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Optical Stimulation and Pacing of the Embryonic Chicken Heart via Thulium Laser Irradiation

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Optical stimulation provides a promising alternative to electrical stimulation to selectively modulate tissue. However, developing noninvasive techniques to directly stimulate excitable tissue without introducing genetic modifications and minimizing cellular stress remains an ongoing challenge. Infrared (IR) light has been used to achieve optical pacing for electrophysiological studies in embryonic quail and mammalian hearts. Here, we demonstrate optical stimulation and pacing of the embryonic chicken heart using a pulsed infrared thulium laser with a wavelength of 1927 nm. By recording stereomicroscope outputs and quantifying heart rates and movements through video processing, we found that heart rate increases instantly following irradiation with a large spot size and high radiant exposure. Targeting the atrium using a smaller spot size and lower radiant exposure achieved pacing, as the heart rate synchronized with the laser to 2 Hz. This study demonstrates the viability of using the 1927 nm thulium laser for cardiac stimulation and optical pacing, expanding the optical parameters and IR lasers that can be used to modulate cardiac dynamics.

Keywords : Optical pacing, Thulium laser, Embryonic chicken heart, Infrared cardiac stimulation
OCIS codes : (000.1430) Biology and medicine; (140.3070) Infrared and far-infrared lasers

I. INTRODUCTION

Optical stimulation of excitable tissue offers significant advantages over electrical stimulation. The use of electric current introduces electrical artifacts, and limits the cellular selectivity and spatial precision of the stimulation site [1-5]. Furthermore, electrical stimulation has low utility in embryonic studies, as direct tissue contact is required and induces tissue damage [6]. However, the use of light overcomes these challenges, and light can be used to noninvasively excite tissue with or without introducing exogenous agents [6, 7]. These advantages have led to multiple studies using optical stimulation to excite neural and cardiac tissue [5-15].

While earlier studies used ultraviolet and visible light to excite cardiac tissue [6, 16], infrared (IR) light has been demonstrated to be safe for basic science and clinical studies, and has recently become a preferred modality in stimulating and inhibiting cardiac tissue [5, 14]. Optical pacing uses pulsed infrared light to rapidly and reversibly initiate heart beats and modulate cardiac rhythm with high spatial precision, thereby enabling noninvasive studies of embryonic cardiac development and diseases. A study in 2010 by Jenkins *et al.* first demonstrated optical pacing of the intact embryonic quail heart *in vivo* using a pulsed infrared laser ($\lambda = 1875$ nm), and a subsequent study by the group in 2013 demonstrated successful optical pacing of rabbit hearts [12, 14].

Although the precise mechanisms underlying the complex light-tissue interactions that enable optical pacing remain unclear, studies coherently support the involvement of light-induced thermal gradients [6, 10, 11, 17-23]. A study by Wells *et al.* suggested that infrared neural stimulation

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(INS) likely relies on the photothermal interactions caused by water absorption [8], and a subsequent study by Shapiro *et al*. found that the absorption of infrared light by water in the tissue reversibly depolarizes target cells by changing the electrical capacitance of their plasma membranes [17]. Wavelengths sufficiently absorbed by water to produce rapid heat transients for action potentials were found to be optimal for INS [24, 25], with the stimulation threshold being inversely proportional to absorption by water. While the breadth of optical parameters applicable for INS *in vivo* still remains largely unexplored [26], infrared stimulation studies have primarily focused on neural stimulation, rather than cardiac stimulation. Studies demonstrating optical cardiac stimulation and pacing are even more limited in both the model organisms and light delivery parameters used. However, as biological tissues are largely comprised of water, the mechanism underlying INS is likely translatable to cardiac stimulation, with infrared energy being absorbed primarily by water in cardiac tissue.

