Abstract: Cardiac arrhythmias are often triggered by ectopic membrane depolarization originating deep inside the myocardial wall. Here we propose a new method utilizing a novel near-infrared voltage-sensitive fluorescent dye DI-4-ANBDQBS to determine the three-dimensional (3D) coordinates of the sources of such depolarization. We tested the method in live preparations of pig left and right ventricular myocardium (thickness 8-18 mm) and phantoms imitating the optical properties of myocardial tissue. The method utilizes an alternating transillumination approach that involves comparing pairs of simultaneously recorded broad-field epifluorescence and transillumination images produced at two alternating directions of illumination. Recordings were taken simultaneously by two CCD cameras facing the endocardial and epicardial surfaces of the heart at a frame rate up to 3KHz. In live preparations, we were able to localize the origin of the depolarization wave with a precision of ± 1.3 mm in the transmural direction and 3 mm in the image plane. The accuracy of detection was independent of the depth of the source inside ventricular wall.

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1. Introduction

Cardiac contraction is triggered by an electrical wave of membrane depolarization rapidly propagating through the entire heart and exciting the ventricles. Normally, such waves originate in the pacemaker region known as the sino-atrial (SA) node. The SA node is located in the right atrium and its firing rate is under the tight control of the sympathetic and parasympathetic nerves [1]. In pathological conditions, however, such waves originate outside the pacemaker area. The sources of such waves, termed “ectopic foci,” often trigger severe and sometimes fatal arrhythmias [2,3].

Here, we describe an optical method that enables the detection of such foci deep inside the ventricular wall and the computation of their spatial coordinates. The information about electrical excitation is provided by voltage-sensitive fluorescent dyes (VSD). These are small molecules that bind to cell membranes and change their fluorescence proportionally to the changes of the transmembrane voltage [4,5]. During the last two decades, VSDs have been used extensively to image electrical waves in the heart. However such imaging, also known as optical mapping has been largely limited to the myocardial surface and subsurface myocardial layers [6–8]. This limitation stems from the fact that the light in the blue-green part of the spectrum used for excitation of conventional VSDs has a sub-millimeter attenuation length [9,10].

To overcome this limitation, we utilize a recently developed near-infrared VSD, which can be excited at 670 nm [11,12]. At this wavelength, the attenuation length of the myocardial tissue is more than 3 times larger than at wavelengths characteristic for conventional VSDs [13]. The development of effective near-infrared VSD, as well as the availability of powerful continuous wave (CW) red lasers in the range 660–680 nm, enabled us to increase the interrogation depth from about 3 mm to the entire thickness of the ventricular wall, reaching 16–20 mm in large animal hearts.
To detect ectopic foci inside the ventricular wall and to determine their spatial coordinates, we utilize an alternating transillumination approach [14,15]. This approach is based on comparing pairs of broad-field epifluorescence and transillumination images recorded at high frame rates for two alternating directions of illumination. Despite conceptual simplicity, the implementation of this method is challenging, mainly for the following reasons:

- The dynamic nature of cardiac excitation requires millisecond time resolution.
- Voltage-sensitive fluorescence representing changes in transmembrane potential constitutes only a small fraction (approximately 10%) of the overall fluorescence.
- Accurate coordinate detection requires capture of the early stages of the excitation when the voltage-dependent signal is less than 0.1% from the overall fluorescence.

All these factors impose significant constraints on the bandwidth, dynamic range, and fidelity of the optical detectors and data acquisition system.

Here we present the first successful implementation of the alternating transillumination method and demonstrate its feasibility for localization of sources of intramural excitation through the entire thickness of ventricular myocardium in coronary-perfused pig ventricular preparations. To validate the method, we emulated ectopic foci using a specially designed multi-polar stimulating plunge needle electrode [16]. Using this electrode, we could initiate electrical excitation at desired depths. Subsequently, we compare the coordinates of the stimulation site with the optically-derived coordinates of the excitation origin.

