MULTISCALE, MULTIMODAL, AND MULTIDIMENSIONAL MICROSCOPY OF CARDIAC DEVELOPMENT

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ABSTRACT

In vivo fluorescence imaging of the embryonic zebrafish heart as it develops and gains function has recently become possible thanks to several breakthroughs in fast microscopy. However, because of the motion of the fast beating heart, volumetric, long term, continuous, and simultaneous characterization of subtle changes in heart morphology at the organ, tissue and cellular level, changes in heart function, or changes in local gene expression all remain major challenges. We have developed tools that aim at addressing the problem of capturing and integrating multi-modal data at different temporal and spatial scales to build a multi-dimensional model of the beating and developing heart. This paper gives an overview of techniques we developed and integrated to follow heart development, spatiotemporally confined hemodynamics, and gene expression. These tools permit quantitative characterization and will allow studying the interactions between genetic and epigenetic factors that affect cardiac development.

Index Terms— Microscopy, registration, optical flow, wavelets, zebrafish

1. INTRODUCTION

The heart is the first functioning organ; in the human, for example, it starts beating three weeks post-fertilization. At this stage it has the shape of a simple tube. As the heart continues to develop, it forms chambers, valves, and vasculature. Most interestingly, the heart is functional and beating throughout the development to ensure blood circulates throughout the developing organism. The presence of fluid flows was shown to be an essential factor for normal heart formation; In zebrafish embryos, Hove *et al.*[1] have shown that preventing flow from circulating through the developing heart by blocking its inflow or outflow tracts with a bead leads to severe malformations. Valves are missing or malformed or the heart does not undergo looping (a morphogenetic process by which the heart goes from a simple tube to a multi-chambered organ). As suggested by studies carried out in vitro on endothelial cells (the cell type that lines, in particular, the inside of vessels and the heart) that were grown in a flow chamber, thereby exposing them to either static or laminar shear stress generated by a flow, showed dramatic changes in cell shape in response to flow. These changes remained after flow was removed [2]. Further experiments, still on cell cultures, have identified a wealth of genes that are either up-regulated or down-regulated in response to applying flow-induced shear-stress on cells, suggesting that flow is an important epigenetic factor for gene regulation [1] [3]. More recently, a study our lab participated in revealed that the oscillatory nature of the flow inside the heart and, in particular, in a region between the atrium and ventricle where flow velocity is particularly high due to a decrease in the diameter of the heart tube, locally activates a series of genes, thereby demonstrating, in vivo, that gene expression can indeed be regulated by flow patterns.

We have undertaken an effort to develop, gather, and integrate a series of tools to further study the interplay between flow, tissue dynamics, cell and organ morphology, and gene expression. We believe that a systematic understanding of heart development will rely on the abil-

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[1] Even though all cells contain the same genetic material, not all genes are expressed at the same level in all cells and at all times, depending on cell type and environmental conditions.

ity to dynamically measure, characterize, and link cardiac *morphology*, *gene-expression*, and *function*, in vivo, and in normal or perturbed conditions (that is, when flow is altered, specific gene expression is suppressed or enhanced, or the sample subjected to pharmacological agents).

Specifically, our efforts have been concentrated on three axes:

1. acquiring multi-dimensional data at high frame-rate
2. merging and segmenting multi-modal data
3. multi-scale imaging to capture morphogenesis and cardiac function.

In the following sections, we give an overview of our work after a brief introduction to the challenges typically encountered in cardiac and fluorescence microscopy.

2. CHALLENGES IN FLUORESCENCE AND CARDIAC IMAGING

We have chosen zebrafish as our model for cardiac development as it offers many advantages over other systems; zebrafish reproduce externally and rapidly, which ensures embryos are easily collected and available in great number, their embryos are relatively transparent, with the developing heart optically accessible, and multiple transgenic fish have been engineered that express fluorescent proteins in specific tissues, reporting the local expression of the genes they are associated with [4]. In transgenic animals, fluorescent proteins are expressed whenever a specific gene is. Imaging fluorescent proteins can therefore reveal the location and time at which genes are expressed, in which tissue they are expressed (e.g. expression can be limited to endothelial cells [5], cadiac muscle cells [6], or can be specifc to entire organs or parts thereof [4]).

While the availability of fluorescently labeled animal models has opened new avenues for imaging, multiple challenges remain. First, fluorescence imaging requires exciting fluorescent molecules using bright illumination, a process that can be toxic to cells. Second, fluorescent molecules have a limited lifetime before they *bleach* and after which they do no longer contribute to the signal. In practice, illumination power is therefore limited as much as possible and only scarce amounts of fluorescence photons are collected with sensitive detectors. Frame-rates for state-of-the-art microscopes and fluorescence cameras range from 1 to 120 512×512-frames per second [7, 8, 9, 10]. Acquiring the three-dimensional distribution of fluorophores in a sample is possible when using confocal or multi-photon microscopes or deconvolution microscopy. However, acquiring volumetric data further reduces already limited frame-rates by one to three orders of magnitude, depending on the number of planes in the third dimension and the speed at which scanning the axial position is possible.

