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Optical assessment of the cardiac rhythm of contracting cardiomyocytes *in vitro* and a pulsating heart *in vivo* for pharmacological screening

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Abstract: Our quest in the pathogenesis and therapies targeting human heart diseases requires assessment of the contractile dynamics of cardiac models of varied complexity, such as isolated cardiomyocytes and the heart of a model animal. It is hence beneficial to have an integral means that can interrogate both cardiomyocytes *in vitro* and a heart *in vivo*. Herein we report an application of dual-beam optical reflectometry to determine noninvasively the rhythm of two representative cardiac models–chick embryonic cardiomyocytes and the heart of zebrafish. We probed self-beating cardiomyocytes and revealed the temporally varying contractile frequency with a short-time Fourier transform. Our unique dual-beam setup uniquely records the atrial and ventricular pulsations of zebrafish simultaneously. To minimize the cross talk between signals associated with atrial and ventricular chambers, we particularly modulated the two probe beams at distinct frequencies and extracted the signals specific to individual cardiac chambers with phase-sensitive detection. With this setup, we determined the atrio-ventricular interval, a parameter that is manifested by the electrical conduction from the atrium to the ventricle. To demonstrate pharmacological applications, we characterized zebrafish treated with various cardioactive and cardiotoxic drugs, and identified abnormal cardiac rhythms and atrioventricular (AV) blocks of varied degree. In light of its potential capability to assess cardiac models both *in vitro* and *in vivo* and to screen drugs with cardioactivity or toxicity, we expect this approach to have broad applications ranging from cardiopharmacology to developmental biology.

OCIS codes: (170.0170) Medical optics and biotechnology; (170.2655) Functional monitoring and imaging.

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1. Introduction

Heart diseases such as arrhythmia, heart failure or myocardial infarction remain a leading cause of death globally, and thus have attracted much effort in contemporary medical and pharmaceutical research [1]. Among research that targets heart diseases, the utility of cardiac models of varied complexity such as cardiomyocytes *in vitro* or a heart of living animals is

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almost inevitable. In particular, isolated cardiomyocytes, the fundamental unit of a heart, are most suitable for basic research that demands detailed assessment of the contractile property of cells with no interference of confounding factors such as neural or hormonal activities, preload and afterload [2,3]. On the other hand, the zebrafish (*Danio rerio*) possesses numerous attractive features such as rapid development, ease of genetic manipulation, convenience of drug delivery and conservation of essential cardiac anatomy and function [4–6], and has become a popular model for cardiac research [7–9]; its notable translucency at the embryonic and larval stages facilitates an interrogation of the internal organs such as the heart *in vivo* with optical means [10–12].

Cardiac rhythm is a manifestation of the electrophysiological function of a heart. In clinical settings, the regularity of heartbeat is generally measured with an electrocardiograph (ECG) [13]. Through simultaneous recording of the atrial (P wave) and ventricular (QRS complex) electrical activities, details of the electrical conduction through the atrio-ventricular node (such as conductivity, conduction velocity et cetera) are revealed, based on which the pathophysiology of heart diseases or the outcome of therapeutic interventions can be assessed. ECG has been applied to zebrafish [14–16], but it is invasive because of a necessity to insert needle electrodes into the body of fish and demands skillful operation especially in small larvae [17,18]. Towards this end, various optical modalities have been developed to characterize the cardiac rhythm of zebrafish and other small animals such as goldfish and molluscs [11,19,20]. Despite these advances, the preceding work has some limitations. For instance, most such work focused on only the heart rate, or the regularity at best. Crucial information such as the interval between the atrial and the ventricular contractions is typically unavailable. The missing information that underlies the atrio-ventricular conduction might hinder an elucidation of the interplay between cardiac rhythm and electrical conductivity, and impede the identification of abnormal atrio- ventricular conduction in varied cardiac dysrhythmic diseases.

