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Original Article

ZebraPace: An Open-Source Method for Cardiac-Rhythm Estimation in Untethered Zebrafish Larvae

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Abstract

For the assessment of cardiac function, heartbeat represents one key parameter. Current methods of heartbeat measurements in the zebrafish larvae usually require larval immobilization, fluorescent transgenic strains and a confocal microscope, costly commercial software for analysis, or strong programming skills if the software is open-source. Here, we present a simple yet powerful method of heartbeat analysis using untethered, unlabeled zebrafish larva using ImageJ (open-source software), which does not require programming skills. We named it as ZebraPace for **Z**ebrafish **P**recise **A**lgorithm for **C**ardiac-rhythm **E**stimation. ZebraPace works directly with AVI videos and requires no image processing steps. ZebraPace uses pixel intensity change in a grayscale video to count the number of beats. We have validated the ZebraPace method by pharmacological alterations of the heartbeat in zebrafish larvae of 48 and 72 hpf stages. We have also determined beat-to-beat interval, which relates to rhythmicity of heartbeat. The results obtained by using ZebraPace corroborates well with the heartbeat values previously reported for similarly aged larvae as determined by using specialized software. We believe that the ZebraPace method is simple, cost-effective, and easy to grasp as it involves fewer steps. It not only reduces the manual workload but also eliminates sample preparation time and researcher subjectivity.

Keywords: zebrafish larvae, heartbeat, cardiovascular, ZebraPace, ImageJ, optical imaging

Introduction

Zebrafish represents an excellent animal model to investigate the mechanisms underlying various cardiovascular diseases.[1–5] Also, it is widely used to investigate toxicological endpoints.[6–10] Heartbeat represents an important parameter that not only provides vital information about cardiovascular diseases but also is an important factor in determining the toxicological endpoint. Historically, scientists have assessed heartbeat manually by counting it from the slow-motion replay of recorded videos.[11,12] The method of manual counting is laborious and suffers from serious drawbacks of being highly subjective, which leads to inaccuracies. We have seen that often experienced researchers are not able to reproduce their own manual counting data (personal observation).

To overcome the drawbacks of manual analysis, several methods have been developed that are automated/semi-automated based on the computer-controlled, image-based heartbeat detection techniques. Such methods allow reproducibility and objectivity of the results.[6,7,12–14] However, these software-based methods have their own limitations. The main problem that is associated with the imaging of heartbeat using software-based methods is the requirement of immobilization of zebrafish larvae (often achieved by the use of anesthesia or physical confinement in methylcellulose or low melting agarose). This physical confinement could limit oxygen supply and can affect heartbeat.[15,16] Therefore, those methods are preferred that do not rely on such anesthetics and physical confinement. In the recent past, few authors have tried to address some of the problems. The study by De Luca *et al.* published ZebraBeat as a flexible platform for heartbeat analysis.[6]

However, the ease of ZebraBeat rests on the pre-requisite of a costly confocal microscope and the use of a resonant scanner to image the heartbeat of fluorescent transgenic zebrafish larvae expressing fluorescent proteins in their heart. Further, the ZebraBeat method requires the use of a large

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image database and uses costly MATLAB software for the data analysis. However, working with massive image database would demand extensive computational resources, which could be cumbersome and limiting to many end-users.

Though their software application is available to users on request, its further development or its custom modification to suit individual need is solely dependent on the source lab or would demand extensive programming skills from the end-users. Such skill sets could be seriously limiting among many zebrafish users. Also, the ZebraBeat method cannot be used for wild-type, non-fluorescent zebrafish larvae. It only works with those zebrafish larvae that are expressing fluorescent proteins in their heart regions. Few or all of these things might be the limiting factors for many zebrafish users around the world to actively employ ZebraBeat in their studies.

Later, Pylatiuk *et al.* also published their automatic method for the analysis of heartbeat. Though their method is an improvement over the existing methods, their study used costly commercial software for the heartbeat analysis. The major limitation of their method is that it is sensitive toward the movement of erythrocytes in the large blood vessels, which could affect the optical signals. Both image resolution and pigmentation also greatly affect their analysis.[12] Based on the outcome, the currently published methods are reliable, but they have some limitations, such as none of these methods are absolutely cost-effective or based on user-friendly open-source software, nor does their algorithm involve fewer steps to assess heartbeat without the need of programming skills.

Therefore, in this study, we present a simple method of heartbeat analysis from the untethered, unanesthetized, and unlabeled zebrafish larva using ImageJ (open-source) software. We named our semi-automatic method as ZebraPace for **Z**ebrafish **P**recise **A**lgorithm for **C**ardiac-rhythm **E**stimation. Our method is truly cost-effective, and it uses an algorithm that involves fewer steps without the requirement of cumbersome image processing and programming steps. It is also highly reproducible across researchers.

ZebraPace produced comparable results of zebrafish heartbeat in a cost-effective manner as produced by the methods requiring costly instruments/software. The benefit of using ZebraPace is that it does not require any image database and works well with the AVI video files. Another advantage is that ZebraPace uses ImageJ, which is freely available and maintained by the active scientific community at the global level, so its further development is not limited to any particular lab.

Materials and Methods

Animals and their maintenance

Adult zebrafish (*Danio rerio*) of mixed gender were purchased from a local commercial supplier. Zebrafish were maintained in transparent rectangular tanks fitted with white light, aeration assembly, and stone pellets for biological filtration. Three-stage purified water was used for the zebrafish maintenance. A 14/10 (light/dark) period was maintained by using automatic switch-assembly as described earlier.[17] Fishes were manually fed *ad libitum* twice a day with a commercial pellet-based diet. All zebrafish underwent at least 4 weeks of acclimatization period in the laboratory environment before being used in the experiments. All animal handling and experiments were performed in accordance with the approved protocols and guidelines prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. No animals were killed at the end of the experiment.

