likely that less than 50% of the photons hitting the camera sensor are converted into electrons (retrievable signal). For a lens system that already loses a lot of light between lenses, like the relay lens splitter, a 50% decrease in signal at the image sensor could be detrimental to recording small signals such as transmembrane potential and calcium transients.

Ideally, the splitter and spectral design used for *ex vivo* hearts could be easily translated to an *in vivo* experimental design. Currently, optical mapping has been accomplished *in vivo*, but only using a single voltage dye.[23, 25, 28] Attempting to visualize calcium's signal in a blood-perfused heart is problematic. Blood and its components strongly absorb blue/green light.[19, 29] These are the same wavelengths that are needed to excite commonly used calcium dyes. Current experiments that involve calcium optical mapping still operate without blood to avoid spectral complications.[1, 11, 30] The calcium dyes used throughout this work would suffer from the same pitfalls as the dyes used in the studies cited above. Thus, simultaneous imaging of Vm and CaT *in vivo* will still be a challenging endeavor.

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# APPENDIX A

# IACUC APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

**MEMORANDUM**

**DATE:** 16-Sep-2021

**TO:** Rogers, Jack M

**FROM:** Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

**SUBJECT:** **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 16-Sep-2021.

| | |
|---|---|
| **Protocol PI:** | Rogers, Jack M |
| **Title:** | Optical Mapping of Cardiac Electromechanics in the In Vivo Setting |
| **Sponsor:** | National Heart, Lung, and Blood Institute/NIH/DHHS |
| **Animal Project Number (APN):** | IACUC-21498 |

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 15-Sep-2024.

**Institutional Animal Care and Use Committee (IACUC)**

403 Community Health on 19th | 933 19th Street South

Mailing Address:
CH19 403 | 1720 2nd Ave South | Birmingham AL 35294-2041

phone: 205.934.7692 | fax: 205.934.1188
www.uab.edu/iacuc | iacuc@uab.edu

APPENDIX B

IACUC PERSONNEL FORM

**Record Number**
IACUC-21498

Done Save

**Optical Mapping of Cardiac Electromechanics in the In Vivo Setting**

**Jack M Rogers - Biomedical Engineering (National Heart, Lung, and Blood Institute/NIH/DHHS)**

Lab Animals
View Mode
Change Project Info

Submissions (8) Linkages (2) Species (1) Summaries ?

Home Summaries Personnel

Summary
**Personnel**

## Research Personnel

Add

All Certifications and Training

| PI | Name | COI | Start Date | | End Date |
|---|---|---|---|---|---|
| ◉ | Jack Rogers - Biomedical Engineering<br>Role: PI | | 21-Sep-2018 | Retire | Remove |
| | Certifications and Training | | | Email | |
| ○ | Sharon Melnick - Med - Cardiovascular Disease<br>Role: | | 21-Sep-2018 | Retire | Remove |
| | Certifications and Training | | | Email | |
| ○ | Haley Patton - Biomedical Engineering<br>Role: | | 10-Oct-2019 | Retire | Remove |
| | Certifications and Training | | | Email | |
| ○ | Shannon Salter - Med - Cardiovascular Disease<br>Role: | | 21-Sep-2018 | Retire | Remove |
| | Certifications and Training | | | Email | |
| ○ | Gregory Walcott - Med - Cardiovascular Disease<br>Role: | | 21-Sep-2018 | Retire | Remove |
| | Certifications and Training | | | Email | |
| ○ | Garrett Wood - Biomedical Engineering<br>Role: | | 04-Sep-2020 | Retire | Remove |
| | Certifications and Training | | | Email | |





| PI | Name | COI | Start Date | | End Date |
|---|---|---|---|---|---|
| ○ | Hanyu Zhang - Biomedical Engineering<br>Role: | | 21-Sep-2018 | Retire | Remove |
| | Certifications and Training | | | Email | |

## Past Research Personnel

**Contacts**

Office/Lab Contacts
**Name**

*No Records Found*