Herein, we report a platform based on dual-beam optical reflectometry for non-contact and noninvasive determination of the cardiac rhythms of two representative models in cardiac research–chick embryonic cardiomyocytes and zebrafish larvae. Our approach is significant in several respects. First, it allows functional assessment of both primary cardiomyocytes *in vitro* and the heart of a popular model animal *in vivo* on the same platform; this feature should benefit translational research that requires characterization of cardiac models of varied complexity. Second, our setup utilizes two laser beams that aim precisely at the atrial and ventricular chambers, which allows simultaneous recording of the atrial and ventricular beatings and determination of the temporal interval between the atrio-ventricular pulsations; the information obtained is particularly important to reveal the electrophysiological dysfunctions of a heart. Third, to obtain signals with minimal cross talk, we modulated the two probe beams at distinct frequencies and extracted signals specific to individual cardiac chambers using phase-sensitive detection. Fourth, we revealed the temporal variation of the contractile frequency of self-beating cardiomyocytes and the heart rate of zebrafish with a short-time Fourier transform. For demonstration, we measured zebrafish treated with various drugs of known cardiac activities and toxicities to human beings, and identified arrhythmia and atrio-ventricular (A-V) blocks of varied degrees that were induced by these drugs. We expect that this approach should benefit not only pharmaceutical development but also biomedical research that requires a detailed evaluation of cardiac rhythm *in vitro* and *in vivo*, such as cardiopharmacology, developmental biology and regenerative therapy.

2. Materials and methods

2.1 Reagents

Epinephrine, esmolol hydrochloride, digoxin, tricaine (Sigma Aldrich), and isoflurane (Baxter) were obtained from the indicated sources.

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2.2 Experimental setup

A schematic diagram of the experimental setup is displayed in Fig. 1(A). Two near-infrared lasers (Excelsior-785C-45, Newport) were employed; their beams were combined with a beam splitter, directed to the back port of an inverted optical microscope (Eclipse Ti, Nikon), and focused onto the sample with a water-immersion objective lens (CFI Plan Apo 60X, N.A. 1.2, Nikon). To illuminate the regions of interest, the two probe beams were adjusted independently with the guidance of a video system. The reflected light was measured with a photodiode (DET110, Thorlabs). To separate signals produced from the two probe beams, a dual-slot optical chopper (SR540, Stanford Research System) modulated the two beams at distinct frequencies (for instance, 2.5 kHz and 3.0 kHz). The signal detected with the photodiode was digitized with data acquisition cards (PXI-4461, National Instruments) and demodulated at the two modulation frequencies using a virtual lock-in amplifier (LabVIEW Lock-In Amplifier Start-Up Kit, National Instruments).

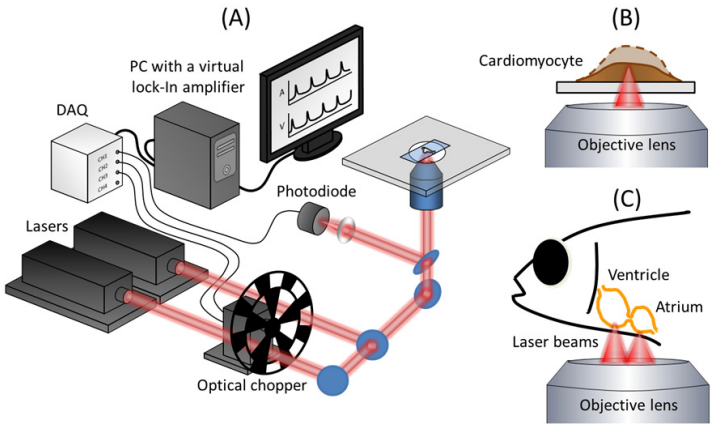

Fig. 1. Schematic of the optical determination of cardiac rhythm. (A) Dual-beam optical reflectometry. (B-C) Cartoons showing determinations of cardiac rhythm of cardiomyocytes and zebrafish larvae.

2.3 Determination of the contraction of primary cardiomyocytes

Chick embryonic cardiomyocytes were prepared according to a reported protocol [21]. Cells cultured for five days to a confluent layer (10^5 cell/dish), and beat synchronously and regularly with a stable rhythm were utilized in this work. Before experiments, a Petri dish that contained cells was enclosed in a mini-incubator (INU-NI-F1, Tokai Hit) maintained at 37 °C and 5% CO_2. The incubator was mounted on the sample stage of the optical microscope. To measure the contraction of cells, one laser beam was blocked and the other beam was adjusted to illuminate a region of interest (Fig. 1(B)). The power of each laser, measured before entry into the microscope, was kept below 4 mW.