Mating of zebrafish and embryo collection

Fertilized eggs were obtained by mating adult zebrafish in a 2:1 ratio (female: male ratio respectively). Selected male and female fishes were placed in a custom-made mating box made of two chambers (upper and bottom) filled with dechlorinated water. The mating chamber was placed in the dark for overnight and in the morning, light was provided. After a maximum of 2 h of light condition, fertilized eggs were collected from the bottom chamber and placed in a Petri dish containing E3 medium with the help of Pasteur pipette. Eggs were washed twice with 1X E3 medium containing (in M) (0.0595 NaCl, 0.021 KCl, 0.039 $CaCl_2.2H_2O$, and 0.048 $MgCl_2.6H_2O$: pH 7.2, sterile). Only live, fertilized eggs were manually sorted and placed in the incubator at 28°C till further use. E-3 medium was replaced every 24 h.

Drug treatment

Zebrafish embryos at 2 or 3 dpf were used for the treatment with Isoproterenol (ISO) (20 μM in E3 medium) for 1 h at 28°C, Tricaine (Tri) (200 mg/L in E3 medium) for 5 min at 28°C, or Nifedipine (Nif) (30 μM in E3 medium) for 30 min at 28°C. Embryos treated with E-3 or solvent only for similar durations were used as the appropriate control (Ctrl) group.

Imaging setup

The imaging setup consisted of an inverted microscope (Olympus IX73 series) equipped with a 10-megapixel camera (ProCAM HS-10 MP). For the heartbeat recordings, zebrafish larvae of appropriate age groups were individually placed on a glass slide in a lateral side-up configuration in a 10 μL volume of their respective solutions (i.e., E3 medium or drug solution). The heart region was focused, and the video files were recorded in AVI format at 30 frames per second (fps) by using H264 (Native) video encoder of a basic free version of ''Debut video capture'' software v2.02 (NCH software) installed on a windows 10 laptop. H264 encoding was preferred because it resulted in a small-sized output of AVI files with better graphics quality.[18]

Videos were captured for 20–30 s with a 10 × objective. All the imaging experiments were performed at 28°C. In our experience, larvae of these age groups showed episodic movements out of which extraction of a continuous period of 10 s, where they showed virtually no motor activity, was easily possible. We used a 10-s length video out of the recorded 20–30-s length video for the validation of the ZebraPace method. For the data included in this article, immobility during the video recording was seen in 280 larvae out of a total of 300, which is a success rate of more than 93%.

ZebraPace algorithm for the heartbeat estimation

Extracting and generating 10 s AVI video files having total motor inactivity of zebrafish larvae. As the current version of ImageJ (v1.51) lacks capabilities to handle H264 encoded video files, the recorded video files were first appropriately

converted into raw AVI format, which can be read by ImageJ software. The technical details of video conversion steps are described elsewhere.[18] Briefly, the recorded video files (H264 encoded videos) were opened in Virtual-Dub v1.10.4 (open-source) software. A continuous period of 10 s (300 frames) for each larva, where larva showed total motor inactivity, was extracted and saved as raw AVI format.

Next, the extracted raw AVI video files from the Virtual-Dub software were opened in ImageJ and again saved as an AVI file, however, after re-compressing them by using JPEG encoder with 30 fps option. This JPEG-compression processing significantly reduces the AVI file size (from few GB to tens of MB). It also ensures proper quality control of the AVI video files used for the heartbeat investigation. For better clarity, events in a single heartbeat rhythm are shown as time-lapse images in Figure 1. An exemplar video of zebrafish larva of 2 dpf recorded for 30 s is shown as Supplementary Video S1 (Supplementary Data are available online at www.liebertpub.com/zeb) to show that larva can stay immobile for at least this much duration.

Extracting the *x-y* coordinates of the region of interest. JPEG compressed AVI video files were opened in ImageJ in the grayscale mode without a virtual stack. A circular region of interest (ROI) was drawn on the AVI video file in the cardiac region of the zebrafish larva, and the time profile of pixel intensity change (as heartbeat) was generated by using ''plot-z-axis profile'' option of ImageJ located under ''stacks'' option of ''image'' tab in the main menu bar. The location of cardiac region for the best z-profile output (as heartbeat) was screened by manually moving the circular ROI using the mouse cursor in the various locations of the pericardial area.

It is important to mention that while screening for the best location, the ''live'' button in the *z*-axis profile window must be activated for the real-time update of the heartbeat profile for the particular region (Fig. 2A; right-hand side panel). Care should be taken to avoid cardiac regions generating a wavy *z*-axis profile in the ''live mode.'' The heartbeat profile (i.e., *z*-axis profile) was saved as X (frame number or time data) and *y*-axis (pixel intensity change) data values by using ''save'' button located close to the ''live'' button at the bottom of the *z*-axis profile window (Fig. 2A; right-hand side panel). These X-Y data values were used for further analysis using OriginPro version 9.0 software (OriginLab, MA) or MS Excel or online Google spreadsheet.

Signal processing. The signal processing steps were performed in OriginPro version 9.0 software. However, as an alternative method to the use of OriginPro, we have also developed a scheme for data analysis by using MS Excel and online Google spreadsheet to make our ZebraPace method truly cost-effective. The algorithm used in the alternative methods (MS Excel and Online Google spreadsheet) is shown in Supplementary Methods. Here, we describe the algorithm used in the OriginPro software. The heartbeat profile was first plotted as a line graph (Fig. 3A) on which an FFT filter (point window 10, cutoff frequency 0.05) was applied to determine the baseline (Fig. 3B). The FFT filter method is accessible through the popup window of ''smooth'' option of ''signal processing'' function located under the ''analysis'' tab of the main menu bar of OriginPro software.

The baseline values obtained from FFT filtering for each time point were subtracted from the corresponding Y data values (change in pixel intensity) of the raw heartbeat profile to produce the resultant data values/profile (Fig. 3C, gray color). Using the FFT filter for smoothening eliminated any minor shifts in the baseline or wavy nature of the heartbeat profile. Next, the resultant heartbeat profile was again smoothened by using the ''adjacent averaging (AAv) filter'' method (points of window 5, without weighted average) (Fig. 3C, dotted dark gray line). Similar to the FFT filter, the AAv filter method is also available as a drop-down option in the popup window of ''smooth'' option of ''signal processing'' function located under the ''analysis'' tab of the main menu bar of OriginPro software. AAv filtering eliminated any notch (split) in the peaks that could account for false counting of peaks. Please note that for FFT and AAv filtering, the graph window should be active.