Nerves and muscles can be directly stimulated by pulsed infrared lasers with wavelengths exceeding 1.5 μm without introducing exogenous genetic or chemical agents, and lasers with wavelengths in the ranges of 1470~1550 nm and 1840~2120 nm can be delivered through an optical fiber, enabling flexibility in minimally invasive light delivery [17, 27]. Within these ranges, water has its most prominent absorption peak between 1930 nm and 1975 nm [28]. Here, we demonstrate optical stimulation and pacing in the chicken embryo as a new model organism using a thulium laser with a wavelength of 1927 nm that corresponds to high water absorption of infrared energy, expanding the toolkit of technologies and optical parameters that can be used to control embryonic cardiac dynamics.

II. METHODS

2.1. Chicken Embryo Preparation

As shown in Fig. 1, chicken eggs were incubated for 60~63 hours at 38.5°C and 65% relative humidity. Embryos were dissected with vascular systems intact using qualitative filter paper (WHATMAN No.1) and washed gently in pre-warmed Howard Ringer Solution to remove excess yolk. They were then transferred into 6-well culture plates filled with pre-warmed Howard Ringer Solution with the filter paper facing downwards. Embryos in the 6-well culture plate were placed underneath the stereomicroscope (Leica S9D) promptly following dissection while using a heating plate to maintain a temperature of 37°C. The solution was removed from each well immediately prior to laser irradiation to prevent absorption of infrared energy by the water in the solution, and thus ensure that the energy is absorbed only by water within the cardiac tissue for effective stimulation. Embryos irradiated in solution demonstrated no change in heart rate. All tools for dissecting and transferring embryos were sterilized, and embryos were carefully maintained at 37°C throughout the experiment.

2.2. Thulium Laser Irradiation

The hand-piece of the thulium laser (WONTECH Corp., Daejeon, South Korea) was disassembled for both cardiac stimulation and optical pacing. For cardiac stimulation, embryonic hearts were irradiated several times with the thulium laser in pulse-mode (pulse duration of 16 ms) at a distance of 50 mm with a spot size of 3.98 mm^2 and radiant exposure of 0.33 J/cm^2 (Fig. 2(a)). The guidance beam attached to the thulium laser that was used to assist

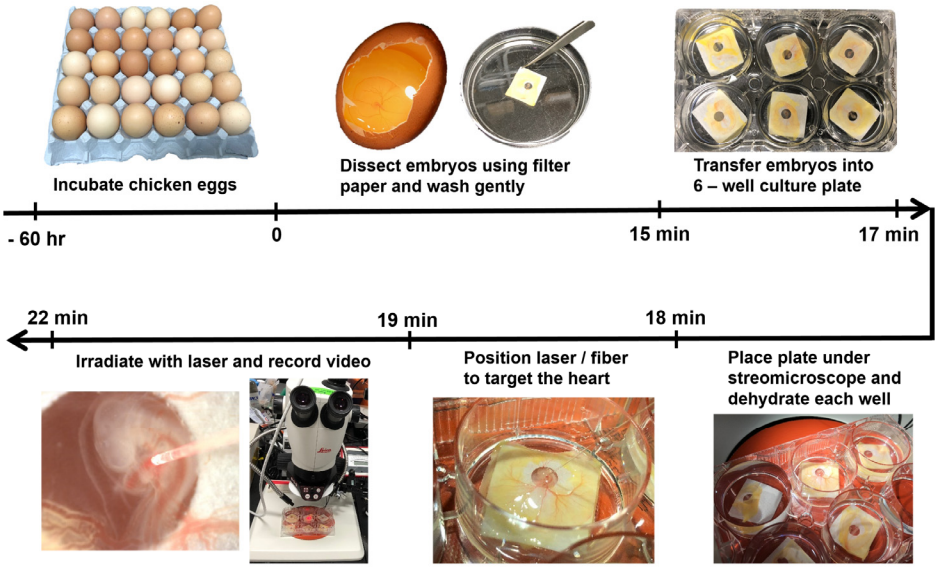

FIG. 1. Experimental pipeline and relative timeline. Approximately 22 minutes are required to complete the experiment for 6 embryos contained in a single 6-well plate. The experiment must be completed in minimal time to avoid introducing tissue damage and inter-sample variability.