2.4 Determination of the atrial and ventricular pulsation of zebrafish

The Animal Investigation Committee of National Chiao Tung University approved this work (Permit No. 0099000). Breeding colonies of AB strain zebrafish (*Danio rerio*), obtained from Taiwan Zebrafish Core Facility at ZeTH, were maintained according to a reported protocol [12]. Larvae at three or six days post fertilization (dpf) were utilized in this work. Before experiments, zebrafish larvae were immersed in a solution containing tricane and isoflurane (100 ppm each) for 10 min for anesthetization [22]. To administer esmolol, an anesthetized larva was placed on a chambered glass slide and immersed in a solution containing esmolol

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and the anesthetic. To administer epinephrine or digoxin, a solution (approximately 1 nL) of the drugs was injected into the retro-orbital vasculature of the anesthetized larva with a microinjector (FemtoJet and InjectMan NI 2, Eppendorf). The larva was then placed on a chambered glass slide and immersed in the anesthetic solution. To measure the pulsation of the atrium and the ventricular, the chambered glass slide was placed on the sample stage of the microscope; the two probe beams were then adjusted to illuminate the two cardiac chambers (Fig. 1(C)). The power of each laser beam, measured before entry into the microscope, was kept below 3 mW. All experiments were performed in a room with the temperature maintained at 24 °C.

3. Results

3.1 Determination of the contractile frequency of primary cardiomyocytes in vitro

We recorded the contraction of self-beating embryonic cardiomyocytes for duration of 10 min. A fraction of the temporal trace is displayed in Fig. 2(A). Each peak of the temporal trace represents a contraction of the cells. To reveal the temporal variation of the contractile frequency, we performed a short-time Fourier transform (STFT) of the temporal trace to obtain a spectrogram [21], displayed in Fig. 2(B). The result shows that the contractile frequency remained stable (1.215 ± 0.025 Hz) throughout the duration of measurements (10 min). This result indicates that, under our experimental conditions, an illumination of the cells with the laser beam exerted no discernible perturbation of the contractile frequency of these cells.

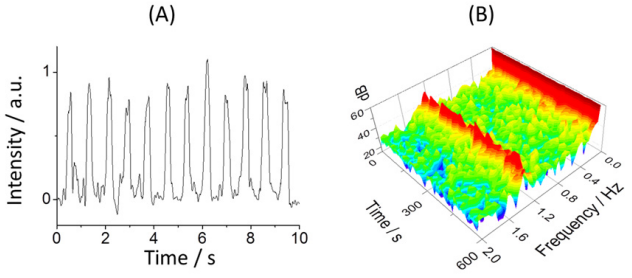

Fig. 2. Determination of the contractile frequency of self-beating cardiomyocytes *in vitro*. (A) Part of a temporal trace recorded from chick embryonic cardiomyocytes cultured *in vitro* showing individual beats. (B) Spectrogram obtained with a short-time Fourier transform of the temporal trace of cardiomyocyte contraction showing that the contractile frequency remained stable for 10 min.

3.2 Determination of the heart rate of zebrafish larvae in vivo and its application to screen drugs of cardiac activity

We proceeded to determine the heart rate of zebrafish larvae *in vivo* and to demonstrate an application to screen cardioactive drugs on zebrafish. We assessed the heart rate of zebrafish larvae (3 dpf) treated with drugs that possess known cardiac activity on human beings. In particular, esmolol hydrochloride, an antagonist of the beta-adrenergic receptor, is known to possess a negative chronotropic effect, whereas epinephrine is an agonist of the receptor with a potent positive chronotropic effect [23].

We recorded continuously the temporal trace of heartbeat on a larva treated with esmolol (2000 ppm), and display two sections of the temporal trace in Fig. 3(A). As shown in the result, the interval between two adjacent pulses increased as time proceeded. To reveal the temporal variation of the heart rate, we performed a STFT on the temporal trace, and display a

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spectrogram in Fig. 3(B). As expected, the heart rate of zebrafish larvae decreased progressively with time after a treatment of esmolol.

