UNEQUIVOCAL CARDIAC PHASE SORTING FROM ALTERNATING RAMP- AND PULSE-ILLUMINATED MICROSCOPY IMAGE SEQUENCES

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ABSTRACT

In vivo microscopy is an important tool to study developing organs such as the heart of the zebrafish embryo but is often limited by slow image frame acquisition speed. While collections of still images of the beating heart at arbitrary phases can be sorted to obtain a virtual heartbeat, the presence of identical heart configurations at two or more heartbeat phases can derail this approach. Here, we propose a dual illumination method to encode movement in alternate frames to disambiguate heartbeat phases in the still frames. We propose to alternately acquire images with a ramp and pulse illumination then sort all successive image pairs based on the ramp-illuminated data but use the pulse-illuminated images for display and analysis. We characterized our method on synthetic data, and show its applicability on experimental data and found that an exposure time of about 7% of the heartbeat or more is necessary to encode the movement reliably in a single heartbeat with a single redundant node. Our method opens the possibility to use sorting algorithms without prior information on the phase, even when the movement presents redundant frames.

Index Terms— Structured illumination, live microscopy, Heartbeat, Periodicity, Asymmetry, Zebrafish

1. INTRODUCTION

Live imaging of organ development is an essential tool to study the beating heart in zebrafish. However, imaging is challenging because the heart is beating starting from early stages of development and requires a fast acquisition frame rate. Fluorescence microscopy is particularly affected as fairly long exposure times are necessary or camera speed and data-transfer bandwidth are limited.

An attractive alternative to increasing the frame rate is to process a series of still images acquired at a slow (compared to the heartbeat) or even irregular frame rate. Several computational solutions to then sort the images into a virtual sequence that depicts the beating heart have been previously proposed [1, 2, 3]. While these methods do not require prior phase information they fail to reconstruct accurate motions when the heart follows a motion, within one heartbeat, that results in two or more identical configurations as multiple equivalent solutions exist (Fig. 1(a)–(d), left column).

While the acquisition cameras are often already pushed to their speed limit, microscopy offers complete control of the illumination, which affords much more flexibility and fewer temporal constraints. Patterned illumination has been employed effectively to imprint a spatial pattern in structured illumination (spatial) super-resolution microscopy[4] or a sub-frame *temporal* pattern to improve the temporal resolution [5] and sampling [6]. Here, we propose to take advantage of an acquisition scheme where we alternate frames in which the heart is illuminated by a single short pulse [7] and frames in which it is illuminated by a temporally asymmetric ramp pattern (Fig. 1(e)). Thereby, heart configurations that appear identical in still frames but differ in their motion can be distinguished based on their differing motion blur in the ramp-illuminated frames (Fig. 1(a)–(d) right column).

In Section 2, we formally describe the proposed image acquisition protocol. In Section 3, we characterize our method in several illumination scenarios using synthetic data and demonstrate the feasibility of our method using an Arduino Uno, an open-source microcontroller board, with our OpenSPIM [8] microscope on fluorescence data of the beating heart. In Section 4, we discuss the performance and limitations of our method and conclude.

2. METHODS

2.1. Imaging model and problem statement

We consider an image series of a sample that moves periodically, with a period T:

$$f(x, y, t) = f(x, y, t + T), \tag{1}$$

where x, y are the sample coordinates and t the time. We further assume that the motion contains a crossing point, as shown in Fig. 1 (a), that is, two times t_a and t_b, $0 \leq t_a < t_b < T$, where the heart takes the same shape:

$$f(x, y, t_a) = f(x, y, t_b). \tag{2}$$

Our goal is to reconstruct an estimate of the discrete image series covering one period:

$$\mathbf{f}[k, \ell, n] = f(k\Delta x, \ell\Delta y, n\Delta t) \tag{3}$$

where Δx and Δy are the pixels width and height, and $(k, \ell) \in \{0, \ldots, K\} \times \{0, \ldots, L\}$ the row and column index pairs, $n \in \{0, \ldots, N-1\}$ the time frame index, and $\Delta t = T/N$ a time interval. As input, the reconstruction takes a series of N image pairs $(\mathbf{g}_\mathrm{p}[:, :, n], \mathbf{g}_\mathrm{r}[:, :, n])$ acquired as:

$$\mathbf{g}_\mathrm{r}[k, \ell, n] = \int_{t_n}^{t_n + a} r(t - t_n) \cdot f(k\Delta x, \ell\Delta y, t)\mathrm{d}t \tag{4}$$

$$\mathbf{g}_\mathrm{p}[k, \ell, n] = \int_{t_n + a}^{t_n + a + \Delta_\mathrm{p}} f(k\Delta x, \ell\Delta y, t)\mathrm{d}t \tag{5}$$

where t_n, $0 \leq n < N$ is the frame acquisition time, occurring at arbitrary phases of the signal and $r(t)$ is an illumination intensity function that follows a ramp pattern composed of N_a discrete steps:

$$r(t) = i_\mathrm{r} \frac{\left\lfloor t \frac{N_a}{a} + 1 \right\rfloor}{N_a} \tag{6}$$

where i_r is the maximal intensity value of the ramp. The $\mathbf{g}_\mathrm{p}[:, :, n]$ correspond to frames acquired with an illumination pulse of duration Δ_p, just following the previous ramp frame. Since the integral of this ramp over the interval of duration a is $\frac{N_a+1}{N_a} a i_\mathrm{r}/2$ and that of the pulse $i_\mathrm{p}\Delta_\mathrm{p}$, we adjust the ramp intensity i_r such as to achieve similar exposure levels in the pulse and ramp frames:

$$i_\mathrm{r} = i_\mathrm{p} \frac{2\Delta_\mathrm{p}}{a} \frac{N_a}{N_a + 1}. \tag{7}$$

2.2. Phase-sorting ramp-, pulse-illuminated image pairs

Since the ramp- and pulse-illuminated image pairs are acquired at nearby times, we can assume that the phase of the ramp-illuminated images are all offset by a constant (equal to a) when compared to the phase of their corresponding pulse-illuminated frame. We therefore propose to apply a phase-sorting method to the ramp-illuminated images $\mathbf{g}[:, :, n]$, $0 \leq n < N$, thereby obtaining a frame permutation $\sigma : \{0, \ldots, N-1\} \to \{0, \ldots, N-1\}$, $n \mapsto \sigma(n)$ (using a traveling salesman method that minimizes the absolute image difference between consecutive frame pairs, which we previously described [1]). Since the ramp-illuminated images contain motion blur, heart configurations that would lead to ambiguous still frames do no longer confuse the method. However, since the blur is detrimental to any further image analysis, we apply the permutation σ to the sharp, pulse-illuminated sequence to obtain the estimated sequence:

$$\tilde{\mathbf{f}}[:, :, n] = \mathbf{g}_\mathrm{pulse}[:, :, \sigma(n)]. \tag{8}$$

