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Heart Synchronization for SPIM Microscopy of Living Zebra Fish

J. M. Taylor[a], C.D. Saunter[a], B. Chaudhry[b], D.J. Henderson[b], G. D. Love[a], J. M. Girkin[a]
[a]Centre for Advanced Instrumentation, Department of Physics, Durham University, South Road, Durham, DH1 3LE, UK
[b]Institute of Human Genetics, Newcastle University, NE1 3BZ, UK

ABSTRACT

We describe work on producing a selective plane illumination microscope for cardiac imaging in zebra fish embryos. The system has a novel synchronization system for imaging oscillating structures (e.g. the heart) and will have adaptive optics for image optimization.

Keywords: Optical Microscopy, Selective Plane Illumination, Synchronization

1. Introduction

The requirement to image biological samples has always played a significant role in the development of optics both at a practical and theoretical level. In the past ten years there has been an explosive growth in new optical methods in microscopy linked closely to the development of methods of genetic manipulation, specifically the incorporation of fluorescent proteins to enable precise visualization of specific biological events. A particular focus of these optical methods has been the desire for *in vivo* methods in a range of samples from bacteria to rats. An area of specific interest is the use of zebra fish, and their use for studying embryo development [1]. At first inspection the idea that a fish may be a good model to study and to understand human physiology may seem strange but it has now been demonstrated that for many areas of research the zebra fish provides a very suitable model. They are comparatively easy to generically manipulate and they mature quickly (they can provide breeding stock in around 3 months). Crucially, in terms of optical microscopy, they are basically transparent, in particular at the embryonic stage of development. It is even possible to generate strains of fish with no pigment, or to inhibit the development of pigmentation through the addition of chemicals to the water.

Their use in cardiac studies would appear to be less obvious as they only have a very simple two chamber heart compared to the apparently more complex four chambered heart of mammals, and, in particular, the human heart. However, the zebra fish provides an excellent model for many aspects of the human heart and especially for the study of early stage development, when many genetic malformations take place in humans. At this stage of development it is also possible to cause controlled local damage to the tissue to observe the recovery of injured tissue.

The original imaging of developing zebra fish was undertaken using conventional wide-field and confocal microscopy, which proved to be very successful. However, in the case of wide-field methods, although real time imaging was possible, the inability to observe three dimensional structure, through optical sectioning was a limitation. The inverse was the case in confocal and non-linear microscopy (multiphoton, second and third harmonic, CARS) where the optical sectioning capability was limited by the speed possible even using spinning disc systems. Thus a novel method was first reported in 2001 [2] with various improvements since [3]. In so called Selective Plane Illumination Microscopy (SPIM), a single sheet of light is used to illuminate the sample and the observations are made normal to this illumination plane. It should be noted at this point that the sample can be kept alive, and embryos develop normally by placing the sample in a transparent medium such as agar, which permits oxygen to permeate whilst restraining the lightly anesthetized fish, so the instrument does not needs to follow a rapidly moving fish through water! One can thus image at a relatively high speed, determined by the light level and sensitivity of the camera, and build up optical sections by moving the illumination beam through the samples, in synchronism with the objective lens, or more normally by moving the sample

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through a fixed optical system. In this way embryo development, in particular of the nervous system, has been followed with high precision for several days [4].

Thus on first inspection zebra fish would appear to be ideal candidates for imaging applications, however, there are complications in particular when moving organs are the biological tissue of interest. In particular the heart which in a normal developing embryo beats at up to 180 beats a minute. Methods are available to slow the heart down through either temperature control or pharmaceutical agents, but these also alter the development of the heart and also its exact movement pattern if used for extended periods of time. This would appear to limit the use of the model for long term *in vivo* heart development studies as the heart would be moving around the illumination plane at high speed making reliable imaging impossible. A further limitation for long term imaging is the fact that even green fluorescent protein will photobleach if illuminated for extended periods of time in live samples. Thus the idea of illuminating the sample whilst continuously recording video images and selecting the frames of interest has considerable disadvantages.