2. Material and methods

2.1 Cardiac Tissue Preparation

Experiments were performed in coronary perfused ventricular preparations. Ten pigs of either sex were anesthetized with sodium Pentothal (35 mg/Kg IV). The animals were intubated and heparin (500 i.U. IV) was injected and the chest was opened. The heart was arrested using a high potassium cardioplegic solution [17] and quickly excised. The right ventricle was perfused through a plastic cannula inserted into the right coronary artery. To provide adequate perfusion of the entire left ventricular wall, we cannulated both the left anterior descending and the left circumflex arteries. Perfusion was performed with oxygenated Tyrode's solution at a constant pressure of 80 mmHg at 37 ± 1°C. The preparations were stretched on a plastic frame and placed in a glass perfusion chamber to expose their endocardial (N) and epicardial (P) surfaces (see Fig. 1a and 1b) to the excitation light and cameras. To optically image electrical activity, the ventricles were stained with the near-infrared voltage sensitive dye DI-4-ANBDQBS by bolus injection (200 µl in 20 ml Tyrode) [12]. Motion artifacts were abolished by adding 2,3-BDM to the perfusate (12 - 16 mmol/L final concentration).

2.2 Electrical stimulation

To initiate electrical waves at different depths, we used a custom-built resin plunge needle electrode with eight leads spaced 5 mm apart [16]. The electrode was inserted diagonally from endocardium (N) to epicardium (P) (Fig. 1c). After 30 - 40 minutes to allow injury currents to subside, the pacing stimuli were delivered through one of the leads. The remaining leads were used to record unipolar extracellular potentials and to monitor the sequence of intramyocardial activation with reference to a far-field earth lead (Fig. 4). For stimulation, we used 2 ms pulses at 1.5x capture threshold applied at base cycle lengths of 408 to 508 ms. Extracellular potentials were amplified by a multichannel amplifier (Model 3500, A-M Systems) and recorded using a PCI-6229 acquisition board (National Instruments).
Fig. 1. (a) Schematic of the experimental setup. The coronary perfused pig ventricular wall preparation is placed in a glass chamber between two CCD cameras facing the epicardium (P) and endocardium (N). The fluorescence is excited by two 671 nm CW lasers, P and N, illuminating the respective surfaces in rapid succession by alternating the light using a mechanical chopper synchronized with the digital recordings. Boxes 1, 2, and 3 show the holographic diffuser, dichroic mirror, and long pass fluorescence filter, respectively. (b) Views of the right ventricular wall preparation from P and N. The gray squares show the fields of view of respective CCD cameras. The white arrow indicates the perfusion cannula. The black arrow shows the multi-lead plunge needle electrode used for initiation and electrical recordings of intramural electrical waves. The projection of the impaled portion of the electrode is shown by the dashed line. (c) Single pixel recordings from P (top) and N (bottom) cameras. The black points indicate the discarded transitional frames and the red points the retained frames. Double letter notations indicate the position of the light and the camera, respectively. (d) Transmural cross section of the ventricular wall with the inserted plunge needle electrode. Green circles indicate the position of each of the eight unipolar leads.

### 2.3 Optical setup

To excite the fluorescence of the dye, we used two 671 nm, 1000 mW CW lasers (Shanghai Dream Lasers) labeled P and N. The beam of laser P was directed towards the epicardial surface (P) whereas the endocardial (N) surface was illuminated by laser N (see Fig. 1a). The direction of illumination was alternated using a mechanical chopper (ThorLabs) at rates up to 750 Hz. To achieve a broad field illumination, the laser beam was expanded using a 20° holographic diffuser (1) and directed onto the surface by a 685 nm long pass dichroic mirror.
To record voltage-sensitive fluorescence, we used two 14 bit fast CCD cameras (SciMeasure Inc) with 80x80 pixels resolution. The cameras were also labeled P and N, camera P facing epicardial and camera N endocardial surfaces. Figure 1b shows the epicardial and endocardial views of the experimental preparation and fields of view of the P (top) and N (bottom) cameras. The fluorescence was collected through a 715 nm long pass filter (3) placed in front of each CCD camera. The recordings were taken simultaneously from an area of 40 × 40 mm at a frame rate of 2000 fps or 3000 fps and they were synchronized with the chopper. The spatial resolution was either 0.5 mm or 1 mm in binning mode. The cameras were aligned to achieve one-to-one pixel correspondence.

