Separation and Pseudo-Coloring of High-Speed Bright-Field Microscopy Images of the Beating Embryonic Heart

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***Abstract*—High-speed, bright-field (BF) microscopy of the beating embryonic zebrafish heart reveals both static background structures as well as the rapid motion of cardiac tissues and red blood cells (RBCs). However, all structures contribute to BF image contrast in a similar way, making labeling and subsequent analysis of these images difficult. Here, we report on our progress to separate cardiac BF images into three separate channels, based on the periodic, aperiodic, or static motion patterns respective to each of the cardiac structures. Specifically, we improve on a previously developed algorithm that was limited to two-class separation only. We show that the three extracted channels can be recombined into a pseudo-colored sequence in which RBCs as well as heart wall and background structures appear in different colors. We anticipate that these improvements will facilitate quantitative characterization of heart function during normal and abnormal cardiac development.**

***Index Terms*—Bright-field microscopy, fluorescence microscopy, multi-channel imaging, source separation, visualization**

I. Introduction

Fluorescence microscopy and bright-field (BF) microscopy are two commonly used methods for imaging biological samples. Fluorescence microscopy, which requires labeling of the sample with fluorescent molecules, allows to selectively image only the cells, tissue types, or organs that contain fluorescent molecules without contributions from neighboring, unlabeled regions. However, the technique suffers from several drawbacks including the additional step required for labeling the samples and the photo-toxicity induced by the intense light fluorescence excitation requires. The most problematic aspect is that fluorescence emission rates are low, requiring sensitive (and expensive) cameras. Even then, typical frame-rates are low, to ensure images have an acceptable signal-to-noise ratio. In bright-field microscopy, contrast is proportional to light absorption by the sample. Images with strong contrast and high signal-to-noise ratio can be acquired at high speeds. However, BF microscopy does not provide the breadth of possibilities for selectively labeling cells, tissues, or organs that fluorescence microscopy does.

Imaging heart development in embryos requires working at high magnification and high frame-rates. At early stages of development, the heart can be as small as a few hundred microns. At the required magnification, even small motions can induce motion blur if the image integration time is too long. The embryonic heart is a highly dynamic organ and this makes cardiac imaging, visualization, and subsequent analysis particularly challenging. The heart starts off as a tube-like structure and morphs into a complex multi-chambered organ during the growth of the embryo. Even at the tube stage of development, the heart is already beating and pumping blood to the embryo. Biologists are in need of tools for quantification of blood flow and tissue deformation that would in turn aid in understanding the influence of genetic and epigenetic factors on cardiac morphogenesis [1]. While techniques have been developed to fluorescently label different parts of the embryonic heart, fluorescence microscopy is too slow to capture the fastest motions of blood cells or heart tissue [2]. In contrast, brightfield microscopy, with frame-rates several orders of magnitude faster than fluorescence microscopy, would be an ideal modality provided red blood cells (RBCs) and heart tissue could be distinguished from the surrounding tissues.

Here, we propose a technique to address the problem of distinguishing several overlapping structures in cardiac BF images, which is an important step for analyzing cardiac morphogenesis and function. The intensity contributions to the original image from the RBCs, heart, and surrounding tissues need to be effectively separated before the images are analyzed, in order to prevent quantification errors due to cross-talk between the structures. In this work we present a technique that separates the input cardiac sequence into its constituent structure sequences based purely on their motion characteristics. The separated sequences can then be visualized and analyzed without cross-talk between the overlapping structures.

We demonstrate our technique on images acquired in zebrafish (*Danio rerio*) embryos, a popular model organisms for studying cardiac morphogenesis. Early stages of heart development are similar in all vertebrates, yet zebrafish offer several advantages, including: (i) their embryos are transparent, which makes them suitable for non-invasive, in-vivo imaging of the heart, (ii) the heart develops rapidly in about 5 days. Microscopic cardiac imaging of zebrafish requires very high frame-rates to capture all the dynamics of the underlying structures without any motion artifacts like blurring [2].