3. MULTI-DIMENSIONAL IMAGING

Imaging at sufficiently high frame-rate is essential for imaging dynamic processes. Motions that appear slow at macroscopic scales require fast imaging capabilities as the magnification and, more importantly, the required resolving power, increase [9]. With velocities that can reach millimeters per second and a required resolution that should be 10μm or better, the developing heart requires imaging upwards from 50 frames per second, something that can barely be achieved for 2D-only imaging. Since cardiac motion is largely periodic, it is possible, however, to sequentially acquire image bursts of at least one heartbeat in length at fixed axial positions throughout the heart before recombining them following synchronization [11, 8]. This procedure results in dynamic 3D images of the beating heart.

Such volumetric measurements can be repeated throughout the course of development of the zebrafish heart. The major steps of development occur over less than a week as the zebrafish heart starts beating about one day post fertilization (when its shape is, like the human heart, tube-like) and has fully formed chambers and valves by five days post fertilization. Using our dynamic 3D imaging technique, changes both in heart morphology and function are made apparent as the heart undergoes development [8].

Besides the limitation in data-transfer speed, the achievable frame-rate is intrinsically limited by the number of photons emitted by the sample in any given interval of time. As a consequence, increasing the frame-rate in a photon-limited situation increases imaging noise. Specifically, assuming a Poisson-statistic the relative noise decreases in $\mathcal{O}\left(N^{-1/2}\right)$, where N is the average number of emitted photons. To overcome this limitation,

and, again, taking advantage of the periodic motion of the heart, we have developed a method to increase the effective integration time by compounding images acquired over multiple periods of the beating heart [12]. Since the heartbeat can vary slightly, sequences from multiple heart beats are elastically registered to compensate for slight variations in the temporal beat pattern. This technique has also proven effective for image modalities other than standard microscopy, in particular for optical coherence tomography [13].

4. MULTI-MODAL IMAGING

Imaging red blood cells transported in the heart and, in particular, tracking them to measure their velocity and eventually estimate fluid velocity and the mechanical forces exerted on the heart wall requires frame-rates much higher than the ones that can be achieved with fluorescence microscopy alone. We therefore use a microscope capable of measuring both fluorescence at the highest possible frame-rate but also take advantage of bright-field microscopy, which, even though it lacks the specificity of fluorescence imaging allows imaging at thousands of frames per second using white light transmitted by the sample. Since acquisition of the brightfield and fluorescence channels is not necessarily carried out simultaneously, we have extended our synchronization algorithms to handle multi-modal data [14]. This allows taking advantage of the specificity of fluorescence imaging (e.g. for segmentation and gene localization purposes) while bright-field fluorescence allows capturing fast processes and estimating the blood flow velocities [15]. In the absence of a fluorescence channel, we can take advantage of the nature of cardiac motion to segment the blood (whose motion is not repetitive) from the cardiac tissue (whose motion is), and thereby recover some of the specificity bright-field microscopy usually lacks[16].

5. MULTI-SCALE IMAGING

Even though heart function can only be captured at the highest frame-rates, changes in tissue morphology occur on time scales several orders of magnitude slower. For organs other than the heart, snapping an image every few minutes is sufficient to capture tissue rearrangement at the cellular level, including how cells migrate or divide. This approach to time-lapse imaging yields poor results when applied in the heart since the phase in the cardiac cycle at which each image is acquired cannot be controlled. Slowing the heart has been explored as a workaround [17] but the use of anesthetics is cumbersome and the ensuing disruption of the blood flow may likely affect normal heart development. Furthermore, acquiring only a single image does not allow evaluating heart function and probe flow. To overcome these limitations, we developed a time-lapse imaging technique that produces development time-lapses of the heart in fixed contraction states and that cover several tens of hours, revealing the dynamics of cellular rearrangement and even cell divisions. The technique proceeds by acquiring bursts of images (covering at least one full heart beat, typically at 30-500 frames per second) at every step in the development (typically every few minutes over several hours or days). Next, the image series are synchronized. Then, extracting the k-th frame from each fast image series and playing it as a movie yields a time-lapse at a fixed contraction state of the heart [15].

6. SUMMARY

Imaging morphogenesis, function and gene expression in highly dynamic organs poses multiple challenges since the requirement for shortening acquisition times to avoid motion blur must be balanced with the necessity to gather enough photons to maintain acceptable signal-to-noise. Fluorescence imaging techniques are particularly affected by this compromise. Taking advantage of the repetitive motion of the heart allows imaging the heart in multiple dimensions, with multiple modalities and at multiple scales. These techniques constitute a step towards reaching a better understanding of heart development and the complex interplay between genetic and epi-genetic factors.

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