Volumetric optical mapping in early embryonic hearts using light-sheet microscopy

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Abstract: Optical mapping (OM) of electrical activity using voltage-sensitive fluorescent dyes is a powerful tool for the investigation of embryonic cardiac electrophysiology. However, because conventional OM integrates the signal in depth and projects it to a two-dimensional plane, information acquired is incomplete and dependent upon the orientation of the sample. This complicates interpretation of data, especially when comparing one heart to another. To overcome this limitation, we present volumetric OM using light-sheet microscopy, which enables high-speed capture of optically sectioned slices. Voltage-sensitive fluorescence images from multiple planes across entire early embryonic quail hearts were acquired, and complete, orientation-independent, four-dimensional maps of transmembrane potential are demonstrated. Volumetric OM data were collected while using optical pacing to control the heart rate, paving the way for physiological measurements and precise manipulation of the heartbeat in the future.

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Abnormal conduction during heart development can lead to congenital heart defects (CHDs) and abnormalities, including defects of the adult cardiac conduction system, abnormal heart rhythms, and abnormal heart chamber sizes. These defects can be caused by a variety of factors, including genetic mutations, environmental exposures, and congenital malformations.

1. Introduction

During heart development, cardiac electrical signals are important for coordinating the heartbeat as well as providing cues for the normal progression of cardiac development [1, 2]. Abnormal conduction during heart development can lead to congenital heart defects (CHDs) and abnormalities, including defects of the adult cardiac conduction system, abnormal heart rhythms, and abnormal heart chamber sizes. These defects can be caused by a variety of factors, including genetic mutations, environmental exposures, and congenital malformations.
morphology (e.g., ventricular septal defects, and conotruncal defects), and abnormal blood flow [1, 3, 4]. Furthermore, electrical activity and heart morphology influence each other as early as cardiac looping stages [5]. Although much is known regarding the general process of conduction system development, the timing and etiology of conduction-related CHDs and the interplay among electrical activity, cardiac structure and dynamics are still poorly understood. Technology that can better characterize and accurately quantify early normal and abnormal electrical activity is needed to improve our understanding of this topic.

Optical mapping (OM) is the leading technology used for investigating embryonic and adult cardiac electrophysiology [6–8]. OM uses a fast camera to image hearts that are stained with voltage-sensitive fluorescent dye [8, 9]. Motion of the hearts is usually fully suppressed using excitation-contraction (EC) uncouplers. OM acquires high-resolution spatial and temporal signals from an entire field of view simultaneously without contacting the tissue, a significant advantage over electrode recordings. With acquired action potential traces, parameters such as activation time, action potential duration and conduction velocity can be computed. With these features, OM has enabled many contributions to the understanding of both embryonic and adult cardiac electrophysiology [7–9]. Genetically encoded voltage indicators (GEVIs) are alternatives to voltage-sensitive dyes to target specific cell types and reduce phototoxicity [10]. However, they are still under development and their applications are limited [10].

Conventional OM is limited due to the fact that signals are integrated in depth and projected to a two-dimensional (2D) plane. As a result, image data are strongly affected by the orientation of the sample and the position of the focus. Signals from tissue out of focus are blurred and typically only a portion of the heart surface provides usable signal. Information from the back side of the heart surface is not accessible. Several efforts have been made to address or partially address this problem [11–14]. In adult hearts or late-stage embryonic hearts, panoramic OM uses several cameras or splits the field of view of the camera to acquire several images over the surface of the heart [11, 12]. However, it is very difficult to apply these methods to small and topologically complex looping-stage embryonic hearts. For looping-stage embryonic quail hearts, we previously integrated OM and optical coherence tomography to correct conduction velocity calculations based on 3D heart geometry [13]. This improves the accuracy of conduction velocity calculations, but does not restore missing information from the back of the heart. In zebrafish hearts with a GEVI, four-dimensional (4D) OM was acquired with a commercial spinning disk confocal microscope [14]. Complete 4D electrophysiology information was acquired in this study. However, this method may not be applicable to embryonic avian and murine hearts, which are often studied because of their similarity to human hearts. These hearts are much larger and the field of view of confocal microscopy is insufficient without multiple data acquisitions and tiling. Furthermore, the availability of GEVIs in these models is largely limited (not available at all in avian models). These hearts can be stained with exogenous voltage-sensitive dyes (e.g., Di-4-ANEPPS), but voltage-sensitive dyes are usually phototoxic [15, 16] and confocal microscopy exposes tissue to very high light irradiance [17]. Phototoxicity and photobleaching will limit the duration of 4D OM imaging sessions using confocal microscopy.