The solution to both of these limitations is the concept of synchronized imaging. Here the heart can be monitored and the SPIM illumination activated when the heart is in the correct part of its cycle to be imaged. The normal method of undertaking this would be through the use of an electrical probe to monitor the sample's ECG and fire the imaging system at the appropriate time. However, the zebra fish embryo is small and although micro-electrode probes could be used they are hard to position and maintain, in addition to perturbing the sample significantly. Previous high speed heart imaging has used a post acquisition image selection algorithm [5].

We have thus developed a novel method of high speed imaging and image analysis which is capable of predicating the heart's motion and firing the imaging system at the correct time. The method constantly monitors the accuracy of its predictions and thus is capable of in effect "locking-on" to the heart motion even when this changes. High speed arrhythmias may not be followed but to date we have not lost "lock" on an i*n vivo* sample. This paper describes the development and operation of this system and its use for SPIM microscopy.

2. Method

2.1. System Description

The optical system concept is shown in figure 1.

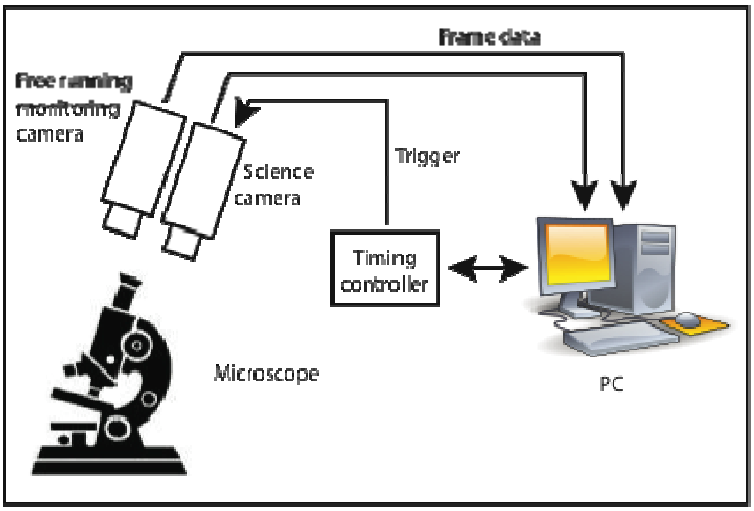

Figure 1. Overall system configuration. The sample is imaged by both a "science camera", which records the final images and a monitoring camera which is used to measure the phase of the heart's cycle and, via the PC and timing controller, to ultimately control the illumination beam.

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A high speed free running camera is connected to one channel of the microscope viewing system and the images sent directly to the control computer. Here the images are analyzed at high speed using custom developed software on a reasonably specified computer. The user can then selected a frame of interest at which the heart is in a particular phase of its cycle. The software then analyzes the continuing video stream and after around three heart beating sequences the system is ready to determine when the heart will be in the selected position. The computer then addresses a dedicated FPGA (field programmable gate array) device which will fire the light source and science camera at the appropriate time. The predictive nature of the system enables it to tolerate communication and image processing latencies within the system. The required images are then sent from the science camera to the computer where they are stored for subsequent use. It is also possible to store the high speed video stream for a period of time for analysis of the system, though under normal biological use this feature is not used. In the full SPIM system to build up three dimensional images, if required, the sample is then moved through the focal plane of the system whilst maintaining the heart synchronization to ensure that the final images of the heart are from the consistent user-selected position in all optical sections.

2.2. Optical Configuration

The optical system outline is shown in figure 2. The SPIM illumination and imaging path is identical to that used in the original SPIM paper [2].