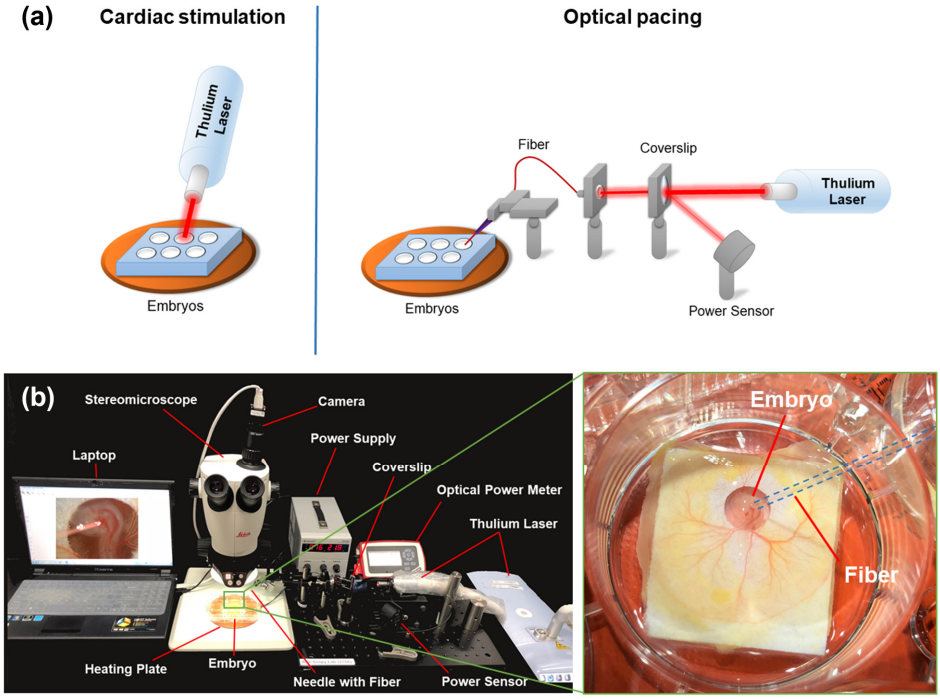

FIG. 2. Experimental setup for optical stimulation and pacing by thulium laser irradiation. (a) Schematic diagrams of cardiac stimulation and optical pacing. (b) Overview of optical pacing. Green box represents magnified view of chicken embryo and fiber.

in targeting the heart had a wavelength of 633 nm. For optical pacing, a fiber-based illumination system was constructed by coupling the output of the thulium laser to a multimode fiber (Thorlabs FG200LEA; 0.22 NA) through a fiber port coupler (Thorlabs PAF-SMA-11D) at an off-axis to prevent burning of the fiber core. The diameters of the core, cladding, and coating of the fiber were 200, 220, and 320 μm, respectively. A coverslip was placed between the fiber and laser to measure the irradiation time frame, and the reflected light was measured using an optical power meter (Thorlabs PM100A) (Fig. 2). The fiber was placed to target the atrium from a minimal distance. The radiant exposure of the laser from the fiber was 1.02 J/cm^2, and the pulse duration was 16 ms. Video recordings of heart pacing were taken using a camera (iDS UI 3240CP-C-HQ) connected to a computer, and heart wall movements were subsequently analyzed by designating a distinct point on the heart wall and tracking its position using Kinovea software (Joan Chartmant & Contrib.).

2.3. Threshold Measurement

The threshold for optical pacing was measured using an experimental setup similar to that shown in Fig. 2(a) neutral-density filter (OD 0.04~3.0) was used to control the power of the thulium laser, while other optical parameters were kept constant. A total of 50 embryos was used for the experiment, and each laser power was tested for pacing in a minimum of three embryos. We subsequently quantified the pacing probability, with a probability of 1 indicating successful pacing instantly following laser irradiation and 0 indicating failure to achieve pacing. The pacing probability plot was fit to a Boltzmann distribution function, and the threshold (50% pacing probability) was determined using Origin Pro (OriginLab, Northampton, MA, USA).