Light-sheet microscopy (LSM) uses a cylindrical lens or a scanning beam to illuminate a sheet of light at a specific plane and collect images of that plane from an orthogonal axis [18–20]. By scanning the light sheet through a volume, one can obtain 3D or 4D images. LSM features large fields of view, high speed, low cost, low photobleaching and low phototoxicity. Photobleaching and phototoxicity are lower due to the efficiency of illuminating only the slice of the sample being imaged, resulting in significantly lower light exposure. LSM has been widely used for imaging a variety of samples [19–22]. The advantages of LSM make it well suited for imaging 4D membrane potentials in embryonic hearts.

Cardiac conduction velocity and action potential duration are highly dependent upon heart rate so it is important to control heart rate when comparing multiple samples [23, 24]. Clinical
cardiac electrophysiology procedures use electrodes for point stimulation to regulate heart rate. Unfortunately, electrical artifacts from point stimulation electrodes obscure signals in small-sized samples. Optical pacing overcomes the limitations of electrical pacing allowing point stimulation in early-stage embryos [25–27].

Here we demonstrate volumetric OM using LSM with optical pacing. This technology enables acquisition of complete, orientation-independent, 4D transmembrane potential maps from looping stage avian embryonic hearts.

2. Materials and methods

2.1 Sample preparation

Fertilized quail eggs (*Coturnix coturnix communis*, Boyd’s Bird Company, Inc., Pullman, WA) were incubated in a humidified, forced draft incubator (G.Q.F. Manufacturing Co., Savannah, GA) at 38°C for 50 hours. Quail embryos were dissected from the yolk. The stage of each embryo was determined by the number of somites observed using a stereomicroscope and the heart was then excised [28].

Fresh voltage-sensitive dye solution (FluoVolt solution) was prepared before each experiment by mixing FluoVolt (Life Technologies, Carlsbad, CA) and PowerLoad concentrate (Life Technologies) into Tyrode’s solution (Sigma-Aldrich, St. Louis, MO) with manufacture-suggested concentrations. The excised embryonic heart was stained in 300 µL of FluoVolt solution for 20 minutes in the dark at room temperature. The embryonic heart was then moved to a cuvette (cat# 14-955-127; Fisher Scientific, Pittsburgh, PA) filled with 1 mL of Tyrode’s solution containing 10 µM of the excitation-contraction uncoupler, cytochalasin D (Sigma-Aldrich), to eliminate myocardial contraction. The cuvette was mounted onto the sample stage of the imaging system. Data from two Hamburger–Hamilton (HH) stage 14 quail embryonic looping hearts are shown as examples in this report.

2.2 Optical system

The imaging system is a custom-built light-sheet microscope, in which the illumination path and the detection path are separated and orthogonal to each other. In the illumination path, a 70 mW, 505 nm diode laser (Vortran Laser Technology Inc, Sacramento, CA) was used as the excitation light source. As shown in Fig. 1, the illumination path of the light-sheet imaging system is composed of a cleanup filter (Chroma Technology, Bellows Falls, VT), a set of relay lenses (LA1255-A, Thorlabs, Inc, Newton, NJ) with a 50 µm pinhole (P50S, Thorlabs, Inc) in between as a spatial beam filter, a galvonometer mirror (6210H, Cambridge technology, Bedford, MA) which scans the light sheet along the detection axis, a beam expander (LA1951-A and LA1708-A, Thorlabs, Inc,) and a cylindrical lens (LJ1695RM-A, Thorlabs, Inc,) to form the light sheet. The galvonometer mirror is placed at a conjugate plane to the back aperture of the cylindrical lens. The light sheet created by this illumination setup was measured to be ~3 µm wide at the focus, which determines the sectioning resolution.

The detection path of the light-sheet imaging system consists of an objective lens (Fluar 5x/0.25, ZEISS, Oberkochen, Germany), an emission filter (535 nm ± 20 nm, Chroma Technology), a tube lens, a set of relay lenses (LA1509-A, Thorlabs, Inc) with an electrically tunable lens (ETL, EL-10-30-C-VIS-LD, Optotune Switzerland AG, Dietikon, Switzerland) in between and an EMCCD camera (iXon Ultra 897, Andor Technology, Belfast, UK). The EMCCD camera acquires images in xy-plane. In this system, the sample and the objective do not need to move in order to acquire multiple z-planes, instead, the galvo-mirror scans the z-position of the illumination plane, and the ETL scans the focus of the camera in correspondence with the position of the illuminated light sheet. The system is similar to a previously published design [29]. The alignment and testing of the ETL were performed as suggested in the same study [29]. The xy-plane resolution is ~7 µm, limited by the pixel size.
The field of view is \( \sim 0.9 \text{ mm} \times 0.9 \text{ mm} \). For OM experiments, the camera acquires images of \( 128 \times 128 \) pixels at 500 frames per second.