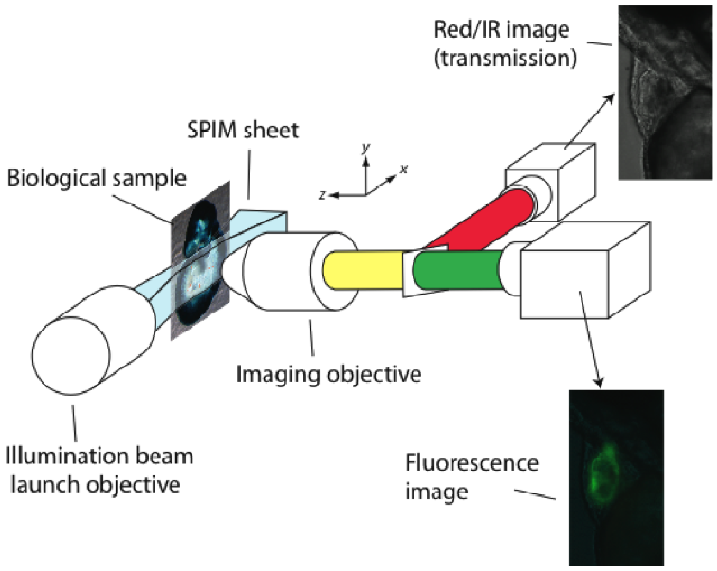

Figure 2. SPIM Imaging optical system, based on a conventional SPIM. An illumination beam is incident on the sample to produce a light sheet orthogonal to the axis of the imaging objective.

Light from a solid state blue laser at 488nm is fiber optically coupled into the system using a single mode optical fiber (Qioptiq Ltd. UK). The resulting beam is then collimated and expanded before passing through a cylindrical lens and being focused into the sample using a x10, 0.3NA water immersion lens (Nikon Ltd, UK). The imaging objective (x16, 0.8 NA long working distance water immersion lens Nikon Ltd UK) is mounted normally to the illumination source and the resulting image passes through a dichroic beam splitter, through an illumination blocking filter (Chroma Inc, USA), and focused onto a sensitive camera (Retiga, Q-Imaging Inc, USA) suitable for fluorescence imaging using a 200 mm lens (the design length for Nikon infinity corrected lenses) though other focal distances are possible to alter the effective magnification of the complete imaging system.

The synchronization image is recorded in the near infrared for two reasons. Firstly to ensure that no light from this part of the system interferes with the fluorescent image on the science camera, and secondly this image is generated in transmission from a light source placed behind the sample. A simple halogen lamp with an appropriate filter is used to direct the light through the sample and the image collected by the objective lens (common to both paths) is relayed onto

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the high speed camera (Prosilica GS650). In the current system a typical frame rate of around 100 Hz has been found sufficient to achieve excellent precision in the synchronization.

2.3. Software Description

The full software algorithm description is currently in preparation [6] with the outline description being given here with the crucial feature being the quantified relationship of the spatial and temporal properties of the heart. The algorithm takes the images of the heart (containing both spatial and temporal information) to build up an accurate model of the temporal behavior. This temporal model is then used to predict when the heart will next be in the correct position for image acquisition. There are thus four building blocks. (i) An initial frame comparison is undertaken using a similarity metric to identify frames which "look the same", and thus are assumed to correspond to same points on the cardiac cycle. (ii) This enables us to build up a set of reference frames corresponding to a single heart cycle, with each one being assigned a phase between 0 and 2π. (iii) We can then perform phase recovery by comparing the current frame against these reference frames in order to quantify the phase of the current frame. This phase development over time can then be extrapolated into the future to anticipate when an imaging frame should be taken. (iv) The real time trigger control system is then used to trigger the actually acquisition of the required frame (or to activate any other device) that is required in synchronization with the heart.

The initial frame comparison uses a simple sum of absolute differences (SAD) method "as its comparison metric," which, despite its simple nature, functions well in this situation. A sample graph is shown in figure 3 illustrating that we have a periodic pattern from our images as expected. These data points are then fitted to a simple V interpolation model when the SAD value is below a certain threshold value. This same comparison metric is used in the phase recovery step, with sub-frame interpolation to determine where the current frame fits into the set of reference frames, and hence establish the phase of the current frame.