Several BF image segmentation techniques have been proposed to classify each pixel in an image as belonging to

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one class or another [3]–[5] but they are not applicable for separating the individual contributions from overlapping cardiac structures in our problem. Source separation techniques usually require making assumptions on the structure, shape and smoothness of the sources to be separated or need prior statistical information about the constituent sources [6], [7]. These techniques cannot be applied directly to cardiac BF imaging since the structure, shape, and smoothness of the structures are highly variable and prior statistical information on intensities is not readily available. Techniques developed for segmentation of MRI [8]–[10] and ultrasound [11], [12] images cannot be used directly for our problem since these macroscopic modalities only resolve the heart (and surrounding tissues) but cannot resolve individual cells in the blood stream.

In this paper we build on our previously developed BF image separation algorithm [13] and discuss a method for separating contributions from the different cardiac structures in bright-field (BF) images, based solely on their motion. The separation is based on the fact that periodic permanent structures return approximately to the same spatial position in every cardiac cycle while transient structures do not. Here, permanent structures are further separated into static structures and periodically moving structures. These separated structures are pseudo-colored and combined into a single composite image for visualization and analysis of the cardiac sequence. In addition, we investigate the quality of the separation on synthetic data with various degrees of overlap between the structures that contribute to image contrast.

This paper is organized as follows. Section II develops the proposed algorithm for separation and highlights the progress made on our previous work [13], [14]. Section III characterizes the separation effectiveness using synthetic data and presents multi-colored visualization results for 2D BF data acquired on a beating embryonic zebrafish heart. Finally, Section IV summarizes the the main features of our separation algorithm and discusses possible applications.

II. The Separation Algorithm

Our imaging and separation algorithm, which extends methods for separation and noise reduction previously presented in [13], [14], operates as follows. We first acquire high-speed BF microscopy images of the embryonic zebrafish heart over several heartbeat periods (see Fig. 2(a)). This image series is then cut into several, shorter series, each covering at least two heartbeat cycles. These shorter image sequences are then temporally registered. Periodically moving structures are thereafter in synchrony in each shorter sequence, while aperiodic features (such as noise or RBCs) are not. At each pixel, a sample median of the corresponding pixels in all synchronized series provides an estimate of the permanent, periodically moving structures. Subtracting this series from the original series results in revealing transient, aperiodically moving structures, such as RBCs (Fig. 2(c)). In this paper, we further propose to separate the permanent structures into period structures (Fig. 2(b)) and static structures (Fig. 2(d)). The latter are obtained by averaging out the images over one full period. Our approach is detailed below.

As done in our previous work [13], we consider that the measured image intensity $I_{\mathrm{m}}(\mathbf{x},t)$ can be modeled as

$$I_{\mathrm{m}}(\mathbf{x},t) = I_{\mathrm{p}}(\mathbf{x},t) + I_{\mathrm{t}}(\mathbf{x},t), \tag{1}$$

with $I_{\mathrm{t}}(\mathbf{x},t)$ the contribution from the transient structures (such as RBCs) and $I_{\mathrm{p}}(\mathbf{x},t)$ the contribution from the permanent structures. In this paper, we further separate these permanent structures as

$$I_{\mathrm{p}}(\mathbf{x},t) = I_{\tilde{p}}(\mathbf{x},t) + I_{\mathrm{s}}(\mathbf{x}), \tag{2}$$

with $I_{\tilde{p}}(\mathbf{x},t)$ the contribution from periodically moving structures (fundamental harmonic and higher; such as heart wall tissue) and $I_{\mathrm{s}}(\mathbf{x})$ the contribution of static structures (background). We assume that the permanent structures contribute to the image contrast in a periodic fashion, that is,

$$I_{\mathrm{p}}(\mathbf{x},t) \approx I_{\mathrm{p}}(\mathbf{x},t+T), \quad \forall t \tag{3}$$

where T is the period of the heart beat.