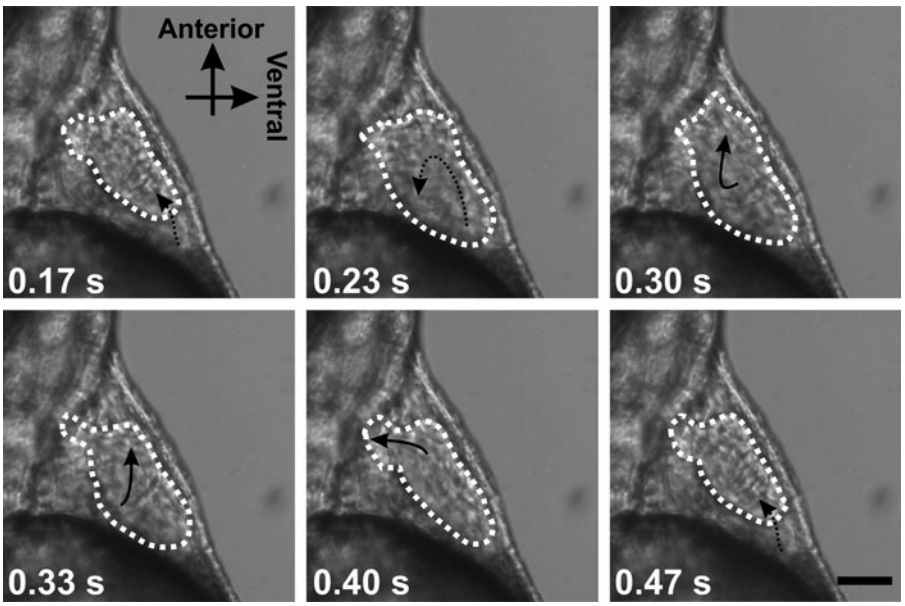

FIG. 1. Time-lapse images of a heartbeat of a zebrafish larva captured from a video. *White dotted line* represents the boundaries of the heart. *Dotted black arrow* in the heart region indicates the blood flow direction towards the atrium. *Solid black arrow* in the heart region indicates the blood flow toward the ventricle and after that toward bulbus arteriosus. The anterior and ventral direction of larva is also marked in the first image. Scale bar represents 50 μm.

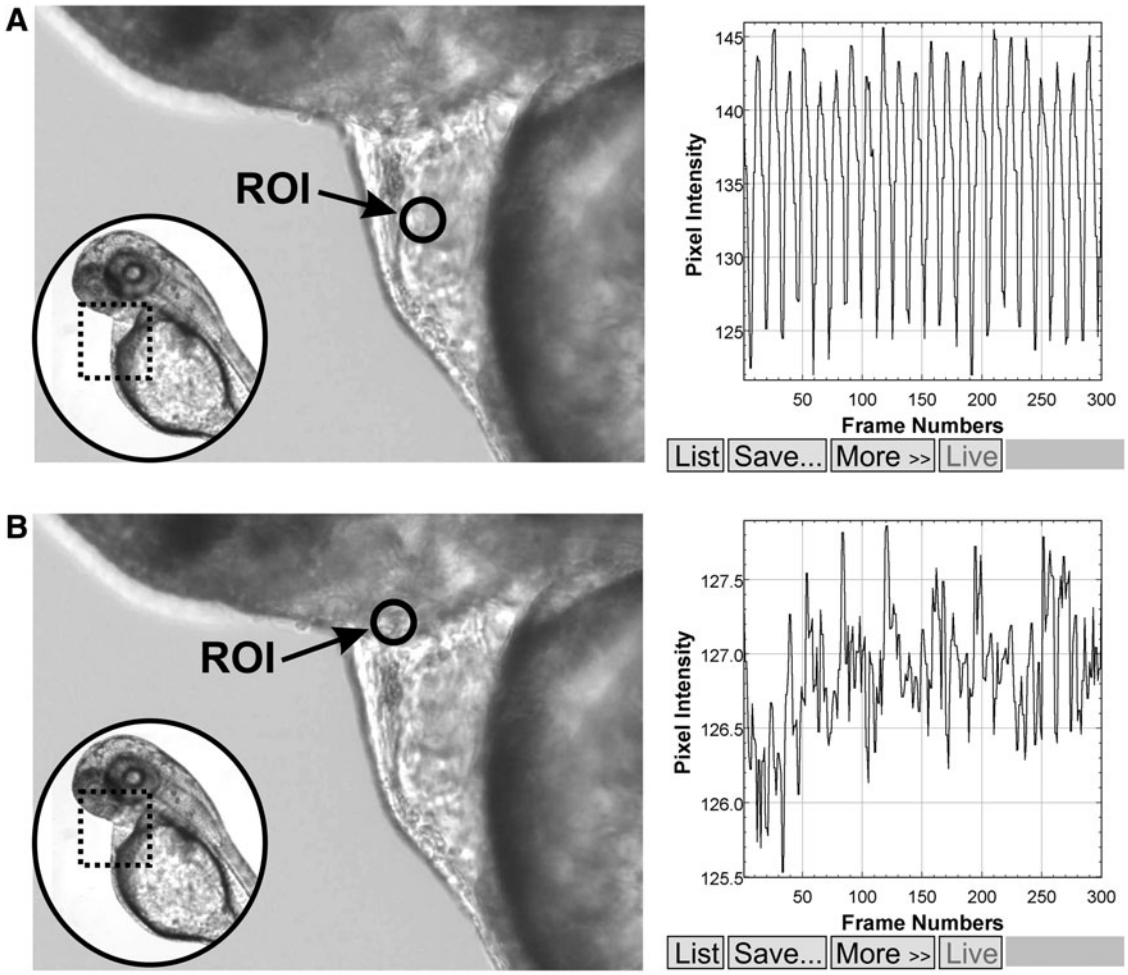

FIG. 2. Effect of ROI selection on heartbeat profile. Beating heart region ROI in **(A)** and non-beating non-heart ROI (**B**) are depicted. Note that rhythmic profile is obtained only when ROI is selected in the heart region. The use of ''Live'' button updates the information of the ROI region in real time. ROI, region of interest.

