Augmented line-scan focal modulation microscopy for multi-dimensional imaging of zebrafish heart \textit{in vivo}

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\textbf{Abstract:} Multi-dimensional fluorescence imaging of live animal models demands strong optical sectioning, high spatial resolution, fast image acquisition, and minimal photobleaching. While conventional laser scanning microscopes are capable of deep penetration and sub-cellular resolution, they are generally too slow and causing excessive photobleaching for volumetric or time-lapse imaging. We demonstrate the performance of an augmented line-scan focal modulation microscope (aLSFMM), a high-speed imaging platform that affords above video-rate imaging speed by the use of line scanning. Exceptional background rejection is accomplished by combining a confocal slit with focal modulation. The image quality is further improved by merging the information from simultaneously acquired focal modulation and confocal images. Such a hybrid imaging scheme makes it possible to use very low power excitation light in high-speed imaging, and therefore leads to reduced photobleaching that is desirable for three-dimensional (3D) and four-dimensional (4D) in vivo image acquisition.

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\textbf{OCIS codes}: (100.0100) Image processing; (120.4570) Optical design of instruments; (120.5060) Phase modulation; (170.2520) Fluorescence microscopy; (180.0180) Microscopy; (290.0290) Scattering.

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1. Introduction

Confocal microscopy, the most common form of laser scanning microscopy, has been a standard imaging tool in modern biomedical researches [1, 2]. With a confocal pinhole rejecting out-of-focus emissions, a confocal microscope is capable of optical sectioning and high spatial resolution in both lateral and axial directions. While conventional confocal microscopy has been successfully applied to two-dimensional imaging of fixed samples, excessive photobleaching and slow scanning process usually make it very challenging to obtain high-quality three-dimensional and four-dimensional (three-dimensional in space and one-dimension in time) imaging data, especially from live samples. The typical frame rate for a commercial confocal microscope is a few Hz, which is limited by the point-to-point scanning mode. Resonant scanners can help increase the frame rate to more than 30 Hz, but at the expense of reduced field of view and non-uniform illumination. Numerical post-processing based background rejection methods, such as structured illumination microscopy [3], HiLo [4], and differential spinning disk microscopy [5], have been reported in the past decade. While these new techniques can provide high image speed, they suffer from limited dynamic range when imaging thick tissues, which is attributed to the fact that no physical device is present to prevent background photons from reaching the imaging sensor.

Selective plane illumination microscopy (SPIM) or light sheet microscopy has emerged as a popular choice for in vivo fluorescence imaging of relatively transparent small animal models, including embryonic zebrafish and fruit fly [6–13]. Optical sectioning in SPIM is mainly achieved by illuminating the sample using a light beam propagating within the focal plane of a detection objective, although a confocal slit is employed in some recently reported setups to further enhance the contrast. Due to selective illumination, SPIM is well known for...
negligible photobleaching in regions above and below the focal plane. Its imaging speed can readily reach 100 frames per second and above, adequate for visualization of fast moving organs in live animals.

Given its plentiful advantages, SPIM may not be the optimal solution for many applications. Side-on illumination is not appropriate when imaging certain samples, e.g., the surface of an extended object. While oblique plane illumination has been demonstrated by a few groups, application of SPIM to \textit{in vivo} mouse brain imaging is less common [14]. Even for animals of the right geometry and optical properties, sample preparation needs to be very careful and the related procedures may have undesirable effects on the living organism [15]. The image quality of SPIM may be globally compromised by local inhomogeneities in tissue optical properties. For example, dense distribution of fluorescence labels, even located outside the field of view, can cause long range streaking artifacts. Local variations in the refractive index deflect and scatter the illumination light, leading to a thickened and distorted light sheet and degraded optical sectioning.

We have recently reported line-scan focal modulation microscopy (LSFMM), an upgraded version of line-scan confocal microscopy (LSCM) [16]. Line-scan confocal microscopy scans the sample line-by-line instead of point-by-point so as to accomplish high frame rates. Similar to SPIM, a cylindrical lens is used in LSCM to form a light sheet condensed in one-dimension, which illuminates the field of view one line at a time. While the illumination line is scanned across the field of view, a line camera behind a confocal slit collects the emissions from focal plane in parallel. Given its high imaging speed, however, LSCM has not been widely used in live animal imaging probably due to its moderate image quality. A confocal slit is less effective in rejecting out-of-focus light in comparison with a confocal pinhole in a standard confocal setup [17]. Consequently, LSCM is not able to provide contrast and axial resolution similar to those of its point-scanning counterpart. In LSFMM, focal modulation is combined with the confocal slit to provide improved background rejection and optical sectioning. Focal modulation is a new optical instrumentation method for introducing periodic modulation of the excitation light intensity confined within the focal plane [18–22]. It has proved to be a very effective way to suppress background emissions in both point scanning and line-scan FMM systems. While the idea of using focal modulation to isolate focal plane signal sounds similar to structured illumination microscopy, LSFMM enables the use of the spatial filter (slit) that is capable of rejecting a significant part of the out-of-focus light. This effectively solves the dynamic range problem and reduces the shot noise.