Given this image model and an estimate of the cardiac period T, we first cut the input image sequence into a set of sequences where each cutout sequence is referred to as a repeat, indexed by r. Following [14], we then temporally register these repeats. This results in R sequences

$$I_{\mathrm{m}}^{r}(\mathbf{x},t), \quad r = 0,\ldots,R-1. \tag{4}$$

Once the registration is complete, the signal contribution from permanent (static and periodically moving) structures occurs at the same space-time position in every aligned repeat. Therefore corresponding pixels in each of the R repeats represent multiple measurements of the same spatial configuration, with an additive contribution from the transient structures. An estimate of the intensity contributions from permanent structures, $\hat{I}_{\mathrm{p}}(\mathbf{x},t)$, can be obtained by computing the sample median along the R repeats

$$\hat{I}_{\mathrm{p}}(\mathbf{x},t) = \mathrm{Median}\left(\{I_{\mathrm{m}}^{r}(\mathbf{x},t)\}_{0\le r<R}\right),\ 0 \le t < T. \tag{5}$$

The estimated permanent structures, $\hat{I}_{\mathrm{p}}(\mathbf{x},t)$, can then be subtracted from the first cardiac period in the input to get an estimate of intensity contributions from transient structures,

$$\hat{I}_{\mathrm{t}}(\mathbf{x},t) = I_{\mathrm{m}}^{0}(\mathbf{x},t) - \hat{I}_{\mathrm{p}}(\mathbf{x},t),\ 0 \le t < T. \tag{6}$$

We further separate the permanent structures as follows. The static background structures $I_{\mathrm{s}}(\mathbf{x})$ can be estimated from the recovered $\hat{I}_{\mathrm{p}}(\mathbf{x},t)$ as

$$\hat{I}_{\mathrm{s}}(\mathbf{x}) = \frac{1}{T}\int_{0}^{T} \hat{I}_{\mathrm{p}}(\mathbf{x},t)\mathrm{d}t. \tag{7}$$

This term can then be subtracted from the permanent structures to estimate the intensity contributions from the non-static (periodic) structures as

$$\hat{I}_{\tilde{p}}(\mathbf{x},t) = \hat{I}_{\mathrm{p}}(\mathbf{x},t) - \hat{I}_{\mathrm{s}}(\mathbf{x}) \tag{8}$$

The separated static structure sequence $\hat{I}_{\mathrm{s}}$, periodically

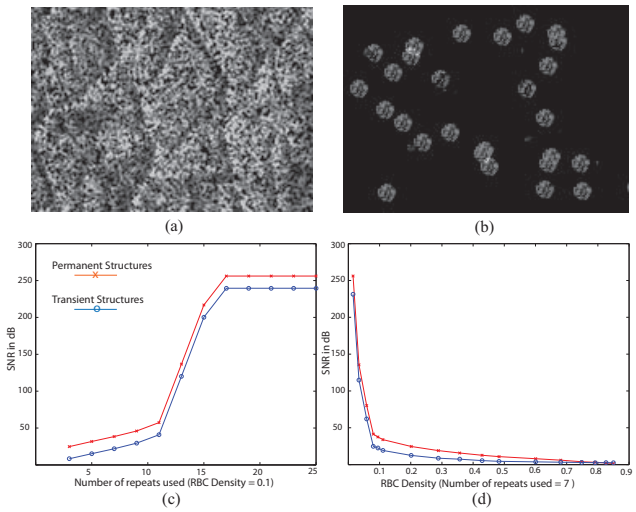

Fig. 1. Separation improves as a result of increasing the number of imaged cardiac cycles or decreasing the spatial density of aperiodically moving features. (a) Frame from the separated permanent structure sequence. (b) Frame from the separated transient structure sequence with RBC density of 12%. (c) SNR of recovered components increases as the number of repeats used increases (fixed RBC density of 10%). (d) The SNR or recovered components decreases as the RBC density increases (images recovered from 7 heart beat cycles).