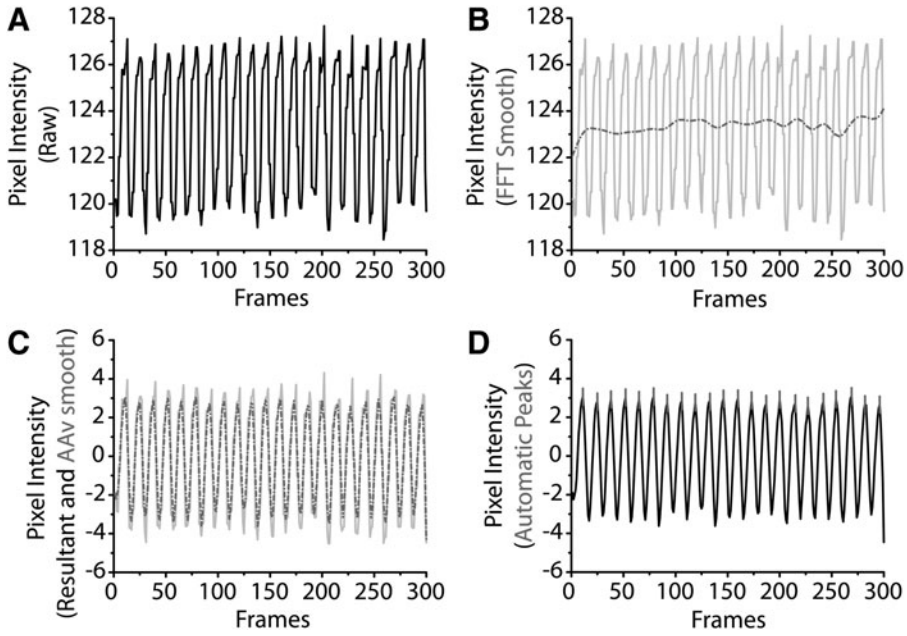

FIG. 3. Signal processing and heartbeat calculation using Zebra-Pace. **(A)** Representative raw profile of heartbeat. **(B)** FFT filter application for baseline correction. The FFT profile is shown in *dotted dark gray line*, whereas the raw profile is shown in *solid light gray line*. **(C)** Baseline corrected profile (resultant, *solid light gray line*) was again smoothened by AAv filter (adjacent averaging AAv, *dotted dark gray line*). **(D)** Automatic detection of peaks using the parameters (as defined in methods) using ''Find Peaks'' function in OriginPro software. Picked peaks are labeled as *light gray ticks*.

Automatic peak detection and counting of heartbeat. Automatic peak detection function was performed on the AAv smoothened heartbeat profile. It was done by using ''peak analyzer'' wizard, which is accessible through ''peaks and baseline'' function located under the ''analysis'' tab of OriginPro software. Please note that for counting peaks the graph window should be active. In the ''peak analyzer'' wizard, first the ''find peaks'' option was selected as the goal. Then, the baseline mode was selected as ''constant'' in which ''mean'' option was chosen. In the next step, the baseline was auto subtracted by using the ''auto subtract baseline'' option. Finally, in the last step of the wizard, the ''enable auto find'' option was chosen. In the same dialogue box, under ''peak finding settings'' one can use either ''local maximum or window search'' as the method for finding ''positive'' direction peaks by using ''auto'' as the default option under ''peak finding settings'' and ''peak filtering'' steps.

For our analysis, we have chosen ''local maximum'' as the method for peak findings and ''by height'' method for peak filtering step along with the ''auto'' options at respective locations. By clicking the ''find'' button (located under the ''enable auto find'' step), all the peaks were automatically detected and labeled for their respective *x*-axis data values. Finishing the wizard will generate a list of all the peaks detected by the software (Fig. 3D, light gray ticks). The total number of peaks represented the number of heartbeats for 10 s in our case, in which, multiplication of six would give us the number of beats per minute (bpm) values for the selected video. OriginPro-based method is robust as it allows the data analysis of those larvae that show slight movement or have a wavy baseline in a simple and user-friendly manner (Supplementary Fig. S1).

Alternative approach for peak analysis. We realized that it is not necessary to use OriginPro for the peak analysis. If the videos were recorded with low noise and good ROI was selected by using ImageJ, then ImageJ was capable of displaying peaks as a graphical display that could be manually counted and accounted.

However, as an alternative method for the automated peak assignment and counting, we have also developed a scheme using MS Excel and online Google spreadsheet. As OriginPro is not an open source, if required, MS Excel or online Google spreadsheet can be used for (1) smoothening of raw heartbeat data to avoid unambiguous peak detection; (2) automatic peak detection and their labeling; and (3) automatic calculation of sum of assigned peaks.

As every software has its own limitation, we observed that the additional smoothening step using the FFT filter is more user-friendly in OriginPro, whereas complex calculations are required in MS Excel or online Google spreadsheet to do the same. Also, an additional smoothening step using the FFT filter was required only when the larva showed slight movement or when the raw heartbeat data baseline was wavy. If the user-acquired data have a stable baseline, then the smoothening step using the FFT filter can be omitted, making both OriginPro and MS Excel or online Google spreadsheet-based methods comparable to each other for automated analysis. Step-by-step details of MS Excel or online Google spreadsheet-based heartbeat detection methods are described in Supplementary Methods.

Calculating beat-to-beat interval. After automatic determination of peaks, the *x*-axis values (frame numbers or time) corresponding to the peaks were collected. Each peak represented the beat. The difference between frames or time between each beat was determined by using the following equation:

$$(Beat\ to\ Beat\ interval) = X_{i+1} - X_i$$

where X_i represents the current frame number, and X_{i+1} represents the next frame number. This was determined by using the ''diff'' command for *x*-axis variables using the ''set column value'' option in OriginPro software. We have also identified a potential application of beat-to-beat interval in determining rhythmicity in the heartbeat, details of which are provided in the Supplementary Methods.

