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Optical pacing of the embryonic heart

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Light has been used to non-invasively alter the excitability of both neural and cardiac tissue[1–10]. Recently, pulsed laser light has been shown to be capable of eliciting action potentials in peripheral nerves and in cultured cardiomyocytes[7–10]. Here, for the first time, we demonstrate optical pacing of an intact heart *in vivo*. Pulsed 1.875-μm infrared laser light was used to lock the heart rate to the pulse frequency of the laser. A laser Doppler velocimetry signal was used to verify the pacing. At low radiant exposures, embryonic quail hearts were reliably paced *in vivo* without detectable damage to the tissue, indicating that optical pacing has great potential as a tool with which to study embryonic cardiac dynamics and development. In particular, optical pacing can be used to control the heart rate, thereby altering stresses and mechanically transduced signalling.

The avian embryo is an important model for studying the mechanisms driving normal development and the abnormalities leading to congenital defects. The embryonic heart is one of the first organs to develop, and in avian models it begins beating at ~40 h of incubation. The progression of a single heart tube into a four-chambered heart (cardiac looping) is shaped by mechanical stimuli from the flowing blood and pumping heart, which influence the molecular and cellular responses that regulate development[11–14]. Much remains unknown about the mechanisms of cardiac looping, in part because of a lack of suitable non-destructive tools with which to study the minute (<2 mm) organ. In the adult heart, the exquisite control achievable using electrical pacing has been critical to advancing our understanding of cardiac electrophysiology. Unfortunately, electrical pacing of the embryonic heart is invasive and difficult to achieve consistently and without tissue damage. A simple non-invasive technique to control heart rate would allow one to manipulate forces applied to cells in early developing embryos, enabling a new class of experiments exploring the roles of mechanotransduction and electrical activation.

It has been shown previously that ultraviolet and visible light can non-invasively increase the excitability of neuronal and myocardial cells[3,4,6]. In particular, Gimeno and colleagues demonstrated that exposure to visible light accelerated the heart rate in the early chick embryo[4]. More recently, Smith and colleagues used focused femtosecond pulses (780 nm) from a Ti:Sapphire laser to induce paced contractions in individual and small groups of cardiomyocytes, with success rates of up to 60% (ref. 7). Unfortunately, the high-powered pulses produce reactive oxygen species that can damage the cell, and optically pacing an entire heart is not currently possible with this method. In 2005, Wells and colleagues demonstrated that pulsed infrared light could reliably elicit compound action potentials in mammalian peripheral nerves in a one-to-one fashion at radiant exposures well below the damage threshold[8,10,15]. The stimulation threshold paralleled the inverse water absorption curve, with ~2 μm light producing the highest damage-to-stimulation threshold ratio. Although the mechanism is not well understood, it has been suggested that temperature gradients caused by infrared light absorption open ion channels[9]. To date, comparable pulsed infrared laser stimulation of cardiomyocytes or cardiac tissue has not been demonstrated. Here, we describe the use of pulsed infrared light to pace hearts of intact quail embryos, suggesting a promising new tool for studying embryonic cardiac dynamics and development.

Figure 1 shows the experimental set-up. A pulsed infrared diode laser ($\lambda = 1.875$ μm; Capella, Lockheed Martin Aculight) coupled light into a 400-μm-diameter multimode fibre. A micromanipulator positioned the fibre in close proximity to (500 μm), but not in contact with the embryo (Fig. 1a), illuminating an area of ~0.3 mm^2 on the inflow region of the heart tube. The stimulation laser included a red HeNe laser coupled into the same fibre for aiming purposes, allowing precise positioning of the stimulation pulses. The optical power of the HeNe laser light illuminating the sample was low (0.9 mW), and the sample was illuminated only briefly (<10 s) to position the fibre. The stimulation laser trigger signal was recorded to document the timing and duration of the stimulation pulses. A laser Doppler velocimeter (LDV, Moor Instruments) was used to monitor the heartbeat of the embryos. The LDV probe was positioned ~1 mm from the heart with a micromanipulator (Fig. 1a). The LDV signal was only intended for heartbeat detection, so the LDV probe was not oriented identically with every heart. No significance should therefore be drawn from the shape of the LDV signals. The trigger from the laser was also directed to a white-light, light-emitting diode (LED), which flashed on and off with the pulsing of the laser and was visible under a video microscope. The output of the video microscope was recorded using a laptop computer for real-time guidance and documentation (Supplementary Movie).