The optical pacing setup includes a 1465 nm laser diode coupled into a single-mode fiber (QPhotonics, Ann Arbor, MI) and a laser diode controller (Newport Corporation, Irvine, CA) [26]. During imaging, hearts were paced at 1 Hz with 20 ms pulses (3.75 mJ/pulse). The pacing fiber was in contact with the inflow portion of the heart tube. The core of the pacing fiber (SMF 28) was 9 \( \mu \text{m} \) with an NA of 0.14.

Galvanometer scanning, ETL focus scanning and optical pacing signals were controlled using a custom Labview program (National Instruments, Austin, TX).

2.3 4D data acquisition and image processing

During an imaging session, an embryonic heart was placed in a cuvette and positioned in the imaging field of view. Under camera guidance, the optical pacing fiber tip was placed in contact with the inflow portion of the heart tube. Once in position, the emission filter was returned to the detection path. While optically pacing the heart, the camera continuously acquired images at 500 frames per second and the light sheet and camera focus was shifted 25-35 \( \mu \text{m} \) every two seconds. For each embryonic heart, 15 – 20 \( z \)-planes were imaged.

2D time-sequence images were reconstructed into a 4D data set based on known time increments between \( z \)-planes and the paced heart rate. The 4D data set was cropped in time to keep one full action potential across the entire heart. Our previously developed action potential fitting software package (Matlab, Mathworks, Natick, MA) [30] was used to analyze the 4D data set. Activation time of each action potential was determined using the fitting software and the results were mapped onto a segmented 3D surface mesh of the heart structure. 3D Gaussian spatial filtering \( (5 \times 5 \times 5, \text{ standard deviation} = 1) \) and interpolation in the \( z \)-plane direction were also carried out in Matlab for data visualization. 2D, 3D and 4D visualizations of transmembrane potential were created from the filtered and interpolated results using Amira (FEI Company, Hillsboro, OR). For visualization of multiplanar
transmembrane potential data sets, the myocardium was segmented by applying a threshold to the gray scale structural images. The relative fluorescence intensity signal was overlayed on the structural images using the myocardium mask.

3. Results

A fluorescence image of a single z-plane and three fluorescence traces from selected pixels in this z-plane are shown in Fig. 2. The myocardium of the heart shows strong staining, with the bottom part stronger than the top part because the bottom shows the side illuminated by the light sheet (indicated in Fig. 2(A)). In this example slice, the bottom side is near the atrial region of the heart and the top side is in the outflow tract. A delay can be clearly observed in Figs. 2(B) and 2(C) with point b activating before point c. The endocardium of the heart was stained, but as expected there was no voltage signal (Fig. 2(D)).

![Fig. 2](image)

Fig. 2. Panel A is a fluorescence image of an embryonic heart with voltage-sensitive dye staining. Yellow diamonds b, c and d are pixels whose traces are plotted in panels B, C and D respectively. Intensity values were normalized. myo: myocardium; endo: endocardium.

With 4D data sets, slices may be visualized in any orientation. Transmembrane potentials displayed in three orthogonal planes from a 4D data set are shown in Fig. 3 and Visualization 1. The imaging plane (xy-plane), shows activation from the bottom of the image to the top, representing conduction from inflow towards outflow at four time points through the course of the heartbeat (Fig. 3(B), 3(F), 3(J), 3(N)). The xz-plane (Fig. 3(C), 3(G), 3(K), 3(O)) is orthogonal to the conduction direction therefore the activation around the heart tube was almost simultaneous. The yz-plane provides a view of the inner and outer curvature of ventricular region of the heart tube. As observed in Fig. 3(D), 3(H), 3(L), and 3(P), conduction along the outer curvature is much faster than the inner curvature which is necessary for coordinated contraction towards the outflow tract. 4D transmembrane potentials of another sample are also shown in Visualization 2 as a time series of the conduction volume rendering overlaid on the structural volume rendering.

An example of a 3D activation map is shown in Fig. 4 and Visualization 3, where activation time is indicated by the color scale. The region in the atrium affected by pacing artifact was removed from analysis because data from this region are not meaningful. The earliest activation times occurred at the site of pacing and propagated toward the outflow tract. The total conduction time from the pacing site in the atrium to the outflow tract was approximately 200 ms.
Fig. 3. Multiplanar reformatting of 4D transmembrane potential data sets. The colored activation wave is overlaid on the grayscale heart tube. A, E, I, M are 3D transmembrane potential volumes at 0.28s, 0.38s, 0.48s and 0.58s respectively and the locations of the three orthogonal planes are indicated. B, F, J and N are xy-planes at corresponding time points. C, G, K and O are xz-planes at corresponding time points. D, H, L and P are yz-planes at corresponding time points. Colormap represents relative fluorescence intensity (normalized). The colormap is shown at the right bottom corner of panel M. AVJ: atrioventricular junction; OFT: outflow tract.