Statistics

All data were analyzed by using GraphPad Prism version 5. All data are presented as Mean ± SEM. Paired or unpaired *t*-test for parametric data and Mann–Whitney test for non-parametric data were performed between two groups as applicable. p value of <0.05 was considered significant, where * denotes $p \leq 0.05$; ** denotes $p \leq 0.005$; and *** denotes $p \leq 0.0005$.

Results

Optical imaging of heartbeat

Time-lapse images showing one cycle of heartbeat extracted from the processed AVI video file with corresponding timestamps are shown in Figure 1. The white dotted line represents the visible boundary of the zebrafish larval heart.

ROI selection and automated detection of heartbeat

The application of the ''live'' option helps in quick finding of the right spot for ROI placement for the heartbeat determination (Fig. 2). Please note that rhythmic profile is abolished on placement of ROI in the non-heart region (Fig. 2B). Filtering/smoothening step of raw heartbeat profile was required to reduce the probability of false positive peaks (e.g., peaks with notch or split) during the automatic peak detection step (Fig. 3).

Manual counting of heartbeat leads to researcher subjectivity

Many published studies report manual counting of the heartbeat. Therefore, in our study, we first assessed whether manual counting of the heartbeat from the recorded videos can produce variability among researchers. In our lab, three independent researchers were asked to manually count the heartbeat from the same videos of 10-s length. The data are plotted as heartbeat (HB) per min in Figure 4A. Though the manual count of heartbeat was different between these researchers, the variability was found to be non-significant. This led us to ask whether the manual counting is sensitive enough to detect any subtle change in the heartbeat induced by pharmacological agents.

We used Isoproterenol (ISO), which is a well-known beta-adrenergic agonist known to increase heartbeat. Videos were recorded both before and after treatment with 20 μM ISO. Surprisingly, manual counting by none of the researchers could detect a significant increase in the heartbeat of ISO-treated

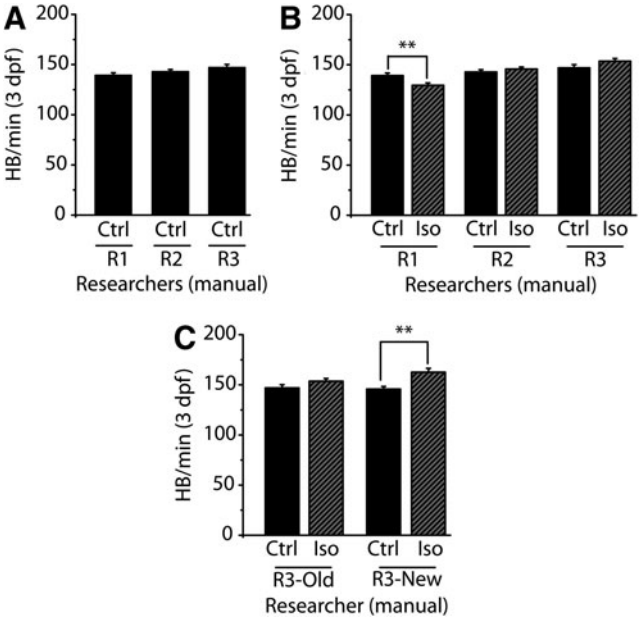

FIG. 4. Researcher variability in manual counting of the heartbeat from the recorded videos in zebrafish larvae at 3 dpf. **(A)** Manual counting of the heartbeat for Ctrl zebrafish larvae by three independent researchers showed variable (but insignificant) differences in HB/min values. **(B)** Manual counting of the heartbeat for Ctrl and ISO-treated zebrafish larvae did not show any increase in the heartbeat when measured by the same three researchers. Researcher 1 reported a significant decrease that contradicts the ISO mode of action. **(C)** Repeat analysis (new analysis) of the same data as in **(B)** (old analysis) by the most experienced researcher 3, showing inconsistent results. The new analysis by manual counting, however, produced a significant difference in the heartbeat. HB/min denotes heartbeat per minute. R1, R2, and R3 denote researchers in which R1 is the most inexperienced researcher whereas R3 is the most experienced researcher. The gap between old analysis and new analysis is ~2–4 weeks. Data represent mean ± SEM of 10 individual zebrafish larvae for each treatment group (paired analysis). $^{**}p \le 0.005$. Ctrl, control; ISO, isoproterenol.

zebrafish larvae (Fig. 4B). On the contrary, one researcher (researcher no. 1, R1) reported a decrease in the heartbeat after ISO treatment. Please note that the data sets analyzed by all three researchers were same. This raised serious concern about the validity of manual counting. Next, we asked whether repeat counting can affect the manual counts; the most experienced researcher (researcher no. 3, R3) was asked to repeat the analysis of the same data set as in Figure 4B, that is, both before and after ISO treatment. This repetition was performed after a gap of ~2–4 weeks. Interestingly, this time the outcome showed a significant increase in the heartbeat values after ISO treatment (Fig. 4C). This clearly showed that manual counting is unreliable and subjective even among experienced researchers and can result in non-reproducible data on repetitive analysis of the same sample by the same researcher.

ZebraPace produced identical results among researchers

The same three researchers (from Fig. 4) were asked to use the ZebraPace method to analyze the same data from Figure 4A.