For comparison, we characterized also untreated larvae (control) and larvae treated with epinephrine (200 ppm) in the same manner, and summarize the final heart rates after the treatments (determined from the temporal traces recorded within the 30th min) in Fig. 3(C). Consistent with the known cardiac activities of esmolol and epinephrine on human beings, our results show clearly that esmolol and epinephrine caused a significant variation of the heart rate of zebrafish larvae relative to the control (2.27 ± 0.05 and 2.78 ± 0.09 beat min^{-1} vs. 2.49 ± 0.07 beat min^{-1}; $p = 0.01$).

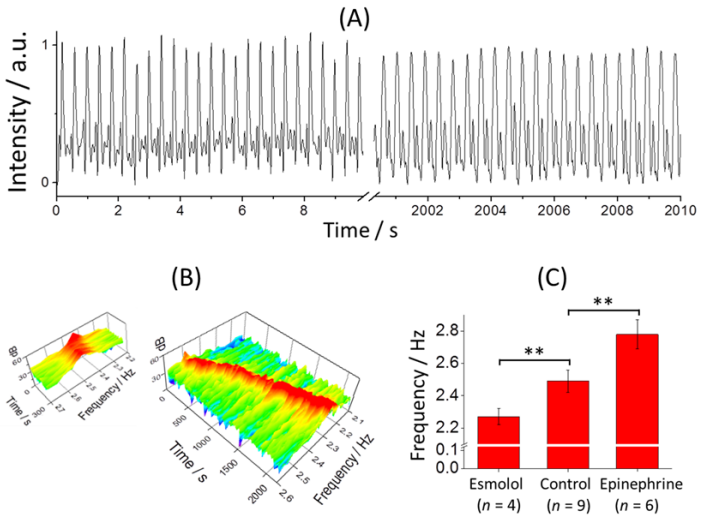

Fig. 3. Assessment of the cardiac rhythm of zebrafish larvae treated with cardioactive drugs. (A) Enlarged sections of the temporal trace of heartbeats recorded from a zebrafish larva (3 dpf) treated with esmolol (2000 ppm). (B) Spectrogram of the entire temporal trace (~2000 s) of heart beats showing the declining heart rate caused by the treatment of esmolol. (C) The heart rates of zebrafish larvae determined after a treatment with either esmolol (2000 ppm) or epinephrine (200 ppm) for 30 min and their comparison with the untreated control. ** $p < 0.01$

3.3 Simultaneous determination of the atrial and ventricular rhythms of the zebrafish heart

Digoxin is widely prescribed to patients with various heart diseases such as arrhythmia or congestive heart failure [24]. Despite the pharmacological benefit, overdoses of digoxin might produce adverse effects of varied severity ranging from bradycardia, atrio-ventricular block and atrial arrhythmia to lethal arrhythmia such as ventricular tachycardia or fibrillation [25,26].

To demonstrate an application of our approach to assess zebrafish models of heart diseases and to screen drugs with unforeseen cardiac toxicity, we treated zebrafish larvae (6 dpf) with digoxin (33 ppm) to induce arrhythmia or atrio-ventricular (A-V) blocks of varied types, and then determined both the atrial and ventricular rhythms of the treated larvae. Our results show that the atrium and the ventricle beat at a comparable rate initially (left part of the temporal traces; Fig. 4(A)), but the ventricular trace altered greatly as time proceeded. The interval between two adjacent ventricular beats increased significantly approximately 60 min after the treatment (right part of the temporal trace; Fig. 4(A)).

To analyze the cardiac rhythm in detail, we analyzed the temporal intervals between adjacent pulses and plotted their distribution. The histogram shows that the intervals obtained from the atrial trace remained nearly constant throughout the measurements (0.55 s; red bars in Fig. 4(B)), whereas those corresponding to the ventricular trace lengthened significantly (from 0.55 s to 1.10 s; blue bars in Fig. 4(B)). As explicitly shown in Fig. 4(C), the ventricular

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beating rate became half the atrial counterpart. This characteristic feature indicates a disturbance in the conduction between the atrium and the ventricle or an atrio-ventricular block, which could result from pathological conditions such as A-V nodal dysfunction.