Figure 1c shows single pixel recordings from cameras P and N. The recordings have a characteristic saw tooth pattern caused by switching the direction of illumination. The larger values labeled PP and NN are recorded in epifluorescence mode when the light and the camera are facing the same surface. The smaller values (PN and NP) correspond to the transillumination mode, when the light and the camera are on the opposite sides of the preparation. Such two-letter notation is used throughout the text. The first letter indicates the position of the light whereas the second letter indicates the position of the camera. The recordings from each camera were de-multiplexed to obtain four quasi-simultaneous movies labeled PP, PN, NP, and NN, which were subsequently used for the localization of the source of intramyocardial activation.

Figure 2 shows sequential snapshots of an expanding excitation wave in a 10 mm thick right ventricle preparation extracted from the PP, PN, NP and NN movies. The wave was initiated by applying electrical stimulation through the lead located 3 mm below the P surface.
The yellow square in each of the images on the left shows the projection of the stimulation site onto the image plane. Note the pronounced elliptically shaped excited area, particularly at early stages of excitation (15-20 ms). This is the result of anisotropy of propagation velocity in the cardiac muscle, which is higher along the myocardial fibers. The orientation of the main axis of the ellipse varies between different views (compare PP and PN vs NP and NP) reflecting the change in orientation of myocardial fibers across the thickness of myocardial wall emphasizing the different layers of the myocardium represented in each view.

2.4 Extraction of voltage sensitive fluorescence

To eliminate non-uniformities of staining and illumination, voltage sensitive signals were normalized to background fluorescence. However, for our coordinate detection algorithm it was essential to extract the voltage dependent signal \( I_{i,j} \) while preserving its absolute amplitude (see below). To achieve this goal, after point-by-point normalization and background subtraction, we recovered the amplitude by multiplying the normalized signal by a constant representing the average background fluorescence over the entire frame:

\[
I_{i,j} = \left( 1 - \frac{\Delta F_{i,j}}{F_{i,j}} \right) \hat{F}
\]

where:
- \( \Delta F_{i,j} \) is voltage sensitive fluorescence for pixel \( i,j \)
- \( F_{i,j} \) is background fluorescence for pixel \( i,j \)
- \( \hat{F} \) is average background fluorescence over the entire frame

2.5 Coordinate detection

To determine the coordinates of the origin of the excitation wave, we employed the algorithm developed by Khait et al. for rectangular slab geometry [15]. The depth of the source was calculated using one of the following equations:

\[
\begin{align*}
J_{PP} &= \frac{\sinh (L - Z + d) / \delta}{\sinh (Z + d) / \delta} \\
J_{PN} &= \frac{\sinh (L - Z + d) / \delta}{\sinh (L + d) / \delta} \quad (2) \\
J_{NN} &= \frac{\sinh (Z + d) / \delta}{\sinh (L + d) / \delta} \\
J_{NP} &= \frac{\sinh (L + d) / \delta}{\sinh (L - Z + d) / \delta} \quad (3)
\end{align*}
\]

Here, \( Z \) is the distance of the source from the P surface; \( J_{PP}, J_{PN}, J_{NN}, \) and \( J_{NP} \), are the spatial integral intensities of the PP, PN, NN, and NP images, respectively. L is the thickness of the slab, \( \delta \) is the attenuation length for the emitted light, and \( d \) is the extrapolation distance that defines the boundary conditions at the tissue bath interface. For the left ventricle, the thickness was measured at the location of the stimulation site. Because of the large thickness variation for right ventricle, we used an average thickness of 9 mm. The attenuation length was \( \delta = 3.3 \) mm [13] and \( d \) was set at 2.5 mm [15]. According to theory, the X and Y coordinates of our source can be determined by identifying the pixel with maximal intensity in any of the four images.