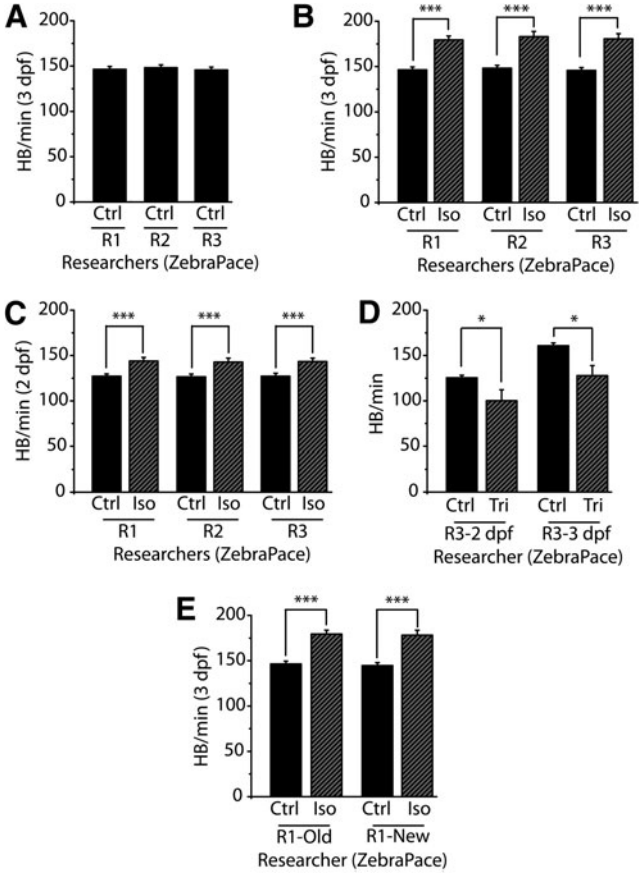

FIG. 5. Heartbeat analysis using the ZebraPace method. **(A)** The heartbeat videos used in Fig. 4A were again analyzed by the same three researchers by using the ZebraPace method. Identical results in heartbeat values of 3 dpf larva were observed for control larvae. **(B)** The heartbeat videos used in Fig. 4B, analyzed by the same three researchers using ZebraPace. All researchers identically reported a significant increase in the heartbeat values for the ISO-treated zebrafish larvae. **(C)** Analysis of 2 dpf zebrafish larva using ZebraPace for before and after treatment with ISO also showed significant changes. Results were consistent among different researchers. **(D)** Analysis of 2 and 3 dpf zebrafish larvae by the researcher 3 (most experienced) using ZebraPace for before and after treatment with Tricaine (Tri, known to reduce heartbeat) produced significant reduction in the treated group. **(E)** Reanalysis of HB/min values using ZebraPace by the researcher 1 (most inexperienced) for the ISO-treated 3 dpf zebrafish larvae produced nearly identical results even after a gap of 1 month. HB/min denotes heartbeat per minute. R1, R2, and R3 denote researchers in which R1 is the most inexperienced researcher whereas R3 is the most experienced researcher. The gap between old analysis and new analysis is ~2–4 weeks. Data represent mean ± SEM of 10 individual zebrafish larvae for each treatment group. $^{*}p \le 0.05$, $^{***}p \le 0.0005$. Tri, tricaine.

Although the data shown in Figure 4A were not significantly different from each other, they had subtle variation in the mean, for example, beats per minute reported by the researchers (R1: 139.2 ± 2.7; R2: 142.8 ± 2.3; R3: 147.0 ± 2.3). Of note, when the same data were analyzed by these researchers using the ZebraPace method, near identical results were obtained, for example, beats per minute obtained by all three researchers (R1: 146.4 ± 3.1; R2: 148.2 ± 3.2; R3: 145.8 ± 3.1, Fig. 5A). This

shows that ZebraPace has successfully eliminated researcher variability, subjectivity and produced more accurate results.

ZebraPace can detect increase or decrease in the heartbeat upon treatment with drugs

Next, we asked whether ZebraPace is sensitive enough to detect changes in the heartbeat on treatment with drugs that are known to modulate heartbeat, for example, ISO to increase the heartbeat. The same data sets from Figure 4B of 3 dpf larvae were analyzed by the same researchers using ZebraPace. This time we observed a significant increase in the heartbeat of zebrafish larvae treated with ISO without any variability among researchers (Fig. 5B). This is in line with the published findings that ISO increases heartbeat in zebrafish embryos.[7]

We have also analyzed the heartbeat of 2 dpf zebrafish larvae by using ZebraPace for before and after treatment with ISO. As shown in Figure 5C, there is a clear increase in the heartbeat on treatment with ISO, with no variability among the researchers. Of note, the heartbeat per minute values of 2 dpf zebrafish larvae obtained from ZebraPace were found to be lower than the 3 dpf (Fig. 5B). These data are also in agreement with the data reported earlier,[6,12,13] further validating our ZebraPace method. We have also validated our method with a drug (e.g., Tricaine) known to reduce the heartbeat.[19,20] The heartbeat from 2 and 3 dpf zebrafish larvae for before and after treatment with 200 mg/L Tricaine was analyzed by the most experienced researcher (researcher 3, R3) using the ZebraPace method.

Figure5D shows a clear decrease in the heartbeat for both age group larvae. These data are consistent with the published findings.[15,21] A decrease in the heartbeat was also observed upon treatment of the larvae with 30 μM Nifedipine (a cardiac drug known to reduce heart rate,[22] Supplementary Fig. S2). Finally, we asked whether the results obtained by the ZebraPace method can be reproduced by the same researcher over time. For this, the most inexperienced researcher (researcher 1, R1) was asked to reanalyze the data of Figure 5B using the ZebraPace method. The results in Figure 5E show that the researcher was able to produce identical results even after a gap of $\sim$4 weeks. This eliminated any effect of researcher subjectivity and variability on the analysis of zebrafish larval heartbeat using the ZebraPace method.

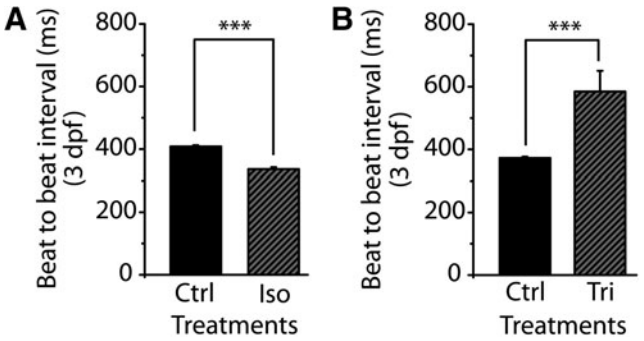

FIG. 6. Beat-to-beat interval from the observed peaks. Beat-to-beat interval was calculated and compared between **(A)** Ctrl and ISO and **(B)** Ctrl and Tri-treated 3 dpf zebrafish larvae. Note that the beat-to-beat interval was significantly reduced on treatment with ISO and significantly increased on treatment with Tri, corroborating with the increased and decreased heart rate respectively. $N=3$ (random). $^{***}p \leq 0.0005$.

