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Optical Mapping of Optically Paced Embryonic Hearts*

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Abstract— **Conduction maps of early embryonic hearts with optical mapping point to heterogeneous conduction patterns that quickly evolve over time. In adult hearts, electrical pacing is utilized to determine the anisotropy of the conduction patterns and the susceptibility of the tissue to arrhythmias. However, studying electrophysiology in developing hearts is limited due to their size. Electrical pacing creates an electrical artifact that obscures recordings from the entirety of early embryonic hearts. In this study, optical point stimulation using a 1440-nm near-infrared diode laser with a 12-µm diameter beam waist was used to pace embryonic quail hearts. Electrical activity was recorded across the surface of the embryonic hearts by high resolution optical mapping using di-4-ANEPPS and cytochalasin D. While there were no electrical artifacts produced by the optical point stimulation, an optical artifact due to thermal lensing did obscure the optical mapping near the point of stimulation. The optical artifact can be minimized by optimizing the stimulation parameters to minimize the energy deposited and can be further reduced by signal processing. Despite the presence of the optical artifact, the electrical activity over the majority of the heart can be obtained.**

I. Introduction

Cardiac electrical activity plays an important role in the proper functioning of the heart. In the fully-developed adult heart, cardiac electrophysiology is a commonly used tool in both research and in the clinic to map electrical conduction across the heart; identify abnormalities in the initiation, propagation, and morphology of action potentials (APs); and treat and induce arrhythmias. In embryos, cardiac electrical activity plays a role in the development of the heart from the initial tubular stage to the mature 4 chambered heart. Electrical propagation has been shown to play a direct role in early development[1-2] and abnormal conduction has been linked with congenital heart defects.[2]

In adult hearts, optical mapping (OM) and electrical pacing are well-established tools used to perform cardiac electrophysiology research. OM is a technique where the heart is stained with a voltage-sensitive dye (VSD) and then contractions are stopped by pharmacological means without stopping electrical activity.[3] The fluorescent signal from the VSD changes with membrane voltage, allowing for surface electrical activity to be recorded with high spatial and temporal resolution over a large field of view. Electrical pacing can be accomplished by placing an electrode on the surface of the heart. It is used to pace *in vitro* cardiac preparations at physiological rates, induce arrhythmias, and with OM, study conduction velocity and AP morphology.

In embryos, OM can be performed in a similar way as with adult hearts.[4-5] The smaller tissue size requires a higher magnification resulting in smaller pixel sizes and lower signal-to-noise ratios. In contrast, electrical pacing has significant issues when applied to small embryonic hearts. Electrodes are difficult to position to achieve consistent pacing without physical damage in such small tissues. The higher current densities required to achieve pacing with small electrodes can also cause tissue damage. Additionally, electrical pacing creates an electrical stimulus artifact that prevents electrical recording near the pacing electrode.[6]

Optical stimulation using near infrared lasers has been shown to be able to initiate propagating APs in a variety of neurons.[7-9] The optical stimuli deposit heat around the pacing site, resulting in a change in membrane capacitance,[10] though the exact mechanism is still under debate. Recently, our group has demonstrated the use of optical pacing in embryonic quail hearts.[11] Enabling pacing of embryonic hearts will allow additional studies into cardiac electrophysiology during development and allow the study of arrhythmias in embryonic models

Here, we describe the use of optical pacing in several embryonic models allowing cardiac pacing at arbitrary locations on the hearts without damage and the use of optical stimuli during OM, allowing the study of activation maps and AP morphology under paced conditions.

II. Methods

A. Animal Models

All animal husbandry and experiments were conducted in compliance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* (NIH publication 85-23, revised 1996) with the approval of the Institutional Animal Care and Use Committee at Case Western Reserve University.

Quail embryos (*Coturnix coturnix*; Boyd's Bird Company, Pullman, WA) were allowed to develop for 48-52 h at 38ºC in a humidified, force draft incubator (G.Q.F Manufacturing, Savannah, GA). Then, the eggshell was removed and the heart dissected out and placed in 1 mL of warm Tyrode solution.