Obtaining high quality in vivo images of live samples, however, is not limited by the signal to background ratio (SBR) alone. An adequately high signal to noise ratio (SNR) has to be maintained so that sample structures are not distorted or masked by noises. The noise level in an imaging system depends on many factors such its work principle, configuration, and operation mode. While LSFMM images enjoy a much better signal to background ratio than that of LSCM images, their noise level is generally higher due to limited modulation depth (typically 30-60%) and coherent speckles generated by the aberrated ballistic excitation photons [23, 24]. In addition, the LSCM images are usually much brighter as scattered illumination photons also contribute to the excitation of focal plane fluorophores.

Here we describe a novel method called augmented line-scan focal modulation microscopy (aLSFMM). It combines the information from both LSFMM and LSCM images to obtain optimal optical image quality and allow the use of low excitation power. The strong background in an LSCM image is dominated by low-frequency components, which can be readily removed by high pass filtering. The remaining high-frequency components provide high-resolution information about focal plane structures. This enables low pass filtering of the LSFMM image acquired simultaneously so as to reduce speckles and noises. The two filtered images are then added together to form the aLSFMM image.
where \( F \) and \( C \) are the LSFMM and LSCM images, respectively, and \( \varsigma \) is the modulation depth. The spatial low-pass filter \( \mathcal{L} \) and high-pass filter \( \mathcal{H} \) are related to each other by

\[
\mathcal{L} + \mathcal{H} = \mathcal{I}
\]

where \( \mathcal{I} \) is an identity operator. A similar hybrid imaging strategy, which combines confocal microscopy with wide-field imaging, has been proved advantageous in our previous study [25]. The choice of cutoff frequency is rather empirical at the moment. A near-optimal value is chosen to compromise between the signal to noise ratio and signal to background ratio in the combined aLSFMM image. The mathematic formulas are almost the same as those used in HiLo image processing, except that in our case the weighting factor \( 1/\varsigma \) is determined by the modulation depth of the LSFMM system and can be experimentally calibrated.

2. Methods

2.1 Sample preparation

To demonstrate the imaging performance of aLSFMM, we have conducted multidimensional imaging experiments with the Tg(phpC31.attP.2A, −0.8myl7: EGFP) transgenic zebrafish, which has the myocardium-specific expression of eGFP under the control of the cardiac myosin light chain 2 (cmlc2) promoter and allows the visualization of myocardium morphogenesis [26]. The zebrafish embryos were collected by natural spawning and maintained at 28°C in fish media. 0.003% phenylthiourea (PTU) was added to the fish media to block pigmentation and ensure that the embryos remained transparent throughout the imaging period spanning from 5 to 15 days post fertilization (dpf). The zebrafish were maintained in the fish facilities at the Department of Biological Sciences, National University of Singapore (NUS) according to established protocols and in compliance with Institutional Animal Care and Use Committee (IACUC) guidelines.

Before imaging experiments, the zebrafish larvae were placed on #0 glass bottomed dishes (MatTek, P35G-0-10-C) and then positioned in a dorsal orientation. 2% low-melt agarose was added to the zebrafish to immobilize them. 0.05% Tricaine was added to the agarose to keep them anesthetized over extended periods. Images acquisition was usually started within an hour of fixing the zebrafish in agarose gel. All images were captured using either an Olympus 20X/0.55 or Carl Zeiss 40X /1.1w (water immersion) objective lens. The beating heart of a zebrafish larva was usually imaged at 50-100 frames per second, as the camera had a maximum full-frame imaging speed of 100 fps. The image depth was controlled by the use of a motorized one-dimensional stage, which enables axial scanning of samples at submicron resolution.