Figure 2 demonstrates optical pacing of an embryonic heart using a pulsed laser. The spikes in the blue traces represent the times when the laser was emitting light, and the red traces show the heart rate of the embryo. Figure 2a presents a recording from a stage-17 quail embryo in a New culture[16] paced at 2 Hz. The dashed box in Fig. 2a is expanded for a close-up view in Fig. 2b. Clearly, the laser pulse and heart rate are synchronized, with each laser pulse eliciting a heartbeat. In Fig. 2a, the time interval between successive heartbeats before laser stimulation was 1.58 ± 0.038 s (0.634 Hz); this interval decreased to 0.4996 ± 0.017 s (2.004 Hz) during pacing. After cessation of the stimulation pulses and stabilization of the heart rate, the time interval between heartbeats increased to 1.44 ± 0.042 s (0.693 Hz). At this point it is not known why the heart rate was slightly higher after pacing than before, but a similar increase occurred in most embryos tested. Although we cannot rule out damage, a physiological explanation for the post-pacing rate increase could be enhanced calcium ion influx during pacing.

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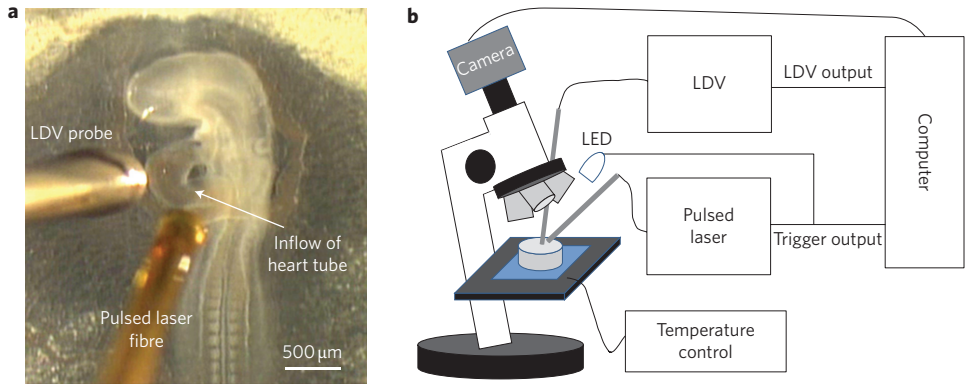

Figure 1 | Optical pacing set-up. a, Photograph of a 53-h quail embryo in a New culture. **b**, Block diagram of the set-up of the optical pacing experiment. Embryos in the petri dish under the microscope were stimulated by a pulsed infrared laser, and a laser Doppler velocimeter probe measured blood flow. The LDV output and trigger pulses from the laser were recorded to verify pacing. The trigger pulse from the laser also activated a white-light LED, which was observable in the video. The video output from the microscope camera was recorded at video rate (29.97 frames per second) by a laptop computer.