For the algorithm to work properly, the images used for detection should be taken at the very early stages of excitation when the size of the excited area is small. In the ideal case, either of the two equations is sufficient to detect the Z coordinate. However, in reality, when the source of excitation is close to the surface only one of the epifluorescence views can be reliably recorded and thus only one of the two equations can be used. Therefore, when the source is closer to P the \( J_{NN} \) and \( J_{NP} \) are very small (well below noise level) and as a result cannot be used for detection. This makes Eq. (3) unusable. Similarly, when the source is
closer to N, the $J_{PP}$ and $J_{PN}$ are very small and Eq. (2) cannot be used. The details of the algorithm are described in more detail in the results section.

2.6 Optical Phantoms

Prior to conducting live tissue experiments, we tested the accuracy of our method using optical phantoms imitating the optical properties of cardiac tissue. The primary goal of phantom experiments was to test the main concept in “ideal conditions” when fluorescent sources are static and when the noise of the camera and the lasers can be largely suppressed by unconstrained time averaging. The phantoms were produced by mixing commercially available intralipid (Fresenius Kabi) as a scatterer and India Ink as an absorber using the protocol described previously [18]. The concentrations of intralipid and India Ink were adjusted in such a way that at 660 nm the phantom would have a reduced scattering coefficient $\mu_s' = 8.33 \, \text{cm}^{-1}$, an absorption coefficient $\mu_a = 0.56 \, \text{cm}^{-1}$, and $\delta_{ex} = 2.5 \, \text{mm}$, which is consistent with the values reported for cardiac tissue at this wavelength [13,19]. To achieve the desired scattering coefficient, the 20% intralipid (IL) stock solution ($\mu_s = 245.7 \, \text{cm}^{-1}$) was further diluted to the final concentration of 0.678%. The absorption coefficient was adjusted by diluting the India ink ($\mu_a = 588.4 \, \text{cm}^{-1}$) to the final concentration of 0.095%. To imitate the background fluorescence of the voltage-sensitive dye, we used Indocyanine Green (ICG) at a concentration of 6.5 µM that gives an emission peak around 750 nm [20]. Agarose powder (10 mg/ml) was used to solidify the phantom.

3. Results

3.1 Phantom Experiments

Equations (2) and (3) used for the coordinate detection algorithm are based on the diffusion approximation of the photon transport equations [21–23]. To determine how well this approximation works in our specific application, we tested its accuracy in phantom experiments replicating optical properties of cardiac tissue at 671 nm (the excitation wavelength of DI-4-ANBDQBS). Inside the phantom we placed a capillary filled with fluorescent dye at various depths ranging from 1 to 9 mm from the P surface. By measuring $J_{PP}$, $J_{PN}$, $J_{NN}$, $J_{NP}$ and solving Eqs. (2) and (3) we determined the depth of the capillary and compare it with its actual location.

![Fig. 3. Validation of the source detection technique in phantom experiments. (a) Reflection (PP, NN) and transillumination (PN, NP) images of a fluorescent capillary placed 3 mm below the P surface. The white bar on the bottom right of the NN image corresponds to 10 mm. (b) Horizontal intensity profiles for the four images in Panel a. Scan lines are shown as dashed lines in Panel a. The profiles in PP and NP images are narrower and have larger amplitudes than those in NN and PN, which reflects the proximity of the capillary to the P surface. (c) The actual depth of the capillary vs the reconstructed depth in two different experiments (white and black circles). The red line is the identity line.](image-url)
The experimental results for different phantoms and Z values are illustrated in Fig. 3. The experimental points with few exceptions lie very close to the identity line (shown in red). Accuracy of detection was high for all Z with the error never exceeding 1.5 mm. This shows that Eqs. (2) and (3) derived using photon diffusion theory give sufficiently accurate results and are appropriate for our application.