2.2 Combining focal modulation with line-scan imaging mode

Figure 1(a) shows the schematic of our optical setup that integrates focal modulation within a line-scan microscope. The fast-scanning microscope includes a 473 nm solid-state laser (not included in the Fig. 1), a scientific CMOS camera (optiMOS, QImaging), a spatiotemporal phase modulator (STPM), a confocal slit, and optical components (beam splitter, lenses, and filters) for illumination and detection. The structure of STPM is similar to the one developed for point-scanning FMM, consisting of an electro optic phase modulator (EOM) followed by a spatial polarizer (SP) and a polarization analyzer (PA) [21]. To be compatible with line illumination, the SP is divided into four translational symmetric (one-dimensional) zones (Fig. 1(b)) with alternating horizontal and vertical polarization directions. It is used to spatially separate the modulated (vertically polarized) and non-modulated (horizontally polarized) components of the excitation beam. The PA projects both components to a 45°
polarization direction so that they can interfere with each other at the focal point. Different from conventional line-scan microscopes, a 2D image sensor (instead of a line camera) is used to detect the fluorescence emission. The optiMOS camera is far more sensitive (<2e read noise) than most commercially available line cameras, which are designed for industrial applications such as machine vision. To take the advantage of the camera’s large sensing area, a slit scanning method is adopted. A detection Galvo mirror scans the image of the confocal slit across the camera, while a synchronized illumination Galvo mirror shifts the illumination line laterally over the sample [27]. Due to spatiotemporal modulation of the illumination beam, the fluorescence image captured by the camera is subject to periodic intensity modulation along the scan direction. Each raw image is then processed to retrieve one LSCM image and one LSFMM image. The raw image size is 1920 x 1080 pixels, while the typical size for LSCM and LSFMM images is 480 x 1080 pixels. When a 20X/0.45 objective is used, the field of view (FOV) is 364 \( \mu \text{m} \times 820 \mu \text{m} \) and the spatial resolutions are around 0.94 \( \mu \text{m} \) and 0.83 \( \mu \text{m} \) along the X- and Y-axes, respectively. The axial resolution is estimated 3.49 \( \mu \text{m} \).

**Fig. 1. LSFMM design.** (a) Schematic of an LSFMM system. EOM: electro-optic phase modulator, whose fast axis is either vertically or horizontally oriented; SP: spatial polarizer; PA: polarization analyzer; G1 and G2: synchronized galvanometer mirrors; CL: cylindrical lens; BS: beam splitter (10/90); L1, L2, L3: lenses; LP1, LP2: lens pairs; EF: emission filter. (b) Polarizing optics. The spatial polarizer (SP) consists of four zones of alternating horizontal and vertical polarization directions. It is followed by the polarization analyzer (PA) with a polarization direction at 45°, parallel to the polarization of the collimated laser beam entering the EOM.

### 2.3 aLSFMM image formation

Due to focal modulation, the raw images captured by line-scan microscope contain intensity undulations, which follow a periodic pattern of a preset frequency. Shown in Fig. 2(a) is one raw image captured from the heart of a 13dpf zebrafish with the Carl Zeiss 40X/1.1 lens. As a typical feature of this zebrafish line, EGFP expression in the ventricle (bottom) appears remarkably stronger than in the atrium. An image segment enclosed in the small yellow rectangle is zoomed in (magnified by 3 times) and included as an inset in the up-right corner, in which periodic intensity modulation along the horizontal direction is clearly visible. Both LSCM and LSFMM images can be retrieved from the raw images via, of course, different image processing methods. Simple low-pass filtering with an appropriate cut-off frequency
can smooth out the fringes while maintaining the spatial resolution. Such a process results in an LSCM image as shown in Fig. 2(b). The LSFMM image is created by a demodulation process, which essentially measures the local amplitudes of the modulated image components. While the slowly changing background is filtered out, the resultant LSFMM image (see Fig. 2(c)) contains very little background contribution. Bearing in mind that the original LSFMM is a few times dimmer than the LSCM image since it only contains in-focus information and the modulation depth is smaller than one. The brightness has been adjusted so as to better compare their quality. The contrast in the LSFMM image is so much better than that of the LSCM image, in which out-of-focus structures cast blurred shadows. Nonetheless, some fine speckles are noticeable, especially in regions of weak signals. Combining the information from both images (see Eq. (1)), we can generate the so-called aLSFMM image (Fig. 2(d)) with high contrast and reduced noise level. As both SBR and SNR are improved, sample structures are better delineated with high-resolution details.

Fig. 2. aLSFMM image formation process. (a) A raw image obtained from a 3 dpf zebrafish. The zoomed-in picture (inset) clearly shows intensity modulation along the horizontal direction. (b) LSCM image and (c) LSFMM image were retrieved from the same raw image. (d) The aLSFMM image that combines information from (b) and (c).