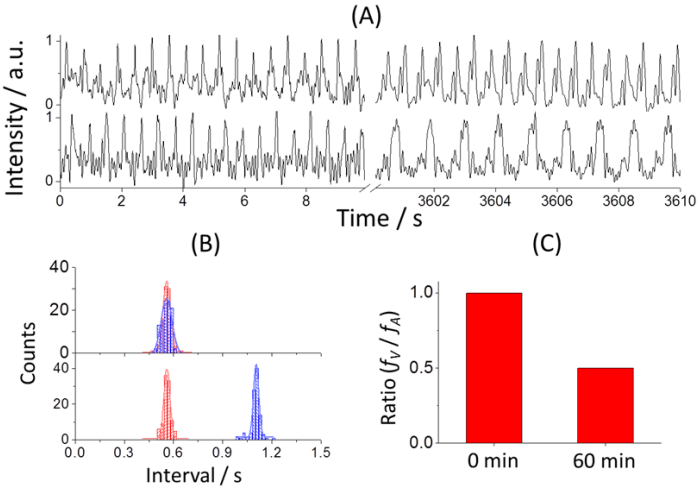

Fig. 4. Simultaneous determination of atrial and ventricular rhythms. (A) Temporal traces corresponding to the atrial (upper trace) and ventricular (lower trace) beatings of a zebrafish larva (6 dpf) treated with digoxin (33 ppm). (B) Histograms showing the distribution of the temporal intervals between two adjacent atrial (red) and ventricular (blue) beats. The upper and lower panels correspond to the histogram analyzed from sections of the temporal traces within the fifth and sixtieth minutes after the treatment. (C) Comparison of the ratios between the ventricular and the atrial beating rates.

3.4 Application to identify atrio-ventricular blocks of varied degree and arrhythmia

We have demonstrated simultaneous and continuous recording of the atrial and ventricular beating traces, and identification of an atrio-ventricular block through frequency analysis. To facilitate an assessment of the regularity of cardiac rhythm and, more specifically, the temporal relation the atrial and the ventricular contraction, we identified the temporal relation between each atrial beat and its subsequent ventricular beat, and display the result in a way similar to the rhythm strip of an electrocardiogram, in which the P-R interval is defined as the interval between the P and R waves.

As the cardiac muscle reflected more light than the blood pool did, the optical reflection became maximum as the cardiac wall overlapped with the laser focus. Facilitated with video imaging, we adjusted the laser focus such that the reflection became maximum at the end of systole. The vertical bars of the strip plot were then located where they matched the intensity maximum of individual pulses in the time trace. Accordingly, the locations of individual vertical bars represent the time-points of the end of systole.

Similar to the P-R interval in an electrocardiogram, we defined an atrio-ventricular (A-V) interval as the lag of the ventricular beat behind the preceding atrial beat from the rhythm strip. Physiologically, this A-V interval is intimately related to the duration required for an electrical pulse to conduct from the atrium through the A-V node, and ultimately to reach the ventricle. An impaired A-V conduction could result from various pathological conditions commonly seen in clinical settings. For instance, A-V nodal dysfunction, electrolyte imbalance and pharmacological or toxicological perturbation could cause an altered A-V interval or even a complete interruption of the A-V conduction. For demonstration, we interfered with the A-V conduction of zebrafish larvae to varied severity with digoxin, and illustrate examples corresponding to abnormal cardiac rhythms of distinct types in the following sections.

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Figures 5(A) and 5(B) display the results obtained immediately after a treatment of digoxin and those obtained 10 min after the treatment. The treatment of digoxin clearly resulted in a decreased atrial beating rate (from 158 beat min^{-1} to 92 beat min^{-1}), and lengthened significantly the A-V intervals (from 0.18 ± 0.02 s to 0.39 ± 0.01 s). The latter increase indicates prolonged electrical conduction from the atrium to the ventricle. Despite the profoundly decreased atrial beating rate and prolonged A-V intervals, the atrium and ventricle exhibited a comparable beating rate. Specifically, the beatings of the two cardiac chambers remained synchronized showing no sign of a missing ventricular beat throughout the measurement. Such lengthened A-V intervals while synchronized A-V contractions with no loss of ventricular beat conform to a first-degree A-V block seen clinically.