3.2 Cardiac tissue experiments

The next step was to test our method in live tissue preparations. As mentioned above, to initiate electrical waves inside ventricular wall in a controllable way, we used a multi-lead plunge needle electrode. Figure 4 shows experimental examples of electrode use. Figure 4a shows the case when the stimulus was delivered through lead 1 located near the N surface (see schematic on the top). The electrical recordings from leads 2 to 6 show monotonic increase of the activation time from 20.2 ms (lead 2) to 54.6 ms (lead 6) consistent with the onset of excitation near lead 1. Figure 4b shows the recordings from the same preparation obtained when the stimulus was delivered through lead 4. Now, the earliest activation is registered by leads 3 and 5, which are closest to lead 4, while activation of leads 6, 2, and 1 occurs with larger delays. This is consistent with the expectation that the excitation indeed originated near lead 4. Plots of distance from the stimulation site vs activation time for left and right ventricle are shown in Fig. 4c and 4d respectively. The Y axis intercept is greater than zero for both left and right ventricle which indicates a slower conduction velocity near the stimulation site. The conduction velocity along the electrode estimated from these plots is 0.4 m/s for the right ventricle and 0.5 m/s for left ventricle.

![Fig. 4. Validation of the wave source detection method in live tissue. Initiation and recording of intramural electrical waves at different depths using a multi-lead plunge needle electrode. Numbers near the extracellular signals indicate the lead from which the unipolar recording was taken. (a) Stimulation applied through lead 1, located near the N surface. A gradual increase of the activation time from lead 2 to 6 can be seen, consistent with excitation of the endocardial surface. (b) Stimulation through lead 4, located 6 mm under the P surface. The earliest activation is detected near leads 3 and 5. The activation time increases as the distance from the recording and stimulation site is increased, consistent with intramural expansion of the excitation front. (c) and (d) show activation time vs distance from the stimulation site for left ventricle (n = 3) and right ventricle (n = 6), respectively.](image-url)
3.3 Periodic excitation

Having the ability to induce intra-myocardial excitation in a controlled way, we proceeded to test how accurately the origin of excitation can be determined using our optical method. As mentioned in the methods section, the algorithm for coordinate detection should give more accurate results when the excitation wave is small. In practice, however, small wave sizes imply low signals and a smaller signal-to-noise ratio (S/N) which can reduce the accuracy of detection.

The task becomes much simpler when the source generates periodic waves. In this case, the signal-to-noise ratio (S/N) can be significantly increased by using ensemble averaging. Another advantage of a periodic regime is that it does not require switching the light every other frame which increases our temporal resolution. In our experiments, we first ensemble-averaged our data over 20-30 pacing cycles with one direction of illumination to obtain PP and PN movies. The light was then switched and the resulting recordings averaged over another 20-30 pacing cycles to obtain NP and NN movies. This protocol increased the effective acquisition rate to 3000 fps and allowed further improvement of S/N ratio by applying time filtering. We used a 3 point conical kernel with a radius of 1.5 ms, which was sufficiently narrow to avoid any detrimental effect on the temporal resolution of our recordings.

![Fig. 5.](image)

(a) Changes in integral intensities of the PP, PN, NP and NN images produced by expanding intramural excitation wave (ensemble average). The stimulus is applied from the lead located 10 mm below P surface. The black arrow at the bottom of the plot indicates the moment of the stimulation. (b) and (c) show signal dynamics during early stages of front expansion corresponding to the grey box in (a) and (b), respectively. The horizontal lines in (c) indicate the integral intensities threshold at two standard deviations above mean background intensity. The depth of the source is detected at the moment indicated by the vertical line.

To determine the depth of the source, we use the ratio of integral intensities \( J_{PP}/J_{PN} \) and \( J_{NN}/J_{NP} \), of the PP, PN, NN, and NP images (see Eqs. (2) and (3)). Figure 5 shows integral intensities \( J_{PP}, J_{PN}, J_{NN}, \) and \( J_{NP}, \) of the PP, PN, NN, and NP images, respectively, as a function of time in one of the experiments (left ventricular wall preparation, thickness \( L = 17 \) mm, stimulation depth \( Z = 10 \) mm). All four integrals have a characteristic bell shape (See Fig. 5a). The growth phase starting at the left of the plot reflects the expansion of the excitation wave, whereas the decay phase represents the repolarization process.