moving structure sequence $\hat{I}_{\tilde{p}}$ and transient structure sequence $\hat{I}_{\mathrm{t}}$ are combined into the different channels of a single multi-channel image as

$$I^c_{\mathrm{vis}}(\mathbf{x},t) = \begin{cases} \hat{I}_{\mathrm{s}}(\mathbf{x}), & c=0 \\ \hat{I}_{\tilde{p}}(\mathbf{x},t), & c=1 \\ \hat{I}_{\mathrm{t}}(\mathbf{x},t), & c=2. \end{cases} \tag{9}$$

The different channels of I_{vis} can be pseudo-colored and visualized using appropriate software [15].

III. Experiments and Results

We first characterized the separation effectiveness of our algorithm on a synthetic heartbeat data set. The procedure was then applied to 2D+time BF microscopy data of a beating embryonic zebrafish heart.

In [13] we have described a technique for generation of synthetic heartbeat data in which the permanent structures move periodically in a linear fashion and transient structures overlap the permanent structures and move at a constant velocity with various densities of the transient features. This synthetic heartbeat data was separated using our technique and the results (Fig. 1(a), (b)) were used to characterize the separation effectiveness of the algorithm as a function of two quantities: (i) the RBC density (ratio of the number of pixels that belongs to RBC particles to the total number of pixels in the synthetic structure sequence) (ii) the number of repeats R over which the sample median is computed for separation of permanent structures. The separation effectiveness was quantified using the SNR metric, which compares the estimated image with the ground truth [13].

In Fig. 1 (c), we see that for a fixed RBC density, the recovered periodic and transient structures are more accurate (as reported by the increase in the SNR value) as we increase the number of repeats used for the sample median. For a fixed number of repeats, the estimated images are less accurate as the RBC density increases (Fig. 1 (d)).

We next proceed to apply our technique on an experimental image sequence. The 2D+time BF microscopy dataset is comprised of several periods of a 100 hours post fertilization (hpf) beating embryonic zebrafish heart acquired with a Photron SA3 camera running in area-of-interest mode (resolution of 256×256 pixels) for fast acquisition (1000 frames per second). The camera was mounted on a Leica DMI 6000B microscope equipped with a $40\times/0.60$ objective. We show one representative frame of the raw dataset and the corresponding frames of the periodic, transient, and static structures in Fig. 2 (a), (b), (c), and (d), respectively. Additionally, we show pseudo-colored images of the RBC and heart wall (Fig. 2 (e)) and the RBC and permanent structures (Fig. 2 (f)). As seen from the results, our algorithm produces a multi-channel visualization of the beating embryonic heart with a higher degree of specificity than input BF image sequence.

IV. Conclusion

In this paper we have proposed a three-channel separation and pseudo-coloring algorithm to effectively separate the contributions from the different cardiac structures based solely on their periodic, aperiodic, or static motion characteristics. We have characterized our algorithm's separation effectiveness as a function of RBC density and changing number of repeats and illustrated the applicability in practice on a BF microscopy dataset. We foresee our technique to be particularly relevant for separate analysis of the motion of the separated features and aid in the study of interactions between tissue development and blood flow, thereby helping to reach a better understanding of cardiac morphogenesis.