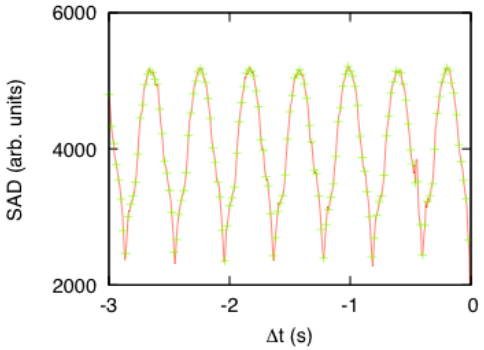

Figure 3. SAD value plotted against time for a beating Zebra fish heart. Here the comparison is between the frame that has just been acquired ($\Delta t = 0$ s) and the frames from the preceding three seconds (up to $\Delta t = -3$ s)

To actually synchronize the image capture and associated light sources the timing available on a personal computer is not accurate enough as the operating system is designed for consumer activities which do not require guaranteed high accuracy timing. We have thus used a dedicated FPGA device (Xilinx Spartan-3 200) based upon previous experience [7]. Any such system has an in-built latency and thus it is crucial that the computer informs the FPGA device sufficiently in advance of requiring the image. This latency must include possible delays in the software algorithm caused, as described above, by other background activity within the host operating system. Occasionally it may not complete in time, but this situation can be identified when it occurs and is reported by the timing controller as an error. Such a protocol ensures that either a frame will be acquired (or laser trigger signal sent, etc) at precisely the desired trigger time; or (occasionally) the frame will not be acquired at all and that error condition will be correctly identified by the code.

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3. RESULTS

The assessment of the performance of the system does not lend itself to the written page as we are looking for small changes in a series of effectively still video images. In the first experimental verification we used our new image selection system to record a series of frames of a beating zebra fish heart and compared these to the streamed images taken at 100 Hz where the frames with the heart in the "same position" were selected from the video sequence. Figure 4 shows two images taken from a video stream and the synchronization method.

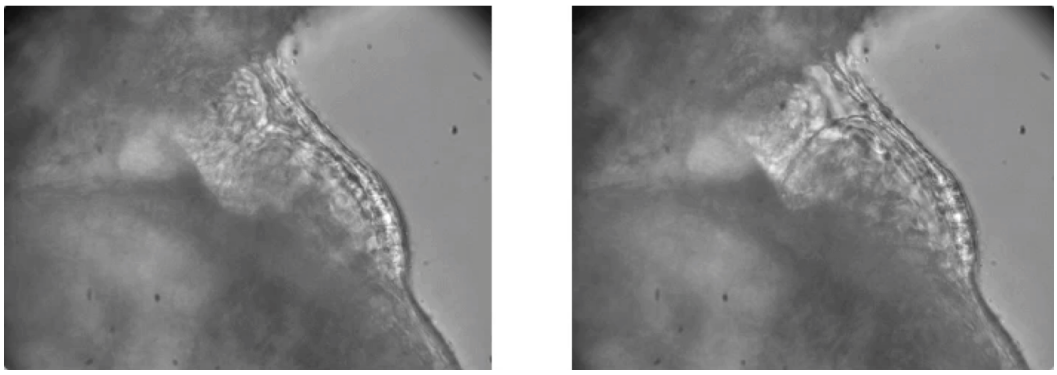

Figure 4. Still image from a movie [6] comparing postprocessing with live synchronization. (Left) A movie frame showing input data used for cardiac gating and (right) a synchronized frame. The original movie shows postprocessing first, followed by live synchronization, which gives more consistent synchronized images