The early stages of expansion are shown with increasing level of detail in Fig. 5a and 5b. As can be seen from Fig. 5b, in this particular experiment the signal can be first detected in NN and NP movies and these were indeed chosen by our algorithm for the detection of the excitation source coordinates. The next task was to select the optimal frame for calculation of the source coordinates.

The criteria for selecting the frame which provides the most consistent results were as follows. First, we chose the frame when the values of both \( J_{NN} \) and \( J_{NP} \) exceeded two standard deviations (\( \sigma \)) of the average background noise spatial integral (\( J^0 \)). The values of \( J^0_{NN}, 2\sigma_{NN}, \)
$J_{NP}^0$, $2\sigma_{NP}$ were calculated individually for each movie over a 20 frame interval preceding the pacing stimulus onset. The signal was allowed to grow for 2 ms to increase S/N ratio and the resulting frame was used for measurements of the 3D coordinates of the source. Figure 5c illustrates the algorithm for frame selection. $J_{NN}$ reaches the threshold first at $t = 2.3$ ms and $J_{NP}$ follows at $t = 6$ ms. The values of $J_{NN}$ and $J_{NP}$ for the selected frame ($t = 8$ ms) were inserted into Eq. (3) to calculate the depth of the source $Z$. In this particular example, solving (3) yields $Z = 10.1$ mm, which is only 0.1 mm from the $Z$ location of the stimulation site.

The X and Y coordinates of the excitation source were calculated from the same images used for the depth detection. For each image, we calculated the coordinates of the center of mass of pixels with intensities above 50% of the maximum intensity and averaged the results to obtain the coordinates of the origin of the excitation wave. Excluding pixels with low intensity minimizes the effect of readout noise on the coordinate detection. In our experience, using the coordinates of the center of mass rather than those of the pixel with maximal intensity is more robust, particularly in the experiments with low S/N.

$$Z = 10 \text{ mm}, \ L = 17 \text{ mm}$$

![Image](image.png)

Fig. 6. PP, PN, NP and NN snapshots of the excitation wave at the moment of detection. The stimulus is applied from the electrode located 10 mm below P surface. The colors show the normalized voltage sensitive fluorescence. The NN image is the brightest, consistent with the wave being initiated in the mid-myocardium, closer to the N surface. The dashed line is the projection of the plunge needle electrode with the stimulating electrode indicated by the square. AR is the radial displacement between the stimulation site coordinates and the reconstructed source coordinates. The white bar on the bottom right corresponds to 10 mm.

Figure 6 shows NN and NP images of the excitation wave used for coordinate detection in the experiment illustrated in Fig. 5. The other two images (PP and PN) that were recorded simultaneously but failed to satisfy the selection criteria are shown for comparison. The PP image shows no signal, which is consistent with the fact that in this experiment the excitation wave originated far away from the epicardial surface. The red asterisk in the NN image is the center of mass assigned by our algorithm as the excitation center. Indeed, it is located in the center of the depolarized region. The X, Y projection of the stimulation site is shown as a black square. In this particular experiment the stimulation site was within a millimeter from the origin of excitation detected by our algorithm.

### 3.4 Single wave detection

In real clinical situations, an ectopic focus may only fire sporadically while remaining silent for long periods of time. This prevents the use of the ensemble averaging procedure described in the previous section. To record all four movies during sporadic events, we must use the acquisition protocol shown in Fig. 1c. In this case the chopper switches the light every other frame, successfully capturing the single wave event in all four movies. Again, as for the case of periodic sources, we obtain PP, PN, NN, and NP movies, but at a lower frame rate (750 frames per second).
fps) due to de-multiplexing. The movies are filtered in time with the smallest possible 3 point kernel, which is now 6.5 ms due to the lower frame rate. The localization procedure is essentially the same as the one described in the previous section.