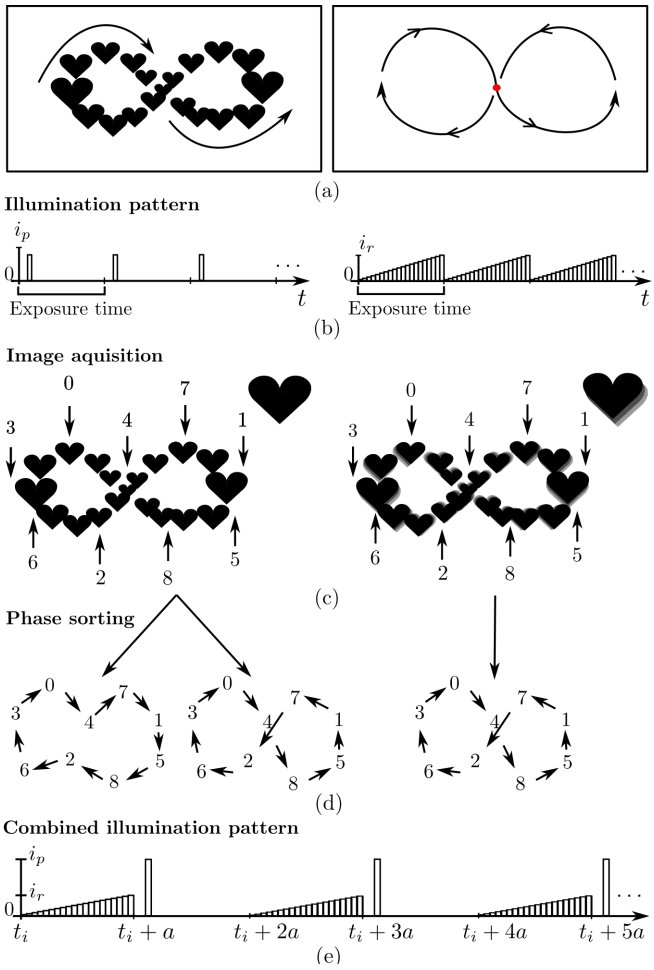

Fig. 1. Structured illumination pattern removes sorting ambiguity. (a) The object movement trajectory is an eight-shaped and periodic. The red dot (on the right) indicates a position appearing twice in the period. (b) Illumination patterns. Left: Pulse illumination, during a fraction of the camera exposure time. Right: Ramp illumination, with increasing intensity during the whole exposure time. (c) Left: The pulse illumination leads to a sharp image. Right: The ramp illumination pattern creates a movement blur in the image. (d) Left: The image sorting process can lead to two different results, as the center of the eight-shaped movement is a point of symmetry. Right: The structured illumination removes the central point ambiguity thanks to the movement blur. (d) We propose an alternating illumination pattern, where the ramp-illuminated images are used to sort the pulse-illuminated images.

3. RESULTS AND DISCUSSION

3.1. Optical flow trajectories of sorted data

To test our proposed acquisition procedure, we simulated synthetic data of a beating heart [9]. We simulated a deformation pattern that roughly resembles a figure-eight deformation pattern, with two phases within one heartbeat in which the heart has the same shape (Fig. 1 (a)) but not the same velocity. We simulated the acquisition of $N = 240$ 256×256-pixel frames, 120 for each illumination method. The period was $T = 6\pi \cdot N_a$ and with the number of ramp steps set to $N_a = 20$ we combined frames such as to end up with a period of $T = 6\pi$ in the final simulated sequence. We further added background intensity and finally considered each pixel to be a realization of a Poisson process.We additionally simulated a ground truth sequence.

We then sorted both the ramp- and pulse-illuminated data. The sorted pulse-illuminated data followed a path different from the ground truth whereas the ramp-illuminated data yielded the correct motion. To better visualize the paths, we tracked keypoints on the image sequences (using the OpenCV implementation of the Lucas-Kanade optical flow method [10] and selected keypoints with the good feature to track method [11]). Fig. 2 shows the trajectories of five points in the ground truth (Fig. 2 (a) and (d)), sorted pulse-illuminated (Fig. 2 (b) and (e)), and ramp-illuminated (Fig. 2 (c) and (f)) sequences. The arrows in Fig. 2 (a), (b), and (c) point towards the point in the data where the trajectories cross. The ground truth (Fig. 2 (a)) and ramp-illuminated (Fig. 2 (c)) data show the correct eight-shaped trajectory. The pulse-illuminated (Fig. 2 (b)) data shows the alternative bow-tie-shaped trajectory. Fig. 2 (d), (e), and (f) shows that the complete trajectories for the ground truth (Fig. 2 (d)), pulse-illuminated (Fig. 2 (e)) and ramp-illuminated (Fig. 2 (f)) data is the same. See also Supplementary Movie [00:00-00:25].

3.2. Ground truth phases of sorted data

In order to characterize the requirements on the ramp length required to disambiguate the signal, we simulated 5 sequences of different ramp duration (exposure time as compared to the duration of one heartbeat) together with the corresponding pulse illumination. We sorted the data and plotted the sorted ground truth phases in Fig. 3. To make the data more readable, we shifted the phases for each sequence such that the minimal phase is at frame $n = 0$. The phases should be in increasing order. Fig. 3 (a) shows the sorted phases of the pulse-illuminated data. Discontinuities in phases (marked by circles) occur at the repeat motion crossing points and show that the sequences are sorted incorrectly. Fig. 3 (b) shows the sorted phases of the ramp-illuminated data. An exposure time $a < 0.066\,T$ leads to errors in sorting (circles), showing that there was not enough shaded blur to encode direction.