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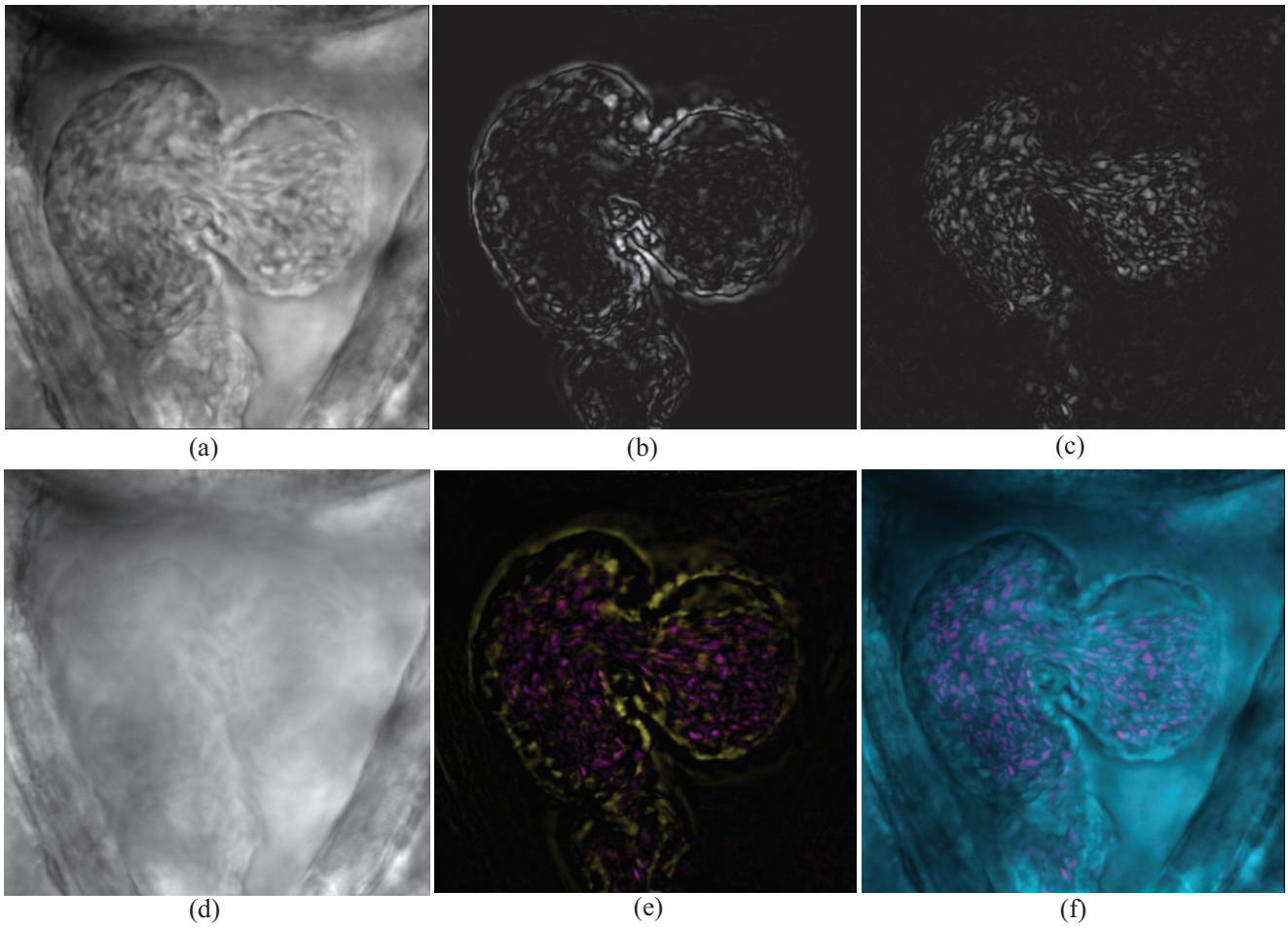

Fig. 2. Multi-colored visualization of the embryonic zebrafish heart with greater degree of specificity than input bright-field data. (a) Raw bright-field microscopy image showing poor contrast between heart-wall, RBCs and static background structures. (b) static structures, (c) periodic structures, (d) transient structures, (e) Frame showing RBCs in pink and the heart-wall in yellow. Note that individual blood cells are visible in pink. (f) Frame showing RBCs in pink and permanent structures in blue.

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