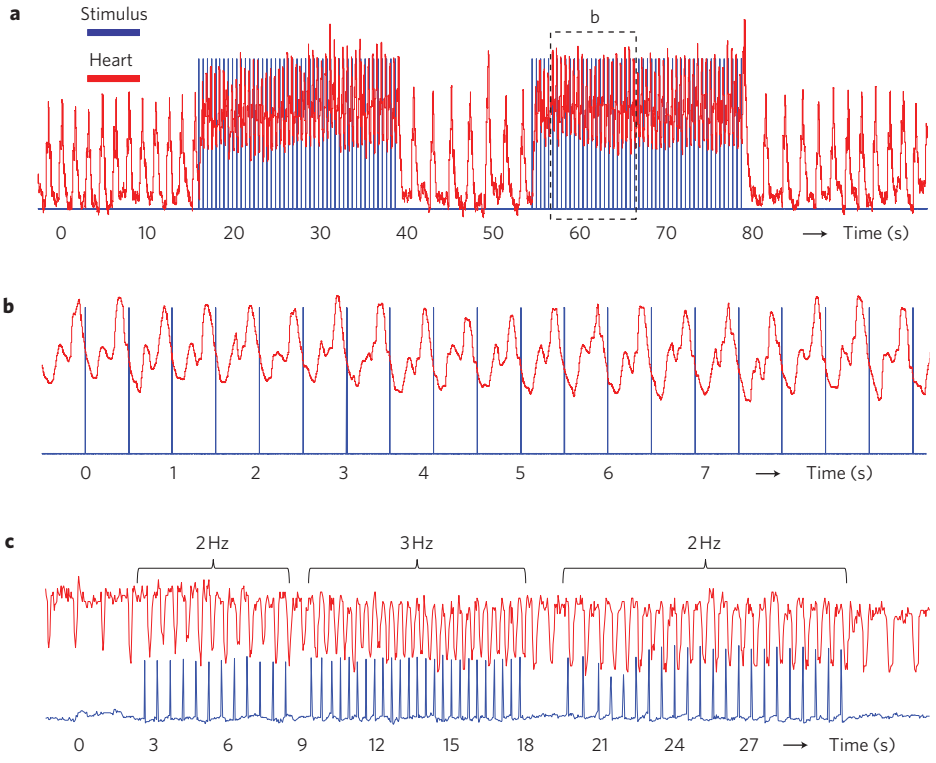

Figure 2 | Pacing of the embryonic quail heart. a,**b**, The trigger pulse (blue) from the pulsed laser is superimposed on the LDV recordings (red) of heart rate. **a**, Recording from a stage-17 (59-h) quail embryo in a New culture paced at 2 Hz. The laser pulse duration was 1 ms and the radiant exposure was 0.92 J cm^{-2} per pulse. The laser pulses were directed toward the inflow region of the heart tube. The laser pulses were turned on and off several times to demonstrate the robustness of optical pacing. The heart rate increased from 0.634 Hz to 2 Hz when the stimulation laser pulses were started and decreased to 0.693 Hz by the end of the trace. **b**, Close-up view of the dashed box in **a**. The trigger pulse and LDV signal were synchronized, with each laser pulse eliciting a heartbeat. **c**, Recording from a stage-14 (53-h) quail embryo in a New culture. The laser pulse duration was 2 ms and the radiant exposure was 0.84 mJ cm^{-2} per pulse. The frequency of the laser pulses was varied from 2 Hz to 3 Hz and back to 2 Hz to demonstrate the ability of the embryo heart to follow the pulse frequency. Laser stimulation (blue) and heart rate (red) traces were calculated from the video by plotting the pixel intensity of LED flashes triggered by the laser and intensity at the edge of the heart wall.

Figure 2c shows the heart beating in synchrony with the laser pulses as the frequency of the pulses is changed between 2 and 3 Hz.

The radiant exposure threshold required to pace a day-2 embryonic quail heart in a New culture was determined by subjecting each embryo to a different radiant exposure level and noting whether pacing had occurred. Successful pacing was defined as one-to-one stimulation, laser pulse to heartbeat, over a 20-s interval. In total, 25 embryos were tested, and the results were fit to a normal cumulative distribution function as shown in Fig. 3. The threshold (50% probability point) was 0.81 ± 0.01 J cm^{-2}, with a t-value of 79.9 and 95% confidence interval between 0.794 and 0.836 J cm^{-2}. The standard deviation of the distribution was 0.036 ± 0.016 with a t-value of 2.32. The t-value measures the importance of the coefficients in the curve fit. Larger values indicate a greater contribution to the fit and higher certainty. In a shorter study we demonstrated that optical pacing is feasible in embryos cultured on the yolk (see