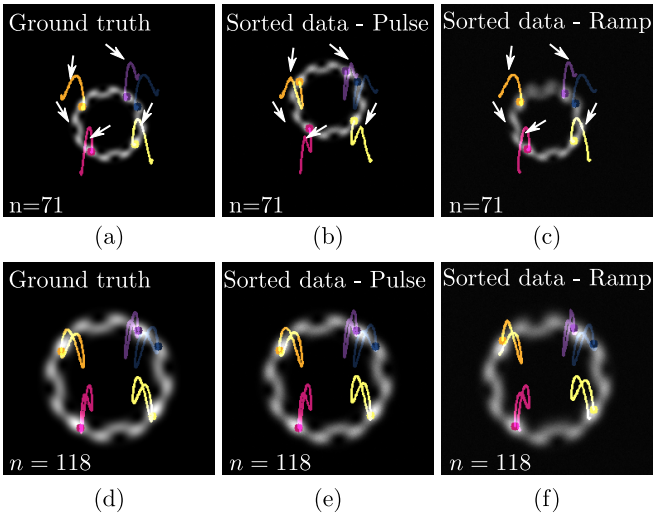

Fig. 2. Simulation of a periodic signal containing a symmetry node. The acquisition time was 9.3% of the signal period. The colored lines are the trajectories of keypoints, forming an eight-shaped trajectory with a symmetry points shown by the arrows. (a)–(c) Frame $n = 71$. (a) Ground truth data, with the expected trajectories. (b) Sorted data of the simulated acquisition with a short pulse. The correct sorting path was missed at the symmetry point. (c) Sorted data of the simulated acquisition with a ramp illumination. The sorting was successful, as the ramp eliminated the ambiguity at the symmetry node. (d)–(f) Last frame $n = 118$. The complete trajectories shown in (d)–(f) all have the same final shape, showing that the error is indeed only located at the symmetry node. See Supplementary Movie [00:00-00:25]

3.3. Application on experimental data

We finally tested whether our method could be implemented in an experimental setting without adversely affecting the image quality. We bred transgenic zebrafish Tg(actb2:LIFEACT-RFP) [12], which express red fluorescent proteins that bind to F-actin fibers. We collected embryos that we grew in E3 medium with added PTU (0.003% 1-phenyl-2-thiourea) at age 24 hours post fertilization (hpf) to avoid pigmentation. At 48 hpf, we anesthetized the hatched embryos with Tricaine at 0.08 mg/ml, pH 7. We embedded the embryos with the anterior side (head) down in a fluorinated ethylene propylene tube in 1% low melting agarose (Promega). We imaged the heart on a OpenSPIM [8] microscope with an UMPLFLN 20XW semi-apochromat water dipping objective lens. We imaged the heart across the ventro-dorsal plane.

We used an Arduino Uno to control the laser illumination pattern and MicroManager [13] to control the camera of our OpenSPIM [8] as well as the image acquisition itself. We set the exposure time to $a = 70$ms for the ramp with $N_a = 8$ steps, and the pulse exposure time to $t_p = 4$ms. We added a delay of 10ms between the image pairs due to hardware