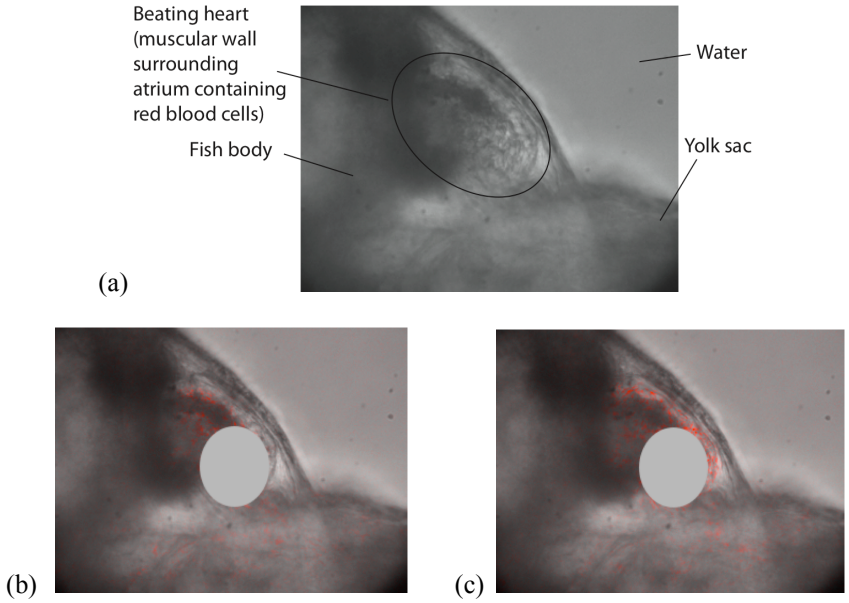

Figure 5. Top 5(a) shows heart structure (field of view around 500 microns) with 5(b) on the left showing the difference of two successive frames (in red) using the new synchronization method and 5(c) (right) showing the differences of two frames selected from a video stream. The center oval is grayed out as blood cells are clearly visible which are not correlated from frame to frame. 5(b), on the left, has less areas of red than the right frame, 5(c) showing that our method produces frames which are more similar than simply collecting a video sequence.

The difference between successive images can also be used to compare the two methods. In figure 5(a) we show a single image of the heart with a field of view of around 500 microns. 5(b) and 5(c) then show two successive frames where the heart should be in the same position, 5(b) from two successive synchronized frames, 5(c) from two frames selected from a video sequence where the heart is a close as possible in the same position. The centre portion of the image has been

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grayed out as red blood cells are clearly visible in the frames and of course have no correlation. Areas where the images differ are shown in red with 5(c) showing a significantly higher level of differences between two frames that are supposed to be the same.

These results (movie and stills) clearly demonstrate the improved consistency that can be achieved using real- time synchronization. This results in improved 3D tomographic data, as well as being the only option in laser targeting applications. Note that while all these examples show white-light transmission images for reasons of clarity, some of the greatest benefits are obtained using a two-camera system, with continuous transmission imaging and triggered fluorescence imaging.

Figure 6 shows a single raw image of a GFP transfected zebra fish heart taken with the SPIM imaging system. No post processing has been undertaken and this is one optical section for a complete series through the heart recorded using the SPIM system described above. This image and others indicate that adaptive optics will be necessary to produce the very high quality images.

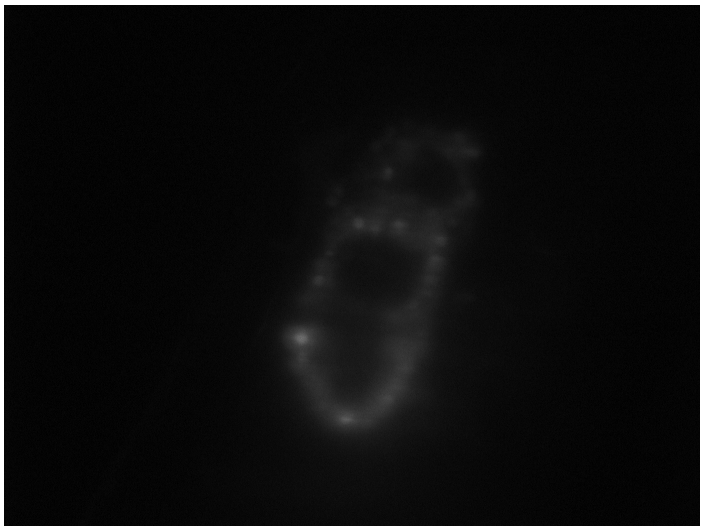

Figure 6. SPIM sectioned image through a zebra fish heart, taken using our system, fitted with the gating system.

4. CONCLUSIONS AND DISCUSSIONS

We have developed a heart synchronization system suitable for use in the live image of structures that follow a repeated pattern such as the heart. The system is suitable for triggering both the illumination source and recording camera in a SPIM microscope but has potential applications for any optical system which requires triggering at a particular moment in a repetitive cycle. We have also undertaken preliminary experiments to trigger a UV laser to cause minor cell damage in specific cells in the heart to study the response to such trauma. Using the system described here to trigger the ablation laser we can be sure of targeting the same position on each heat cycle. We believe that the system also has applications for various forms of microscopy where it is crucial that images are recorded in the same physical position on each occasion.

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