Fig. 4. 3D activation map of an HH stage 14 embryonic heart. AVJ: atrioventricular junction; V: ventricle; OFT: outflow tract. The region missing colormap in the atrium was the region affected by pacing artifact and it was removed from analysis.
4. Discussion

We have demonstrated 4D optical conduction mapping in avian embryonic hearts using voltage-sensitive dyes, light-sheet microscopy, and optical pacing. This provides complete, orientation-independent electrophysiology information from a majority of the heart tube. Acquiring complete information from one sample reduces the number of samples needed to investigate multiple regions of interest. This technique enables more direct and accurate comparisons between individual animals reducing the number of animals needed to achieve statistical significance. This LSM-based 4D OM method may also be adapted to study the hearts of other animal models.

For embryonic cardiac conduction, previous knowledge and data acquired with conventional OM may serve as validation of our newly developed 4D OM. The new system was able to acquire clearly interpretable single action potential traces (Fig. 2). At early looping stages, avian embryonic hearts present a unidirectional conduction pattern, with slower conduction at the atrioventricular junction and the outflow region, and faster conduction at the ventricle region [2, 13, 26, 31–33]. Furthermore, within the ventricle the outer curvature conducts faster than the inner curvature [2, 13, 26, 31–33]. Our results are consistent with these previously observed features, as shown in Fig. 3, Fig. 4, Visualization 1, Visualization 2, and Visualization 3. The total conduction time of the sample shown in Fig. 4 is approximately 200 ms, which is also very consistent with our previous reports of similar stage embryos imaged with conventional OM [13, 26].

In designing the 4D imaging system, SNR and imaging speed were especially critical. OM of embryonic hearts suffers from low SNR due to the small region interrogated by each camera pixel and small signal change with conventional dyes such as Di-4-ANEPPS [30]. Furthermore, optical sectioning reduces the number of fluorophores imaged by each camera pixel thus reducing the fluorescence signal. The newly available voltage-sensitive dye, FluoVolt, provides a more than 5 fold increase in SNR when compared to Di-4-ANEPPS [8]. Here, our light-sheet imaging system provided sufficient SNR (Fig. 2) to analyze the data using our previously developed fitting algorithm [30]. Both the new dye and the new analysis algorithm were necessary to enable imaging of action potentials with light-sheet imaging. 4D OM imaging not only requires a high-speed camera to sample the fast action potentials, but the total time of each imaging session should be minimized to maintain the health of the heart and avoid phototoxicity [16, 34]. The light-sheet microscopy system described here meets these criteria by using a high-speed camera, scanning the light sheet instead of the sample, and pacing the heart so that only 2 heartbeats are needed at each z-plane.

Optical pacing is a reliable point stimulation technique. It is important to control the heart rate when comparing multiple embryonic hearts because electrophysiological parameters are rate dependent. Unfortunately, pacing produces an artifact at the pacing site. The artifact is caused by thermal lensing, as described previously [26]. While this does not affect the visualization and analysis of the rest of the heart tube, we believe that the size of the artifact region may be further reduced by optimizing the delivery of light to decrease the pacing threshold. Also, the hearts were not maintained at body temperature which increased the pacing threshold and enlarged the artifact. In this demonstration, we also used optical pacing to synchronize z- slices into a volume, however, this is not the only available synchronization method. If an experiment requires that a heart be imaged while intrinsically beating, retrospective gating can also achieve synchronization [35].

Embryonic hearts were imaged at room temperature in this demonstration. Development of a temperature-controlled imaging chamber will enable experiments under more physiological conditions, and may extend imaging sessions by maintaining the health of the beating heart and reduce pacing artifacts.

In summary, we have proposed an LSM-based 4D OM imaging method and demonstrated its utility by imaging early embryonic quail hearts. LSM-based 4D OM is capable of providing full characterization and phenotyping of early embryonic cardiac...
electrophysiology. It will be a useful tool for comparing normal and abnormal cardiac conduction in developmental models. Early cardiac electrophysiology has been shown to be influenced by early cardiac structure [5]. 4D transmembrane potential mapping may potentially enable more accurate experiments to address key questions about the interplay among cardiac structure, hemodynamics, mechanical function, gene expression and conduction.

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