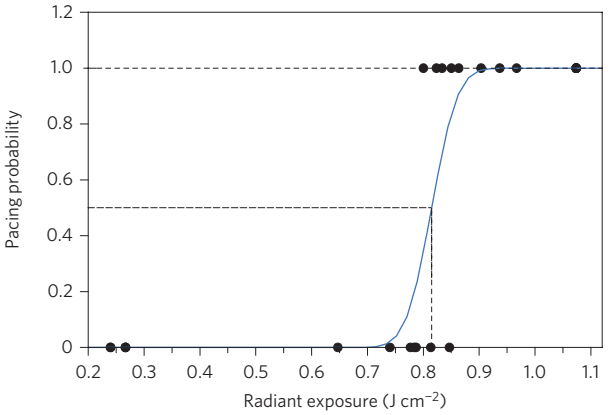

Figure 3 | Threshold measurement. Twenty-five embryos in New cultures were exposed to varying radiant exposures, and the results were plotted in terms of pacing probability (successful pacing = 1, unsuccessful pacing = 0). The data were fitted to a normal cumulative distribution function (blue line). The threshold (50% probability point) was 0.81 ± 0.01 J cm^{-2} with a *t*-value of 79.9 and 95% confidence interval between 0.794 and 0.836 J cm^{-2}. The standard deviation of the distribution was 0.036 ± 0.016 with a *t*-value of 2.32.

Supplementary Information). This method of embryo culture is compatible with longer viability of the embryos and would be useful for following the long-term effects of optical pacing.

Several approaches were used to determine if optical pacing damaged the embryo. Standard transmission electron microscopy (TEM) was used to examine the ultrastructural detail of cardiomyocytes in the inflow region of the heart tube where the laser was aimed. Figure 4 shows a control embryo (same experimental protocol, but no light exposure, Fig. 4a), an embryo paced slightly above threshold (0.88 J cm^{-2}, Fig. 4b) and an embryo paced well above threshold (4.33 J cm^{-2}, Fig. 4c). In hearts paced well above threshold, the mitochondria are vacuolated and the nuclear envelope and rough endoplasmic reticulum are slightly expanded, whereas the ultrastructure of the cardiomyocytes of embryos paced slightly above threshold look similar to that of the controls. In all three examples, several regions of the heart tube were examined in detail to look for anomalies. No clear indications of cellular damage were identified in the embryo paced slightly above threshold. Some subtle abnormalities in the mitochondrial cristae, small spaces within the cristae, are present in the control and threshold embryo, and are probably the result of necessary embryo manipulations during harvest before fixing. Other damage assays showed no structural damage to embryo heart cardiomyocytes paced at threshold levels (see Supplementary Information). At this point we did not detect evidence of damage resulting from optical pacing, but further study is warranted.

The mechanism of the observed phenomenon is at this point unclear. However, given the laser parameters used (wavelength, pulse duration and peak power), some inferences can be drawn. Gimeno and colleagues used continuous visible light to increase the heart rate, with a maximum effect at 475 nm (photon energy, 2.61 eV) (ref. 4). Proposed mechanisms included acetylcholine sensitivity to light and inhibition of ATPase. In a different study, Smith and colleagues showed that a focused near-infrared femtosecond laser caused contraction in cultured neonatal rat cardiomyocytes[7]. A window for this effect was found to occur at an average power between 15 and 30 mW for an 80-fs, 82-MHz pulse train at 780 nm, using 8-ms exposures applied periodically at 1–2 Hz. Mechanistically this effect was attributed to the laser-induced release of intracellular calcium, which has been shown in various cell types for these laser parameters. In contrast, our study used near-infrared pulses ($\lambda = 1{,}870$ nm) and relatively long (millisecond) pulses of continuous laser light. The photon energy (0.66 eV) is therefore insufficient to drive photochemistry directly, as was the case in the work by Gimeno, and the irradiance (W cm^{-2}) is too low for multiphoton effects, as in the experiments by Smith (irradiance from the femtosecond pulses and diffraction-limited spot size is approximately eight orders of magnitude higher than we used). It is more likely that the mechanisms responsible for the effects seen in our study align with those described by Wells and colleagues, who used identical laser parameters in the rat sciatic nerve and concluded that the laser-induced spatio-temporal temperature gradient was responsible for the induction of action potentials in excitable tissues[8,9]. It has been shown that non-absorbing wavelengths with otherwise similar parameters do not result in