III. RESULTS & DISCUSSION

3.1. Cardiac Stimulation by Thulium Laser Irradiation

After testing various optical parameters with the thulium laser, the parameters shown in Fig. 3(a) were used for effective cardiac stimulation of embryonic chicken hearts. Following irradiation with the thulium laser, 92.3% of embryos (HH 17~19) instantly experienced an increase in heart rate (Fig. 3(b)). Heart rate returned to the basal rate within seconds when the laser was turned off and immediately increased again when the laser was turned on again. Each embryonic heart was stimulated with the laser three or four times to confirm successful and reversible cardiac stimulation. For six of the embryos, minor bleeding was observed near the edge of the vascular system at a considerable distance from the embryo body 1~2 hours following stimulation, presumably due to dehydration.

We suspect this bleeding resulted from dryness and does not indicate tissue damage caused by prolonged laser exposure or stimulation. Embryos had to be irradiated outside of solution, and thus briefly remained in dry conditions when the Howard Ringer Solution was removed from the culture plate. The solution is isotonic to embryonic body fluids and is necessary to preserve tissue health and

(a)

Power	831 mW
Pulse Duration	16 ms
Spot Size	3.98 mm^2
Frequency	2 Hz
Radiant Exposure	0.33 J/cm^2

(b)

	Acceleration in heart rate	Constant basal heart rate
Number of Embryos	24 (92.3%)	2 (7.7%)

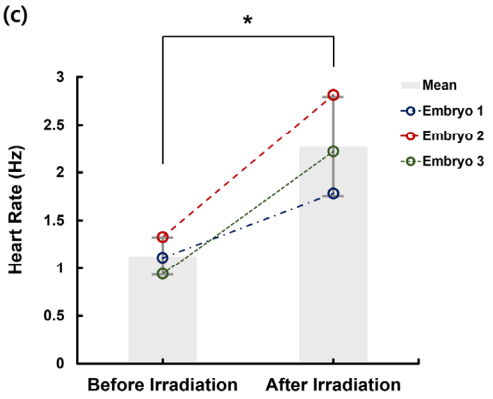

FIG. 3. The 1927 nm thulium laser effectively stimulates cardiac tissue in chicken embryos. (a) Optical parameters used for cardiac stimulation. (b) Hearts were directly illuminated with the laser, and heart rate instantly increased in 92.3% of embryonic chicken hearts (HH 17-19) after thulium laser irradiation (n = 26). (c) Increase in heart rate following thulium laser irradiation (n = 3). Mean heart rate of chicken embryos increased from 1.1 Hz (SD = 0.20) to 2.3 Hz (SD = 0.52) (*p = 0.04, paired t-test). Heart rates were determined through video analysis.

integrity, and maintaining proper humidity is essential when incubating chicken embryos and working with various *ex ovo* culture systems. Furthermore, we observed bleeding in an embryo that failed to be stimulated and was thus exposed to the laser only for a relatively short period of time. Collectively, we presume that the temporary exposure to dry conditions immediately prior to irradiation, and not the laser and stimulation themselves, led to bleeding in some embryos. Nevertheless, further research is necessary to conclude that the thulium laser does not cause bleeding in the embryos.

While we also examined the survival of embryos in different buffer solutions such as PBS or HBSS, Howard Ringer Solution enabled the longest survival period. When placed in Howard Ringer Solution under optimal conditions inside the cell incubator (37°C, 5% CO_2), embryos maintained normal heart beating rates for more than 3 hours, and no bleeding was observed. However, the solution had to be removed prior to irradiation, as we found that irradiating the embryo in the solution failed to achieve stimulation and pacing, likely due to absorption of the infrared laser energy by the surrounding water. As the embryo had to be irradiated under dry conditions that are detrimental to embryo health, we ensured that the subsequent steps were completed within the shortest time frame possible. Regardless of minor bleeding, embryos in dry conditions maintained at a physiological temperature of 37°C via a heating plate sustained basal heart rates for up to an hour, whereas embryos at room temperature experienced heart failure sooner. This highlights the importance of developing strategies to maintain favorable environmental conditions for embryos during optical stimulation experiments.