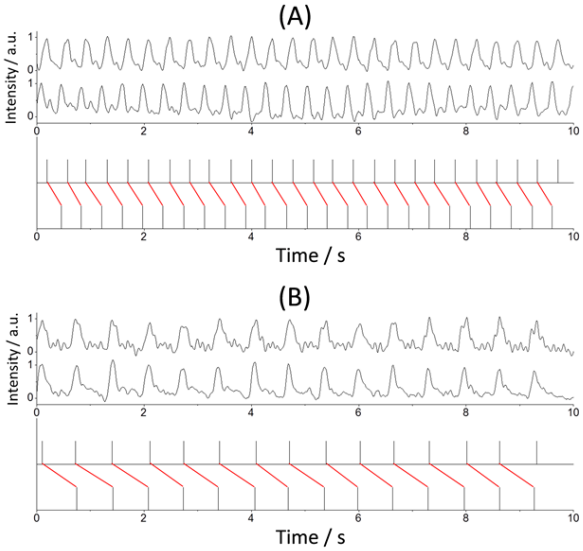

Fig. 5. Identification of an atrio-ventricular block of first degree. (A) Temporal traces and the corresponding strip plot obtained from a zebrafish larva recorded immediately after an administration of digoxin (33 ppm). Upper panel: temporal traces showing atrial (upper trace) and ventricular (lower trace) beating. Lower panel: strip plot highlighting the temporal relation between each atrial beat and its subsequent ventricular beat. (B) Temporal traces and the corresponding strip plot obtained 10 min after a treatment of digoxin showing characteristic features of an atrio- ventricular block of first degree.

Beyond the first-degree A-V block, we observed occasionally also A-V blocks of greater degree or atrial arrhythmia on treating zebrafish larvae with digoxin of the same dose. The results are illustrated below.

Distinct from the preceding result that represents only prolongation of the A-V conduction, Fig. 6(A) shows another situation in which the atrial rate decreased slightly (from 142 beat min^{-1} to 131 beat min^{-1}), but, most profoundly, the ventricle failed to beat synchronously with the atrium. The corresponding rhythm strip reveals further that the A-V conduction was blocked intermittently. This condition conforms to a second-degree A-V block.

The result displayed in Fig. 6(B) illustrates arrhythmia of another type. Grossly, it appeared that some ventricular contractions were lost and that there was a dis-synchronization between the atrial and ventricular contractions. Inspection showed that the atrial contraction also appeared to lose its regularity. Intermittent premature atrial contractions mingled with the regular pattern of atrial beats. In particular, these premature atrial contractions were not followed by corresponding ventricular contractions. From a pathophysiological point of view, it is very likely that these premature atrial contractions may

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fall within the refractory periods of the A-V node, which then precludes the conduction of the impulse through the A-V node and leads to a missing of the ventricular beat.

It is well known that digoxin can slow the heart rate, interfere the conduction of the A-V node, and disturb the cardiac rhythm. As an example to apply our system for toxicological screening or to investigate pharmacological intervention, we determined the cardiac rhythm of zebrafish treated with digoxin. As demonstrated in the results, our dual-beam system is able to trace simultaneously the atrial and the ventricular beats, which further strengthens the capability of our approach. Not only the heart rate and the cardiac rhythm can be well recognized, but also the interval of A-V conduction and a loss of A-V synchrony can be delicately shown and recorded. Based on which, the underlying electrophysiological mechanisms and pathophysiological significance can be explored in more details.