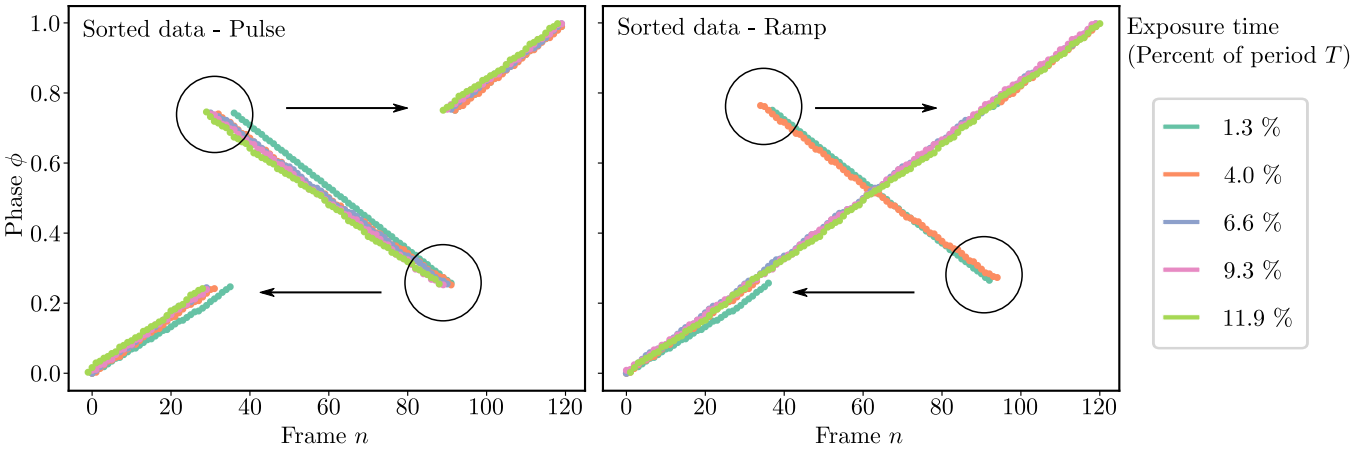

Fig. 3. Characterization of the acquisition time a required to disambiguate the signal in percent of period T, shown as numbers in the graph legends. (a) Phases of the pulse acquisition sorted according to the sorting results of the pulse acquisition simulation. The sorting was not successful at the symmetry point (circles), as can be seen by the discontinuity and change in direction of the phase. The arrows point toward the correct phase position. (b) Phases of the ramp acquisition sorted according to the sorting results of the ramp acquisition simulation. The sorting was successful when the ramp acquisition time a was longer or equal to 6.6% of the signal period and unsuccessful for shorter ramps as can be seen by the discontinuity in the phases (circles).

limitations. We sorted the ramp-illuminated data and applied the solution to the pulse-illuminated data. Fig. 4 shows the acquisition of a beating zebrafish embryo heart with our alternating illumination. Fig. 4 (a) shows an image acquired with the ramp illumination, and Fig. 4 (b) shows the image acquired with the pulse illumination. The arrows point toward single cells that can be seen in the pulse-illuminated image but cannot be seen in the ramp-illuminated image due to motion blur. The acquired data can be seen in Supplementary Movie [00:26-00:58], and the sorted data can be seen in Supplementary Movie [00:59-01:12].

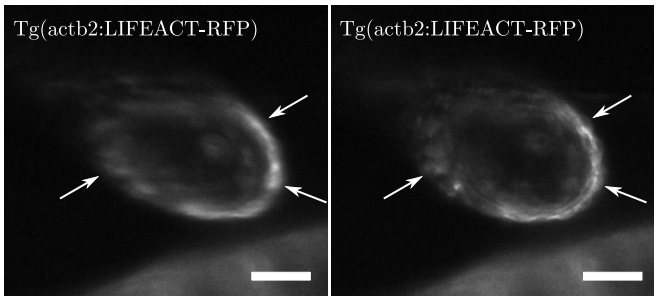

Fig. 4. Beating zebrafish heart acquired using alternating structured illumination. (a) Image acquired with a 70ms ramp illumination. (b) Image acquired with a 4ms pulse. The arrows point toward single cells that are visible in the pulse-illuminated data, but cannot be seen in the blurred ramp-illuminated data. Scalebar is 30μm. See Supplementary Movie [00:26-00:58] and [00:59-01:12]

4. CONCLUSION

Our method allows sorting still images of the beating heart even when it contains repeat shape configuration, without prior phase information, simply by encoding the movement in every other image with shaded motion blur through ramp illumination. We showed that the ramp illumination duration should be around 7% or longer than the signal period. We showed its applicability by implementing the proposed structured illumination patterns on the Arduino Uno open source microcontroller and showed the shaded motion blur on alternating images of the acquired data. Our method requires the implementation of a custom illumination pattern and the synchronization with a camera.

5. COMPLIANCE WITH ETHICAL STANDARDS

Zebrafish breeding and procedures were carried out within the scope of an institutional protocol approved by the Veterinary Office of the Canton of Bern, Switzerland.

6. ACKNOWLEDGMENTS

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