To quantify the heart rates of the chicken embryos, heart wall movements were tracked by processing video recordings of the embryonic hearts. Each embryo demonstrated a significant increase in heart rate following irradiation with the thulium laser ($p = 0.04$, paired t-test); the increased heart rate lasted for a brief time period on the order of seconds, and subsequently returned to the basal heart rate (Fig. 3(c)). The mean heart rate of the embryos increased from 1.1 Hz to 2.3 Hz ($n = 3$). Our results demonstrate that irradiation with the 1927 nm thulium laser successfully stimulates cardiac tissue in embryonic chicken hearts.

3.2. Optical Pacing by Thulium Laser Irradiation

While instant cardiac stimulation is desirable for applications requiring cardiopulmonary resuscitation, tuning and locking heart rates through pacing provides significant advantages for studies of heart development and cardiac diseases, and may open avenues for therapeutic modalities. To achieve optical pacing of embryonic chicken hearts using the thulium laser, the laser was connected to a fiber and turned on and off to irradiate the heart for various time intervals, while using video processing to track a position on the heart wall and quantify heart rate (Methods 2.2). After calibrating the distance scales in the video to real distance units using Kinovea, the tracking path and time were exported, and the positions of a distinct point on the heart wall were plotted with respect to time. The laser was coupled to a fiber, as the relatively large spot size of the thulium laser precluded precise targeting of the heart, and hearts were repeatedly paced for different time intervals of prolonged laser exposure to demonstrate the robustness of pacing. As the fiber was placed as close to the heart as possible while avoiding contact, we calculated spot size assuming that the diameter of the laser spot is equivalent to the diameter of the fiber core.

Heart rate increased to 2 Hz and thus synchronized with the thulium laser during irradiation, and returned to the basal rate when the laser was turned off, demonstrating that the thulium laser achieves optical pacing in embryonic chicken hearts. Using the optical parameters shown in Fig. 4(a), all 20 embryos used for this experiment demonstrated

successful optical pacing. Two representative samples are shown in Figs. 4(b) and 4(c). No significant tissue damage was observed, and the minor bleeding observed in some embryos following cardiac stimulation was absent, likely because the embryos were directly exposed to the air without solution only for a time period under 30 minutes and as embryo temperatures were maintained at 37°C.

For embryo (b), an initial heart rate of 1.0 Hz increased to 2.2 Hz while being irradiated with the laser and decreased to 1.1 Hz after the laser was turned off. For embryo (c), an initial heart rate of 1.1 Hz increased to 1.8 Hz during laser irradiation and returned to its basal rate of 1.1 Hz after the laser was turned off. The variations in vertical axis values likely resulted from the tracking program's limited displacement resolution of 5 μm. Nevertheless, we visually identified and tracked the movements of the selected position on the heart wall in conjunction with using the program, and verified that the tracking path synchronized well with the heart wall movements. Further analyses using a tracking program with a higher displacement resolution may elucidate whether these variations are due to motion artifacts or natural fluctuations in cardiac dynamics.

A sequence of video frames showing pacing and corresponding to the dashed box in Fig. 4(b) is shown in Fig. 4(d). The position of the black point was analyzed through video processing to quantify heart rates. The movements of the point are visually evident in the video, and a 0~15 μm range of the point's movements is shown in the captured frames. The path tracked throughout the time period shown in Fig. 4(b) had a 0~21 μm range of motion and is represented by the gray region overlaid with the black point. The pumping of blood by the heart is visible in the figure, and pumping frequency increased during irradiation. The increased frequency indicates escalated heart rate, and the lower amplitudes of diastolic expansions and systolic contractions indicate decreased stroke volume (Figs. 4(b) and 4(c)). This phenomenon was