Fig. 7. (a) Changes in integral intensities of the PP, PN, NP and NN images produced by a single intramural excitation wave for the same preparation as in Fig. 5. The black arrow at the bottom of the plot indicates the moment of the stimulation. (b) and (c) show signal dynamics during early stages of front expansion corresponding to the grey box in (a) and (b) respectively. The red vertical line in (c) indicates the moment of detection for the periodic wave. The black vertical line indicates the time of detection for a single wave.

Figure 7 shows integral intensities $J_{PP}$, $J_{PN}$, $J_{NN}$, and $J_{NP}$, of the PP, PN, NN, and NP images for a single propagating wave (the same preparation and the stimulation site as in Fig. 5). At low magnification the figures look very similar. However, at higher magnification the differences become more apparent (Fig. 7c). Indeed, the standard deviation of the background noise determined for $J_{NN}$ and $J_{NP}$ signals in the case of single waves was almost an order of magnitude greater than the respective values determined in the case of the periodic waves. Because of the increased noise, it takes longer for $J_{NN}$ and $J_{NP}$ to exceed two standard deviations above the background noise.

In this particular example, the signals have become strong enough to satisfy the criterion for coordinate detection only 17 ms after the application of the stimulus. This is 9 ms later than in the case of periodic waves. The later detection implies a greater wave size at the moment of detection which can cause a greater error in the detection of the source origin. In this particular example, the error increased by 0.4 mm when compared to the localization of periodic source.

3.5 The accuracy localization

To assess the accuracy of our algorithm, we conducted 63 measurements in nine pig hearts. Three of these were left ventricles (thickness $L = 15$-18 mm) and six were right ventricles ($L = 8$-10 mm). The depth of stimulation varied from 0 to 9 mm below the epicardial surface in right ventricle and 1 to 15 mm in left ventricle. The results of these experiments are summarized in Fig. 8 and Table 1. The accuracy of the method was quantified by measuring of the vertical $\Delta Z$ and radial $\Delta R$ displacements between the stimulation site and the detected center of excitation (see Fig. 8a). Figure 8b and 8c show plots of the $Z$ coordinate of the stimulating lead versus detected $Z$ for periodic and single source respectively.
Fig. 8. Accuracy of the reconstruction of the wave origin from the dynamic integral intensities. (a) The 3D image illustrates the reconstructed origin of the excitation wave (red sphere), the actual stimulation site (green) and the position of plunge needle electrode inside a right ventricle preparation. The size of the preparation is 24 × 24 × 10 mm (b) The depth of stimulation site versus the reconstructed depth for periodic sources. (c) The depth of stimulation site versus reconstructed depth for single wave sources. Different colors indicate different experiments. Open and closed circles indicate right and left ventricle experiments respectively.

As can be seen from plots 8b and 8c, in the experiments with periodic sources the detected depths show significantly smaller errors and much less scatter than in case of single wave sources. The average $\Delta Z$ values for periodic sources was 1.3 ± 1.1 mm (see Table 1), which is close to the accuracy we achieved in phantom experiments. The average $\Delta Z$ in the case of single wave sources is almost two times larger and shows twice as much scatter.

|                    | Periodic source | Single source |
|--------------------|-----------------|---------------|
| $\Delta Z$ (mm)    | 1.3 ± 1.1       | 2.1 ± 2.0     |
| $\Delta R$ (mm)    | 3.3 ± 2.0       | 3.3 ± 2.1     |
| Time of detection (ms) | 13.9 ± 5.5   | 25.8 ± 6.7    |

In the case of periodic sources, we were able to detect excitation much earlier than in the case of single wave sources. The average detection time was 13.9 ms after the stimulus for periodic waves and 25.8 ms for single wave sources. For both periodic and single sources, the accuracy in the image plane $\Delta R$ was ~3.3 mm and did not depend on stimulation depth. This
is noticeably less than the accuracy in ΔZ, particularly for periodic sources where ΔZ is almost three times smaller.