Beat-to-beat interval as an index of rhythmicity

As our method precisely labeled the peaks (Fig. 3D), we asked whether the ZebraPace method can be used to find beat-to-beat interval. We determined beat-to-beat interval as the time interval between the peaks because in our method each peak represented a beat. Figure 6A shows the average beat-to-beat interval of three same zebrafish larvae of 3 dpf age group both before and after treatment with ISO. The data were found to be significantly different from each other when tested by using paired analysis. It is as expected because the beat-to-beat interval should reduce on treatment of the larva with ISO, indicating a faster heartbeat (Figs. 5B and 6A).

Next, we also determined the beat-to-beat interval in larvae treated with Tricaine. Because Tricaine reduces heartbeat, we observed a corresponding significant increase in the beat-to-beat interval (Fig. 6B), further validating our method. A potential application of the beat-to-beat interval could be to determine rhythmicity in the heart. A hypothetical example is provided in the Supplementary Methods and Supplementary Figure S3.

Discussion

Cardiovascular studies in zebrafish are of prime importance owing to the similarity of zebrafish heart to human heart.[23] Assessment of heartbeat and rhythmicity is a key parameter of the cardiac function. Historically, heartbeat has been assessed manually[11,12] and it is still being continued.[24] Manual counting of heartbeat is not only time intensive but also suffers from researcher subjectivity, which could lead to inconsistent results (Fig. 4). Often, researchers are not able to reproduce their own data in manual counting (Fig. 4C). Therefore, an automated method of heartbeat detection is required, which will allow gains in time, reproducibility, and objectivity of the results. This will make the data useful for further statistical or modeling studies.

Studies in the past have successfully attempted to automate the method of cardiac rate determination by using complex image processing and power spectral analysis.[6,12,13,25] Majority of the automated methods relied on the blood flow and, therefore, suffer from limitations such as requirement of a specific age group of zebrafish larvae,[13] sensitive to genetic modification affecting blood flow,[7,13] complex spectral analysis sensitive to larval movement,[7] sensitive to movement of erythrocytes in the large blood vessels,[12] or requirement of high-end microscope and speed cameras.[6] Few other methods (automatic and semi-automatic) were also proposed that focused on image processing and attempted to determine several parameters from the same images/videos. Unfortunately, such methods also suffer from the limitation of being too complex for simple tasks such as heartbeat determination, often involved strong programming skills, costly commercial software, larval embedding steps, or mandatory requirement of transgenic fluorescent line.[26,27]

Therefore, we present a significant update over the existing methods based on the semi-automated mode of determination of heartbeat. We named our method as ZebraPace for

Zebrafish Precise Algorithm for Cardiac-rhythm Estimation. ZebraPace offers the following advantages over existing methods: (1) No sample preparation steps such as larval embedding or anesthetization are required, which may affect the heartbeat. (2) ZebraPace is capable of reliably analyzing heartbeat values from as short as 10-s videos. In our experience, we can find a larva immotile during a 10-s interval, eliminating the need of physical confinement. The only requirement is positioning of the larva under the microscope with the lateral side-up configuration (which is relatively easy to perform). (3) ZebraPace does not essentially require fluorescent transgenic lines, meaning it can be readily used for toxicological assessment using both wild-type and any transgenic zebrafish line.

(4) ZebraPace is not dependent on image processing at all; we only processed the heartbeat signals as a change in pixel intensities over time using standard data analysis and graphing software, readily available in almost all scientific labs or even free of cost through Google Docs. (5) Our method works directly on the AVI video files without the requirement of cumbersome image databases. (6) ZebraPace is based on the scientific ImageJ software (NIH) readily available to everyone under an open-source license. As the maintenance and development of ImageJ software is done by a wide scientific community at a global level rather than by an individual lab or a single user, it ensures timely updates and constant enrichment of ImageJ capabilities.

(7) We have also demonstrated that the ZebraPace method does not demand high-resolution data as compared with the method proposed by Pylatiuk *et al.*[12] (8) The ZebraPace method is capable of analyzing cardiac rhythmicity as beat-to-beat interval and, therefore, is much suited for arrhythmia research. (9) As nearly all the methods of heartbeat analysis are based on image processing, therefore, any jerk movement of zebrafish larva within the period of data acquisition could seriously affect the ability of the method to extract useful data. This often demands a higher number of zebrafish larvae, which may pose ethical issues.

Contrary to this, we demonstrate here that our ZebraPace method is robust enough to extract useful data from such videos, thus complying with the principle of 3Rs (Replacement, Reduction, and Refinement) (Supplementary Fig. S1). In this manner, the data are not wasted from precious scarce samples. As shown in Supplementary Figure S1, we observed a wavy profile when the larva showed jerk movement during video recording. However, the ZebraPace method was capable of extracting heartbeat data within an error limit of 1 beat for the stated period. One beat that is missed during the automatic detection can be added manually (if required).

Future Development

Currently, our ZebraPace method offers a powerful yet simple method to detect heartbeat by using open-source ImageJ and standard data analysis software OriginPro or MS Excel or online Google spreadsheet, but it remains to be seen how ZebraPace would perform in high-throughput data analysis. As the method is based on open-source software, we expect further advancement of its capabilities through plugins for the detailed assessments of cardiac function such as ECG waveforms, ejection fraction, stroke volume etc, which will contribute effectively to cardiac function assessment.

Author Contributions

Idea and conceptualization by A.B. and Y.B.; software algorithm development by Y.B.; experiments by H.G., S.N.; data analysis by H.G., S.P., N.P., A.B., and Y.B.; data visualization by A.B. and Y.B.; original draft preparation by A.B., review and editing by all authors; and supervision, project administration, and funding acquisition by A.B. and Y.B.