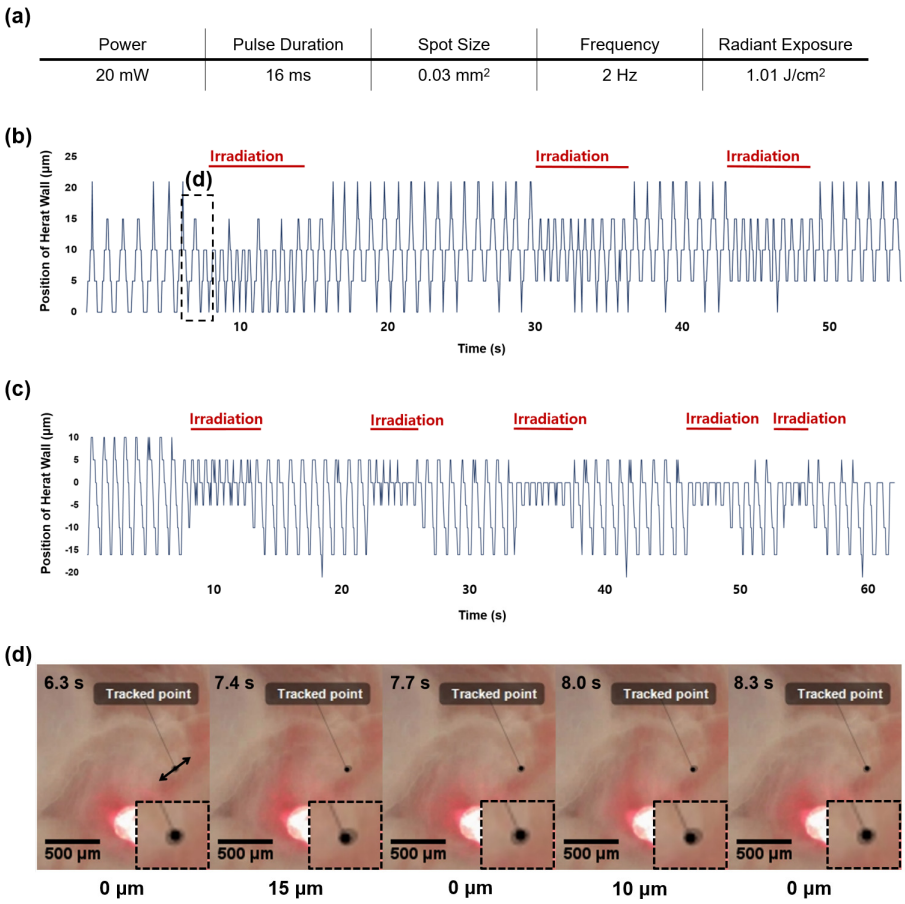

FIG. 4. Optical pacing of embryonic chicken hearts. (a) Optical parameters used for pacing. (b) and (c) show different representative embryo samples selected from a total of 20 embryos. Irradiation synchronized heart rate to the frequency of the thulium laser, and heart rate returned to the basal rate when the laser was turned off. The positions of a distinct point on the heart wall were examined through video analysis to quantify heart rate. (d) Sequence of video frames corresponding to the dashed box in (b), and their respective times and heart wall positions. The tracked point that was analyzed through video processing is indicated by a black point, and the overlaid gray region surrounding the point represents the path tracked for the entire time period shown in (b). An enlarged view of the point is shown inside the dashed box for each frame. The arrow in the first frame (6.3 s) indicates the tracked point's axis of displacement.

also observed in the study by Jenkins *et al*. [13]. Further hemodynamic studies measuring the blood flow in and out of the heart during laser irradiation are necessary to assess whether pacing maintains consistent blood circulation. Such studies would confirm whether pacing does not cause abnormal decreases in stroke volume and increases cardiac output as expected with escalated heart rates.