Timed development of the mouse embryos (mixed backgrounds: used for unrelated experiments) were determined at E 0.5 days if a vaginal plug was found after overnight mating. Embryos were harvested by a caesarean-driven method at E 9.5 days. The hearts were excised and then placed in 1 mL of warm Tyrode solution.

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Fertilized zebrafish eggs were collected after group mating. The embryos were raised until 72 h after fertilization, and then the hearts were dissected out and placed in 1 mL of warm Tyrode solution.

In all models, the hearts were maintained at a temperature of 37 ± 1 ºC using a custom-built heated chamber and a heated stage (Warner Instruments, Hamden, CT) during staining, pharmacological intervention, and imaging.

B. *Pacing*

A 200 mW 1440 nm diode laser (QPhotonics, Ann Arbor, MI) was coupled with single-mode fiber into a scanner consisting of a collimating lens (ThorLabs, Newton, NJ), an x-y scan head (Cambridge Technologies, Bedford, MA), and a focusing lens (Thorlabs). The scanner focuses the laser to a 12-µm beam waist and allows for the arbitrary positioning of the laser in the x-y plane. An arbitrary waveform generator (Fluke, Everett, WA) was used to provide square-wave pulse trains of the desired frequency, intensity, and pulse widths to a laser diode controller (ILX Lightwave, Bozeman, MT). Laser intensity was controlled by 0.1 V steps from the arbitrary waveform generator, which has been measured to correspond to 4.6 mW steps of power out of the scanner, to a maximum of 177 mW. The scanner was placed below the stage, allowing optical pacing from below the sample while simultaneously recording optical mapping from above.

For experiments quantifying the optical artifact, the laser spot was placed on the ventricle of the heart tube. For all other experiments, the laser spot was positioned on the atrium. For spot size experiments, pulse width was maintained at 20 ms. For pulse width experiments, spot size was set to 12 µm.

C. *Imaging*

The imaging apparatus that was built around an Axio Scope.A1 with a 10X, 0.45 NA objective (Carl Zeiss Microscopy, Thornwood, NY). A SOLA Light Engine (Lumencor, Beaverton, OR) was used to provide illumination and an iXon3 860 EMCCD camera (Andor Technology, South Windsor, CT) with a zoom lens (Zeiss) was used to capture 128x128 images at 500 Hz via Andor SOLIS software.

Optical mapping was performed by staining excised hearts with 10 µM di-4-ANEPPS (Life Technologies, Carlsbad, CA) for 8 min and then placing the stained hearts into 1 mL of Tyrode solution with 20 µM Cytochalasin D (CtyoD; Sigma-Aldrich). A custom di-4-ANEPPS filter cube (excitation: 510/80 nm, dichroic mirror: 560 nm, emission: 685/80 nm; Chroma Technology, Bellows Falls, VT) was used.

Analysis of optical mapping data was performed with custom MATLAB (MathWorks, Natick, MA) software. Signal processing of the optical mapping traces was done with a combination of filtering and curve fitting to remove the signal noise. Activation maps were created by determining the temporal midpoint of the upstroke. AP duration (APD90) were obtained by measuring the time between the upstroke midpoint and when the signal recovered by 90% from the maximal depolarization back to the baseline.

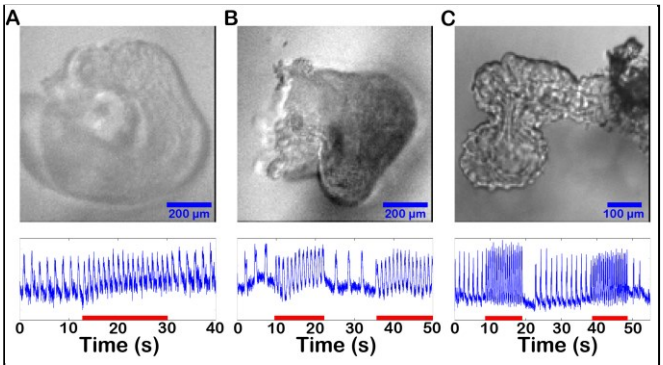

Figure 1. Optical pacing in multiple embryonic models. Examples of optical pacing in (A) 2 day quail, (B) 9.5 day mouse, and (C) 2 day zebrafish embryos. Blue traces show contraction induced intensity changes at a randomly selected pixel on the hearts. Red bars indicate optical pacing.