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Disclosure Statement

The authors declare that they have no competing financial interests.

References

1. Bakkers J. Zebrafish as a model to study cardiac development and human cardiac disease. Cardiovasc Res 2011;91: 279–288.
2. Chico TJ, Ingham PW, Crossman DC. Modeling cardiovascular disease in the zebrafish. Trends Cardiovasc Med 2008;18:150–155.
3. Nguyen CT, Lu Q, Wang Y, Chen JN. Zebrafish as a model for cardiovascular development and disease. Drug Discov Today Dis Models 2008;5:135–140.
4. Sehnert AJ, Stainier DY. A window to the heart: can zebrafish mutants help us understand heart disease in humans? Trends Genet 2002;18:491–494.
5. Dahme T, Katus HA, Rottbauer W. Fishing for the genetic basis of cardiovascular disease. Dis Model Mech 2009;2: 18–22.
6. De Luca E, GM Zaccaria, M Hadhoud, G Rizzo, R Ponzini, U Morbiducci, MM Santoro. ZebraBeat: a flexible platform for the analysis of the cardiac rate in zebrafish embryos. Sci Rep 2014;4:4898.
7. Milan DJ, Peterson TA, Ruskin JN, Peterson RT, MacRae CA. Drugs that induce repolarization abnormalities cause bradycardia in zebrafish. Circulation 2003;107:1355–1358.
8. Hassel D, Dahme T, Erdmann J, Meder B, Huge A, Stoll M, *et al.* Nexilin mutations destabilize cardiac Z-disks and lead to dilated cardiomyopathy. Nat Med 2009;15:1281–1288.
9. Hoffmann S, Berger IM, Glaser A, Bacon C, Li L, Gretz N, *et al.* Islet1 is a direct transcriptional target of the homeodomain transcription factor Shox2 and rescues the Shox2-mediated bradycardia. Basic Res Cardiol 2013;108:339.
10. Meder B, Scholz EP, Hassel D, Wolff C, Just S, Berger IM, *et al.* Reconstitution of defective protein trafficking rescues Long-QT syndrome in zebrafish. Biochem Biophys Res Commun 2011;408:218–224.
11. Paternostro G, Vignola C, Bartsch DU, Omens JH, McCulloch AD, Reed JC. Age-associated cardiac dysfunction in *Drosophila melanogaster*. Circ Res 2001;88: 1053–1058.

12. Pylatiuk C, Sanchez D, Mikut R, Alshut R, Reischl M, Hirth S, *et al.* Automatic zebrafish heartbeat detection and analysis for zebrafish embryos. Zebrafish 2014;11:379–383.
13. Chan PK, Lin CC, Cheng SH. Noninvasive technique for measurement of heartbeat regularity in zebrafish (*Danio rerio*) embryos. BMC Biotechnol 2009;9:11.
14. Simoneschi D, Simoneschi F, Todd NE. Assessment of cardiotoxicity and effects of malathion on the early development of zebrafish (*Danio rerio*) using computer vision for heart rate quantification. Zebrafish 2014;11: 275–280.
15. Muntean BS, Horvat CM, Behler JH, Aboualaiwi WA, Nauli AM, Williams FE, *et al.* A comparative study of embedded and anesthetized zebrafish in vivo on myocardiac calcium oscillation and heart muscle contraction. Front Pharmacol 2010;1:139.
16. Schwerte T, Uberbacher D, Pelster B. Non-invasive imaging of blood cell concentration and blood distribution in zebrafish *Danio rerio* incubated in hypoxic conditions in vivo. J Exp Biol 2003;206:1299–1307.
17. Nema S, Bhargava Y: Open-RAC. Open-design, recirculating and auto-cleaning zebrafish maintenance system. Zebrafish 2017;14:371–378.
18. Nema S, Hasan W, Bhargava A, Bhargava Y. A novel method for automated tracking and quantification of adult zebrafish behaviour during anxiety. J Neurosci Methods 2016;271:65–75.
19. Matthews M, Varga ZM. Anesthesia and euthanasia in zebrafish. ILAR J 2012;53:192–204.
20. Nordgreen J, Tahamtani FM, Janczak AM, Horsberg TE. Behavioural effects of the commonly used fish anaesthetic tricaine methanesulfonate (MS-222) on zebrafish (*Danio rerio*) and its relevance for the acetic acid pain test. PLoS One 2014;9:e92116.
21. Denvir MA, Tucker CS, Mullins JJ. Systolic and diastolic ventricular function in zebrafish embryos: influence of norepenephrine, MS-222 and temperature. BMC Biotechnol 2008;8:21.
22. Langheinrich U, Vacun G, Wagner T. Zebrafish embryos express an orthologue of HERG and are sensitive toward a range of QT-prolonging drugs inducing severe arrhythmia. Toxicol Appl Pharmacol 2003;193:370–382.
23. Nemtsas P, Wettwer E, Christ T, Weidinger G, Ravens U. Adult zebrafish heart as a model for human heart? An electrophysiological study. J Mol Cell Cardiol 2010;48: 161–171.
24. Dambal VY, Selvan KP, Lite C, Barathi S, Santosh W. Developmental toxicity and induction of vitellogenin in embryo-larval stages of zebrafish (*Danio rerio*) exposed to methyl Paraben. Ecotoxicol Environ Saf 2017;141:113–118.
25. Fink M, Callol-Massot C, Chu A, Ruiz-Lozano P, Izpisua Belmonte JC, Giles W, *et al.* A new method for detection and quantification of heartbeat parameters in Drosophila, zebrafish, and embryonic mouse hearts. Biotechniques 2009;46:101–113.
26. Burns CG, Milan DJ, Grande EJ, Rottbauer W, MacRae CA, Fishman MC. High-throughput assay for small molecules that modulate zebrafish embryonic heart rate. Nat Chem Biol 2005;1:263–264.
27. Ocorr K, Fink M, Cammarato A, Bernstein S, Bodmer R. Semi-automated optical heartbeat analysis of small hearts. J Vis Exp 2009;31:pii: 1435.

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