4. Discussion

As mentioned in the introduction, in pathological conditions, certain myocardial cells become sources of abnormal excitation (ectopic foci) triggering severe and sometimes lethal arrhythmias [2,3]. Here we present the first non-destructive optical imaging method that enables the detection of ectopic foci deep inside myocardial wall and reliably determines their coordinates. The method utilizes recently developed near-infrared fluorescent voltage-sensitive dye D1-4-ANBDQBS [11,12] and alternating transillumination approach [14,15]. Using the new method, we were able to detect sources of excitation at any depth in 16-20 mm thick pig left ventricular myocardium and in 7-9 mm right ventricular myocardium.

The new method has several major advantages over existing three-dimensional electrophysiological mapping techniques utilizing high-density multi-lead plunge needle electrodes [24–26] and optrodes [27,28]:

• It allows simultaneous high-resolution interrogation of large volumes of myocardial wall, without impaling multiple electrodes or optrodes.

• It facilitates changing the viewing area (no need to move electrodes/optrodes around)

• It simplifies the registration of the electrical activation with the surface features of the heart in the image plane

The new method will likely become a useful tool for arrhythmia research and it can be used for identifying anatomical substrates for ectopic foci. This information can be used in clinical applications where these substrates can be targeted for radio-frequency ablation.

Our phantom experiments reproducing the optical properties of myocardial tissue show that the new method can provide very high accuracy of coordinate detection with error never exceeding 1 mm, independent of the depth of the source. In live tissue preparations the accuracy of depth detection was around 1 mm for the periodic sources and around 2 mm for single wave detection (Table 1). In the case of periodic sources, we used ensemble averaging and were able to achieve high S/N ratios. This resulted in earlier detection times that significantly improved the accuracy of detection as compared to single wave recordings when ensemble averaging was prohibited (see Table 1). The decreased accuracy for single wave detection is determined by the characteristics of the hardware and thus is not fundamental. With improved technology (more powerful and less noisy lasers, faster switches etc.) the single wave detection should be attainable with accuracy at least as good as what has been achieved for periodic waves.

We discovered that despite the fact that the right ventricular wall is almost two times thinner than the left ventricle and yields a 3-4 fold stronger transillumination signal, this does not improve the accuracy of detection. Considering that relative variations in wall thickness are considerably larger in the right ventricle than in the left ventricle, it would be reasonable to assume that thickness variations could be a significant factor limiting the accuracy of detection in the right ventricle.

It is interesting that the error in depth detection ΔZ is consistently smaller than ΔR (see Table 1). However, this difference is not coincidental and stems from the differences in the algorithms used for the reconstruction of respective coordinates. For Z detection we use a ratio of integral intensities (see Eqs. (2) and (3)). This makes Z detection very robust with regard to illumination (laser) noise and spatial heterogeneities of staining and thickness of ventricular wall. In contrast, to determine the coordinates of the wave source in the image plane we have to deal with spatial intensity distributions which are more sensitive to noise and tissue heterogeneity.

For our reconstructions in live tissue preparations we used the value of attenuation length for pig ventricular wall (δ = 3.3 mm) that was measured in a different study [13]. It is reasonable to assume that this value can vary from sample to sample. However, our analysis
shows that variations of δ by as much as ±0.5 mm (±15%) has very little effect on reconstructed depth: changing it by less than ±0.1 mm.

It is worth noting that the accuracy of our algorithm was assessed under the assumption that the stimulation site is the origin of the excitation wave. However, the experimental studies utilizing the same type of electrodes show that actual origin of the wave can be 1 to 2 millimeters away from the stimulating lead location [26]. It is likely that a similar shift was present in our experiments as well. Indeed, this is consistent with the fact that ΔR was not smaller at shallow stimulation depths when the signal can be detected very early and thus should not produce a significant error. The shift of excitation origin with regard to the stimulating lead location suggests that the error in coordinate detection may be smaller than we estimate.

In this study, we present a non-destructive optical method that allows the extraction of 3D information about electrical excitation waves deep inside myocardial wall. Here we use the method to localize the origin of focal excitation. Theoretically, the alternating illumination data can be used not only for localization of the origin of excitation but also for measuring the size of the excited region as the wave expands and for localizing multiple sources sufficiently separated in space [15]. Experimental exploration of such cases will be subject of future studies.

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