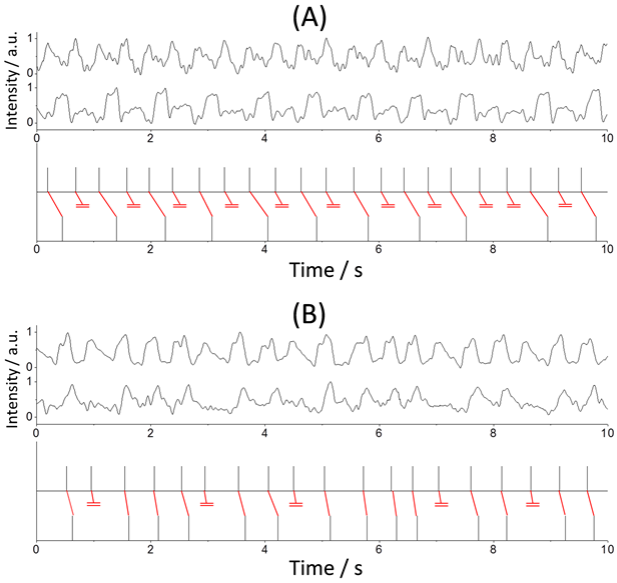

Fig. 6. Demonstration of an atrio-ventricular block of second degree and arrhythmia of another type. (A) Temporal traces and the corresponding strip plot showing characteristic features of an atrio-ventricular block of second degree. (B) Temporal traces and the corresponding strip plot showing arrhythmia of a distinct type that exhibited premature atrial beats with no subsequent ventricular beats.

4. Discussion

We have developed dual-beam optical reflectometry (Fig. 1) to determine the cardiac rhythm of cardiomyocytes *in vitro* and zebrafish larvae *in vivo*. In particular, we measured the contractile frequency of self-beating cardiomyocytes and analyzed the temporal variation of the contractile frequency with a spectrogram (Fig. 2). We determined also the heart rate of zebrafish larvae, and evaluated the effect of two commonly described drugs (esmolol and epinephrine) on the cardiac rhythm of zebrafish larvae (Fig. 3). These results demonstrate a capability of our approach to assess cardiac models of varied complexity. Our approach allows also the simultaneous and continuous recording of both atrial and ventricular pulsations of zebrafish larvae (Fig. 4). We illustrated in particular the temporal relation between each atrial beat and its subsequent ventricular beat with rhythm strips (Fig. 5), and demonstrated an identification of a dysfunctional A-V conduction of distinct types (Fig. 6). In light of a growing interest to utilize zebrafish as a model animal for pharmaceutical development and for screening of drugs with unforeseen cardiotoxicity, these results are particularly significant.

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With two laser beams independently adjusted, we probed two regions (such as atrium and ventricle) flexibly chosen by users. Moreover, we modulated the two probe beams at two distinct frequencies and incorporated phase-sensitive detection to enhance the ratio of signal to noise. This unique setup allowed the separation of signals specific to the atrium and the ventricle free of cross talk, and enabled a determination of the temporal interval between the atrial and ventricular beats. This information is potentially useful to help identify abnormal A-V conduction of varied types.

It is well known that ECG records directly the electrical activity of the heart and provides information of more electrophysiological relevance and clinical significance in comparison with our optical means. However, our approach has some advantage in comparison with conventional electrophysiological methods. For instance, it could reveal delicately the electromechanical coupling of individual atrial and ventricular contractions. Besides, as the optical means makes no physical contact, it is less invasive. These features should facilitate its applications that utilize zebrafish larvae, for instance, in screening of chemicals that act as suppressants or stimulants of cardiac activities *in vivo*. The employment of micro-electrodes for electrophysiological measurements requires operators with sophisticated skills, which might thus hinder adaption of this technique in many laboratories. The necessity to insert electrodes into particular regions of the body of zebrafish makes automation difficult, a prerequisite for application in high-throughput screening. In contrast, our approach might become automated and replicated for high-throughput application, and has an increased tolerance to alignment.

Our approach has some technical essence in common with digital imaging. Although the temporal resolution of our current setup (10 ms) is comparable with that of high-speed digital imaging (100 frames s^{-1}), we note that the temporal resolution of our approach could potentially be improved on modulating the lasers at greater frequencies (for instance, using either electro-optical modulation of the lasers or digital modulation of the driver of the diode lasers). Besides, we recognize that our approach requires addition of specialized apparatus on a video imaging system. Our approach however possesses a subtle advantage. For the same duration of measurements, the data produced with our approach are significantly fewer (by more than two orders of magnitude) than those produced with digital imaging. The decreased extent of data can be remarkable especially when long-term observation is necessary, and can become critical when the capacity of data storage is limited.

5. Conclusions

We report the development of dual-beam optical reflectometry for optical determination of cardiac rhythm on cardiomyocytes and zebrafish. We envisage this approach to benefit research that requires a detailed assessment of cardiac rhythm and has a potential to become a cost-effective platform for high-throughput pharmaceutical screening.

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