3. Results

3.1 Image quality comparison with SPIM

To demonstrate the advantages of our approach over established in vivo animal imaging methods, we have conducted experiments to compare the imaging performances, in terms of contrast and effective spatial resolution, between aLSFMM and SPIM. Figure 3(a) shows one of image frames obtained from a 13 dpf zebrafish heart by using Carl Zeiss Lightsheet Z.1, a well established and sophisticated SPIM system. The corresponding image sequence contains 100 frames acquired at 100 fps, which are included in Visualization 1 (replayed at 10 fps). An aLSFMM image of another zebrafish of the similar developmental stage is compared in Fig. 3(b). It is the last frame in Visualization 2, which was also acquired at 100 fps. It is
interesting to notice that there is minimal background in both cases in areas outside the EGFP labeled heart. This is an improvement over LSCM and is attributed to better suppression of contributions from out-of-focus regions. In areas near the EGFP labeled cells, however, the SPIM image shows elevated background level and blurring. In addition, the image brightness and quality are noticeably inhomogeneous, as the light sheet enters the zebrafish from the left side and gets scattered, aberrated, and attenuated when propagating through the tissue. Streaking artifacts can be noticed on the right-hand side of the ventricular wall when watching the corresponding movie (Visualization 1). In contrast, the aLSFMM image reveals much-detailed high-resolution features and the image quality is uniform across the entire field of view. Shown in Figs. 3(c) and 3(d) are intensity profiles along the yellow lines in Fig. 3(a) and 3(b), respectively. For the SPIM intensity profile, there exists a relatively high background level even within the ventricle cavity. The rising and falling edges feature rather slow slopes and spread over a distance around 10 microns. On the other hand, the aLSFMM intensity becomes almost zero within the ventricle cavity. The intensity transitions over a short distance around 1 micron can be readily identified, implying one order of magnitude improvement in the effective spatial resolution over SPIM. Bearing in mind, however, the performance comparison between SPIM and aLSFMM is based on our preliminary results. More rigorous experimental validations are warranted to fully quantify the advantage and disadvantages of both modalities.

![Image](image.jpg)

**Fig. 3.** Image quality comparison. (a) Ventricle and atrium of a 13 dpf zebrafish larva visualized with a SPIM system. (b) aLSFMM heart image of another 13 dpf zebrafish larva. (c) Intensity profile along the yellow line in (a). (d) Intensity profile along the yellow line in (b).

### 3.2 3D and 4D image acquisition and rendering

While it is straightforward to acquire two-dimensional aLSFMM image sequences from a live animal, 3D and 4D image acquisition are more complicated. For constantly moving animal
organs such as the heart, it is very challenging to directly obtain the 3D images even for a microscope operated at 100 fps. However, it is possible to reconstruct good 3D and 4D imaging data sets from 2D movies when the heart beats regularly and the structural deformation is periodic.

To demonstrate the feasibility of reconstructing 3D and 4D models of zebrafish heart, we acquired a series of fluorescence image sequences with a 3dpf zebrafish. With an Olympus 20X/0.55 objective, the zebrafish was imaged repeatedly at a 2.66-micron depth increment for a total number of 77 imaging depths. A movie consisting of 80 continuous frames were captured for each imaging depth at an imaging speed of 100 fps. It was found that each cardiac cycle lasted for 23 frames, equivalent to a heart rate of roughly 261 beats per minute. As the movie series from different depths were not initially synchronized, Pearson’s correlation was computed for neighboring depths so as to find the timing information to align them properly along the time axis [10]. Once the time alignment is done, a subset of 23 frames was chosen for each depth to form a synchronized 2D1T (two-dimensional space and 1-dimensional time) data set. Each one of the 23 frames was tagged with a specific cardiac phase. For any given cardiac phase, we formed a 3D volumetric model of the heart by retrieving 77 frames of the same phase from all 2D1T data sets. The 23 time-resolved 3D models of various cardiac phases formed a complete 4D model of the heart, which contained all the structural and dynamic information.

One of the 3D models, which was related to a ventricular filling (diastolic) phase, was processed with ImageJ for image rendering. ImageJ 3D viewer was used to record a volume view of the model and record a 360-degree rotation movie (see Visualization 3). Shown in Figs. 4(a)-4(c)] are representative frames from the movie, corresponding to rotation angles 0, 60, and 140 degrees, respectively. For the same 3D model, ImageJ 3D Projection was then used to create a series of projections of the model by using the Brightest Point method (Visualization 4). The rotation angle range was 0 to 360 degrees and the rotation increment was 2 degree. Shown in Fig. 4(d)-4(f) are projections for rotation angles 0, 60, and 140 degrees, respectively.