III. Results

A. *Demonstration of Pacing*

Optical pacing was attempted on 3 embryonic heart models: quail, mouse, and zebrafish. In all cases, 1:1 pacing was achieved above the unpaced rate as shown in Fig. 1.

B. *Initial Optical Mapping*

Optical mapping was performed on quail embryo hearts under unpaced conditions and while optically pacing on the atrium (Fig. 2). Activation maps (Fig. 2 A and C) show the conduction pattern and velocity. APD90 maps (Fig. 2 B and D) show that optical pacing reduced APD90 in the ventricles and the outflow tract.

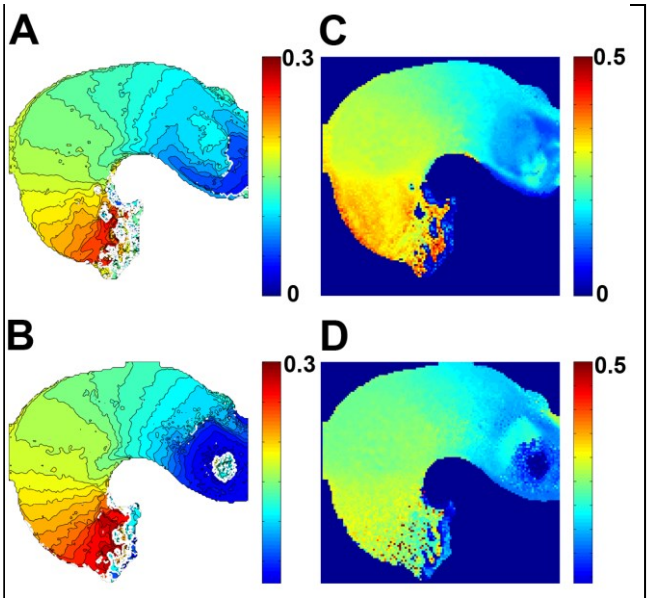

Figure 2. **Optical mapping of optically paced hearts.** (**A,C**) Activation maps and (**B,D**) APD90 maps while (**A,B**) unpaced and (**C,D**) under optical pacing of the atrium.

The optical mapping data in the atrium of the optically paced hearts was obscured by an optical artifact. This artifact caused our image processing software to return either no or bad values for activation time and APD90.

C. *Optical Artifact*

The artifact was a reduction is fluorescence around the pacing site. Sub-threshold optical pacing of the ventricles showed that the artifact had a Gaussian spatial distribution with the peak fluorescence change dependent on the power of the stimulation pulses. Temporally, the artifact showed an approximately exponential decay after each laser pulse.

To reduce the effect of the optical artifact, the optical pacing threshold was determined as a function of spot size and pulse width. Spot size was increased by moving the heart away from the scanner (n = 7). No significant change in the total power delivered by the pacing laser was observed from 12 µm to 240 µm spot sizes, but there was a significant effect when increasing the spot size to 360 or 480 µm (Fig. 3 A). Radiant exposure was calculated by dividing the energy by area of the spot size. There were significant decreases in the radiant exposure for each step up to a spot size of 360 µm and no significant change between 360 and 480 µm (Fig. 3 B). At 600 µm spot size, pacing could only be achieved in 3 hearts (43%) at or below the maximum output of the pacing laser.

Varying the pulse width (n = 3) showed that there were significant increases in the power required to pace the hearts below 20 ms (Fig 3 C). Pulse widths of 40 ms or greater showed a statistically significant reduction in energy required compared to 20 ms, but the difference was small. Radiant exposure showed that increasing pulse width generally resulted in increased radiant exposures.