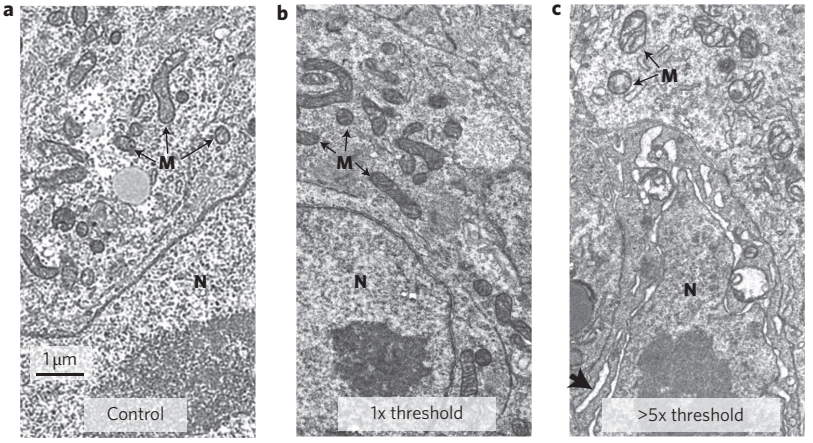

Figure 4 | TEM images after the optical pacing procedure. a–c, TEM images showing a typical cardiomyocyte in the inflow (sinoatrial) region of the heart tube (where laser light was directed) of three different quail embryos. Each embryo was between 52 and 58 h of development. The torso of each embryo was excised and fixed immediately after optical pacing. **a**, Control embryo. The embryo was exposed to the optical pacing experimental procedure, but the pacing laser was not turned on during the experiment. **b**, Embryo paced slightly above threshold. The embryo heart was paced for ~20 s with 2-ms pulses (2.64 mJ pulse^{-1}) at 2 Hz. **c**, Embryo paced well above threshold. The embryo heart was paced for ~20 s with 4-ms pulses (13 mJ pulse^{-1}) at 2 Hz. The micrograph image was taken from a border region separating severely damaged tissue (partially ablated) from apparently healthy tissue. The mitochondria are vacuolated and the nuclear envelope is swollen, as are elements of the rough endoplasmic reticulum (large arrow). The cells and mitochondria in **a** and **b** have a similar appearance and show no signs of damage from the pulsed laser light. Some subtle abnormalities in the cristae present in cells from both threshold paced and unpaced control hearts are due to the unavoidable delay in excising the embryonic torso. N, nucleus; M, mitochondria.

action potentials in the peripheral nerve, and pulse durations that violate the conditions of thermal confinement are similarly unable to induce action potentials. We have shown here and in previous studies that optical stimulation with these laser parameters is feasible without inducing thermal damage[15]. The question of how the laser-induced thermal gradient ultimately results in the opening of ion channels remains unanswered, although several hypotheses are currently under investigation.

Here, we have demonstrated a non-invasive method to pace embryonic hearts with pulsed infrared light. Compared to electrical pacing, optical pacing does not require contact, has high spatial precision, and avoids stimulation artifacts in electrode recordings. Optical pacing was consistently achieved with quail embryos ranging from stage 12 to stage 18 (looped heart stages before septation) in both New cultures and those cultured on the yolk at radiant exposures well below levels at which damage was apparent. In the future we will make use of optical pacing together with advanced imaging to study mechanotransduction (that is, shear stress on the endocardium) by controlling the heart rate of the embryo, thereby manipulating mechanical stress[17–20]. Optical pacing will not only enable a new class of experiments in developmental cardiology, but may also become a useful tool for investigating cardiac electrophysiology, single-cell (cardiomyocyte) dynamics and cardiac tissue engineering. Furthermore, optical pacing may potentially be capable of pacing the adult heart, which could lead to clinical applications.