To visualize the dynamic heart beating process, 3D projections of all time-resolved 3D models with the same rotation angle can be combined to create a movie. Shown in Fig. 5 is a montage of the 3dpf zebrafish heart for a time period identical to a cardiac cycle (Visualization 5). The ventricle contraction started from the first time point (0.01s) while the atrium began to fill, and the ventricular volume was gradually reduced to the minimum at 0.12s. From time points 0.13s to 0.23s, the ventricle underwent the expanding process, which seemed slightly faster than the contractile process. Due to the high imaging speed, the heart morphological changes were captured with very a high time resolution and motion artifacts were essentially absent.

![3D image rendering](image_url)

Fig. 4. 3D image rendering. (a), (b), and (c), 3D zebrafish heart visualized with ImageJ 3D viewer. (d), (e), and (f), ImageJ 3D projections of the same 3D model.
4. Discussion and conclusion

The high imaging speed and high image quality afforded by aLSFMM makes it an excellent microscopic tool for basic biological research, translational research, as well as medical diagnosis. Compared with conventional LSCM, aLSFMM has significantly improved background rejection while maintaining the signal to noise ratio. By the use of focal modulation, LSFMM can achieve a much better signal to background ratio than both LSCM and SPIM. Taking advantage of the useful information from LSCM images, aLSFMM is able to accomplish enhanced signal to noise ratio than LSFMM alone. In general, the noise level in an imaging system depends on the imaging speed and the light source intensity. The signal to noise ratio could be improved with increased integration time and/or increased excitation light intensity. For in vivo imaging of live animals, however, both approaches have their limitations. An increased integration time means reduced imaging speed, which might lead to unacceptable motion artifacts and other adverse consequences. A major problem with increased excitation light intensity is elevated photobleaching of fluorophores and photodamage to the sample. These challenges highlight the importance of aLSFMM, which enables the use of relatively low excitation intensity at a very high imaging speed. Judging from reconstructed 3D and 4D imaging data, we found negligible photobleaching after
scanning the samples for a prolonged time period. For the zebrafish heart shown in Figs. 4 and 5, it took 0.8 second to acquire 80 frames of 2D images for each depth. The total number of depth was 77, leading to a total camera exposure time of 61.6 seconds. The total image acquisition time, however, was about 7 minutes that included the time for axially shifting the sample stage and transferring images from the camera to a computer. The excitation laser was maintained at a constant output during the entire process. The main reason for reduced photobleaching was the low excitation power, which was about 0.1 mW after the objective. The sample received an energy density of 0.339 kJ/m² for each captured image frame. In comparison, a spinning disk confocal setup used an excitation power (at the objective) of 1 mW and an integration time of 0.1 second to image zebrafish in vivo [28]. The energy density deposited on the sample was 3kJ/m², which is one order of magnitude higher than that in our case.

Albeit the significant improvement in aLSFMM, SPIM is believed to fare better in photobleaching minimization in many application scenarios. Nonetheless, aLSFMM outperforms aLSFMM in many other aspects such as the ease of sample handling, effective spatial resolution, and optical sectioning capability. Generally speaking, aLSFMM is a versatile imaging platform that can deal with samples of various size and geometry.

There is room for further improving the current aLSFMM system. For example, the relatively slow translation stage for axial scanning can be replaced by a piezo objective mount and the image acquisition software can be further optimized to expedite the image transfer process. These modifications can help reduce the total amount of time for acquiring 3D and 4D imaging data sets. This is important, as it is very often difficult to keep a live animal motion free for an extended period of time. For the 13-dpf zebrafish mentioned previously, spontaneous body movement could happen during the process of acquiring multiple image stacks. Proper acquisition and reconstruction of multi-dimensional models of the entire heart are currently limited to younger fish models (e.g., 3-6dpf), which are less likely to cause such a problem. Reduction of the overall image acquisition time, therefore, is desirable for cover a wider range of animal models. Another issue with the current line scan setup is that the illumination line intensity is Gaussian distributed. To improve the FOV homogeneousness, it might be better to replace the cylindrical lens with a Powell lens to generate a more uniform illumination line.

To conclude, we have developed aLSFMM and applied it to multi-dimensional imaging of zebrafish heart. The superior imaging performance of aLSFMM, including its high speed, reduced background, reduced noise, and minimal photobleaching, were achieved simultaneously. It is expected that aLSFMM will evolve into a real-time in-vivo imaging platform suitable for many other animal models.

Funding
National Medical Research Council (NMRC) (NMRC/CBRG/0036/2013 and NMRC/CBRG/0100/2015).

Acknowledgments
We thank Prof. Christoph Wolfram Winkler and Ms. Shermaine Tay (Department of Biological Sciences, National University of Singapore) for providing zebrafish embryos and their feedback on image quality.

Disclosures
The authors declare that there are no conflicts of interest related to this article.