Successful and stable optical pacing was achieved only when the fiber was positioned to target the atrium of the heart, suggesting that precise targeting of the stimulation site is crucial for infrared cardiac stimulation. Hearts remained at a constant basal heart rate when the ventricle was targeted, and targeting the center of the heart between the atrium and the ventricle successfully paced some but not all embryonic hearts. Heart rates synchronized well with the laser, even with very brief irradiation and inter-irradiation times of approximately three seconds. Collectively, our results demonstrate that the thulium laser can be used for optical pacing to noninvasively alter cardiac dynamics with high spatiotemporal precision in chicken embryos. Optical pacing was achieved with all twenty samples, indicating that this system for optical pacing using the thulium laser is highly reliable under the aforementioned experimental conditions and optical parameters.

3.3. Threshold of Optical Pacing

The threshold for optical pacing was measured by irradiating embryos with different radiant exposures and assessing whether pacing was achieved (Methods 2.3). A sample size of 50 embryos was used to quantify the threshold, and embryos were irradiated with 15~30 second intervals between exposures. As shown in Fig. 5, the threshold (50% pacing probability) was found to be 0.99 J/cm^2, and the data were fit to a Boltzmann distribution function ($\chi^2 = 0.05$, $R^2_{adj} = 0.79$). While immediate and successful pacing was easily identifiable, some embryos paced after a varying number of heart beats (pink region in Fig. 5). In our demonstration of optical pacing, we achieved 100% pacing using a radiant exposure of 1.01 J/cm^2 that is slightly above the threshold, implicating that carefully selecting the appropriate radiant exposure is crucial for effective and reliable optical pacing.

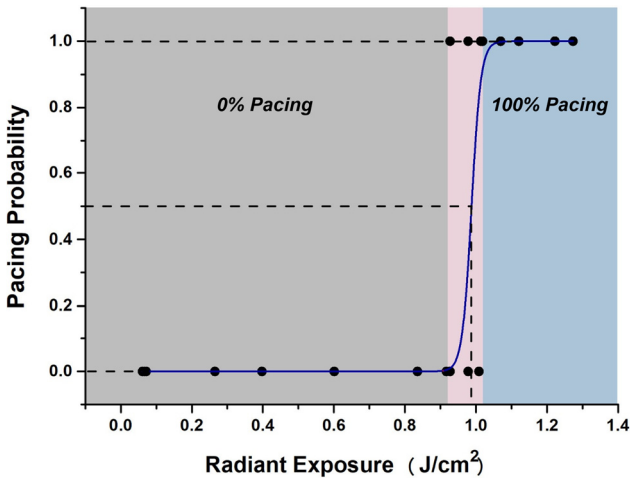

FIG. 5. Stimulation threshold for optical pacing. The stimulation threshold was measured by irradiating 50 embryos using different radiant exposures, and the pacing probability with respect to radiant exposure is shown, where pacing probabilities of 0 and 1 indicate no pacing and successful pacing, respectively. The threshold (50% probability) was 0.99 J/cm^2. Radiant exposures in the gray region (below 0.93 J/cm^2) failed to pace; those in the pink region paced some embryos instantly and others after several heart beats; those in the blue region (from 1.02 J/cm^2) achieved 100% successful pacing. The data were fit to a Boltzmann distribution function ($\chi^2 = 0.05$, $R^2_{adj} = 0.79$).

IV. CONCLUSION

Optical pacing using infrared light has emerged as an effective approach to modulate cardiac dynamics with high spatial precision without introducing artifacts or tissue damage. However, the light delivery methods and optical parameters that can be used to achieve pacing remain largely uninvestigated. Here, we have demonstrated that the 1927 nm thulium laser successfully stimulates cardiac tissue and achieves pacing of the embryonic chicken heart. This study establishes the thulium laser as a viable technology for optical stimulation and pacing using the chicken embryo as a new model organism, and expands the toolkit of infrared lasers and optical parameters that can be used to modulate cardiac dynamics. Further electrophysiological and hemodynamic studies may provide additional insight into the utility of this laser for optical pacing. The advancement of optical pacing technologies may open avenues for more intricate research on cardiac developmental dynamics and disease pathology, and also inspire novel therapeutic interventions.

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