Methods

Egg preparation. Fertilized quail (*Coturnix conturnix*) eggs were removed between 48 and 60 h post-fertilization from a humidified, forced-draft incubator (38 °C). Embryos were removed from the egg and either cultured on the yolk in a sterilized 3.5-cm-diameter petri dish or cultured using a New culture, in which the embryo is inverted on an egg–agar substrate[16,21]. The New culture fully exposes the heart, which facilitates interventions, but reduces the lifespan to a few days, whereas embryos cultured on the yolk can survive through gestation. Both techniques are routinely used in the field. Figure 1a shows a healthy 53-h embryo in a New culture. For pacing experiments, embryos were placed on a temperature-controlled heating plate (ATC 1000, WPI), which maintained the temperature at 38 °C.

Radiant exposure calculations. Radiant exposures (J cm^{-2}) were calculated by dividing the pulse energies by the laser spot size. Pulse energies were measured using a pyroelectric energy meter (PE50BB, Ophir), and spot size was computed using the numerical aperture, fibre diameter and fibre distance from the tissue. Laser light was delivered to the tissue through a 400 ± 8-μm-diameter flat-polished multimode optical fibre (Ocean Optics) with a numerical aperture of 0.22 ± 0.02. The fibre-to-heart distance was held constant at ∼500 μm for each trial. The tissue was assumed to be perpendicular to the fibre. Although the fibre was oriented at an angle to the membrane of ∼37 ° from the perpendicular, the curvature of the membrane covering the heart was assumed to offset the angle of the fibre sufficiently to make these effects negligible.

Threshold measurements. Twenty-five embryos in New cultures were tested to determine the radiant exposure level needed to induce optical pacing. To quickly find the precise location for optimal stimulation, the laser was set to a level slightly above threshold and the position of the fibre optimized, which normally took less than 10 s. The optical fibre was positioned 500 μm from the embryo. Because the LDV fibre took a significant time to accurately place, for this experiment we relied on the LED flashes and heart motion in the microscope video to determine successful pacing. After a brief waiting period (∼1 min) with the laser off, the radiant exposure was adjusted and the laser turned on for 20–30 s. Embryos were then either fixed for further experiments or discarded. For each embryo we determined whether there was successful pacing (1) or failed pacing (0), and plotted the results in Fig. 3. Some embryos took two or three beats to synchronize to the laser pulses, but were counted as successful pacing if the heart rate was consistent after the first couple beats. Conversely, if the heart followed the laser pulses, but skipped more than one beat, it was scored unsuccessful. The difference between successful pacing and unsuccessful pacing was easy to distinguish by closely observing the videos.

TEM preparation. The torso of the embryo was excised and immediately fixed by immersion in the triple aldehyde–DMSO mixture of Kalt and Tandler[22]. After rinsing, the tissues were post-fixed in ferrocyanide-reduced osmium tetroxide[23]. After rinsing again, they were soaked overnight in acidified uranyl acetate[24]. Thin sections were sequentially stained with acidified uranyl acetate[24], followed by Sato's triple lead stain as modified by Hanaichi and colleagues[25], and examined in a JEOL 1200 electron microscope.

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Author contributions

M.W.J. conceived the original idea, performed and designed the experiments, analysed data and wrote the paper. A.R.D and S.G. performed and designed experiments and analysed data. Y.D. prepared and performed histology on the embryos and helped in preparing the embryos for TEM. H.J.C designed experiments and analysed data. H.F. supervised analysis and the creation of micrographs. M.W. supervised damage studies and embryo handling. E.D.J. and A.M.R. supervised optical pacing experiments. All authors helped to edit the paper. All authors except H.F. and Y.D. discussed the results and implications at all stages.

Additional information

The authors declare no competing financial interests. Supplementary information accompanies this paper at www.nature.com/naturephotonics. Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/. Correspondence and requests for materials should be addressed to A.M.R.