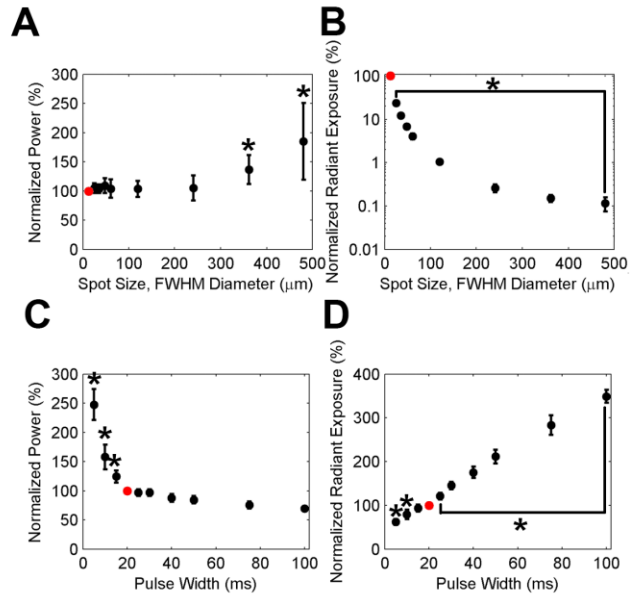

Figure 3. **Stimulation parameter optimization.** The effect of spot size (FWHM of the gaussian beam profile) on (**A**) power and (**B**) radiant exposure at 20 ms pulse widths. The effect of pulse width on (**C**) power and (**D**) radiant exposure at a 12 µm spot size. All values are normalized to that of 12 µm spots with 20 ms pulse widths (100%, red circles). Error bars represent ± 1 standard deviation from the mean. * $p < 0.05$ compared to 100%.

IV. Conclusion

Optical pacing has many advantages over traditional electrical pacing, including higher spatial precision, contact-free pacing, and no electrical artifact. These advantages make it ideally suited for use in electrophysiological studies of small embryonic hearts.

Previously, our group demonstrated optical pacing of embryonic quail hearts using a multimode fiber laser.[11] In this study, we demonstrate the use of a single-mode fiber laser that allows much smaller spot sizes and more precise positioning. We demonstrate that optical pacing is a viable technique for not only quail, but also in mouse and in zebrafish embryos, both of which are commonly used models for studying the effects of genetic modification.

While optical pacing does not generate an electrical artifact, it does cause an optical artifact that attenuates the optical mapping signal. The temporal distribution of the artifact is similar to the temperature changes observed by other groups studying infrared neural stimulation,[7] indicating that the artifact is caused by thermal lensing. This hypothesis is further supported by the visibility of the artifact in the bright field when optical pacing is applied to a sample of water. However, the optical artifact is much smaller than electrical pacing artifacts that can obscure recording for millimeters from the pacing site.[6] The optical artifact would not be expected to interfere with electrode recordings.

Even with the presence of the optical artifact, optical mapping can be achieved for almost the entire heart tube. The change of conduction velocity between unpaced and paced hearts is similar as that observed in adult heart models, where increased heart rates affect sodium channels to result in slower conduction velocities.[12]

The optical mapping data at the edges of the artifact likely can be recovered by improving our signal processing algorithm to subtract away the relatively small artifact. Furthermore, the optical artifact can be minimized by optimizing the pacing parameters to reduce the radiant exposure to the sample being studied. This study indicates that increasing spot size from 12 to 200 µm does not affect the energy threshold for 1:1 capture. This suggests that the increased number of cells illuminated balances the decreased radiant exposure of individual cells. Our results suggest that a spot size of 200-300 µm and a pulse width of 20 ms would be ideal for quail embryos, though these values are likely to vary with animal model and also with the developmental stage. Furthermore, though increasing spot size may reduce the radiant exposure, it also reduces the spatial precision of the pacing. Additional optimizations are being studied, including the effects of pacing wavelength, position, and frequency.

Optical pacing opens up new avenues of study related to electrophysiology of embryonic hearts. It allows the use of pacing to obtain conduction velocity, conduction anisotropy, APD90, and AP morphology in both normal and

pathological hearts, as is commonly done in adult hearts. Additionally, it allows pacing of the heart during development, which can be used to alter heart development.

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