Imaging of post-embryonic stage model-organisms at high resolution using high resolution using multi orientation optoacoustic mesoscopy

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ABSTRACT

Model organisms such as zebrafish play an important role for developmental biologists and experimental geneticists. Still, as they grow into their post-embryonic stage of development it becomes more and more difficult to image them because of high light scattering inside biological tissue. Optoacoustic mesoscopy based on spherically focused, high frequency, ultrasound detectors offers an alternative, where it relies on the focusing capabilities of the ultrasound detectors in generating the image rather than on the focusing of light. Nonetheless, because of the limited numerical aperture the resolution is not isotropic, and many structures, especially elongated ones, such as blood vessels and other organs, are either invisible, or not clearly identifiable on the final image. Herein, based on high frequency ultrasound detectors at 100 MHz and 50 MHz we introduce multi orientation (view) optoacoustic mesoscopy. We collect a rich amount of signals from multiple directions and combine them using a weighted sum in the Fourier domain and a Wiener deconvolution into a single high resolution three-dimensional image. The new system achieves isotropic resolutions on the order of 10 μm in-plane, 40 μm axially, and SNR enhancement of 15 dB compared to the single orientation case. To showcase the system we imaged a juvenile zebrafish *ex vivo*, which is too large to image using optical microscopic techniques, the reconstructed images show unprecedented performance in terms of SNR, resolution, and clarity of the observed structures. Using the system we see the inner organs of the zebrafish, the pigmentation, and the vessels with unprecedented clarity.

Keywords: Optoacoustics, beam-forming, mesoscopy, microscopy, Multiview

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1. INTRODUCTION

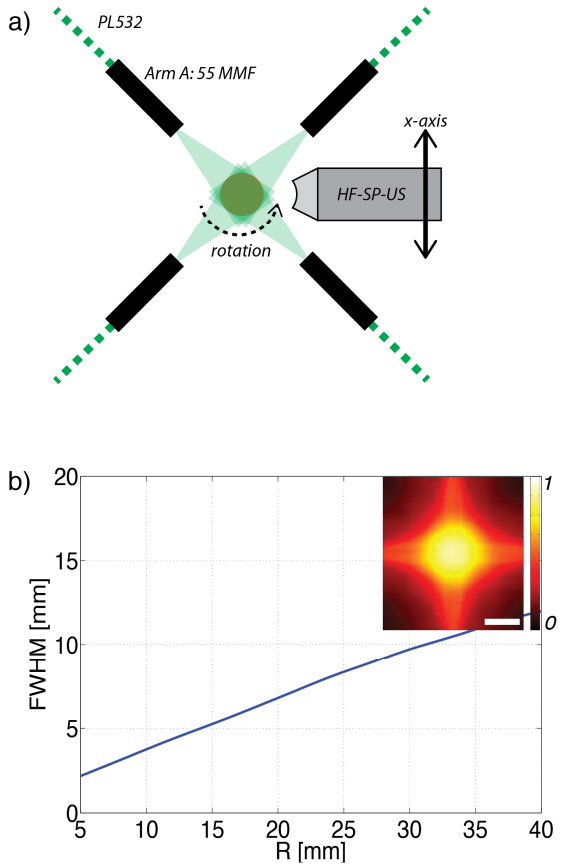

Figure 1: experimental setup, showing the sample illumination, the sample positioning, and the scanning detector (a). The illumination could be adjusted to accommodate different diameters of samples, for the sample used in this manuscript a distance of 14 mm was chosen, this way samples with a maximum diameter of 4.5 mm could be illuminated with a homogeneous illumination. The inset shows a simulation of the illumination in the xy-plane, scale bar 5 mm (b).

Raster scan optoacoustic mesoscopy (RSOM) is a new modality which can generate high resolution images of biological samples ~20 µm[1,2], at depths beyond the diffusion limit[3]. Because of its capabilities it has been used so far to image cancer development in a mouse model of melanoma cancer[4], the human skin at high resolution[5,6], and was successfully combined with optical and optoacoustic microscopy for multiscale imaging of biological samples[7,8].

Although RSOM can provide high resolution images of biological tissues, and it is becoming a mainstream technology for biomedical research, one area where RSOM didn't have a large success was in imaging model organisms, such as zebrafish and *drosophila melanogaster* larvae. Although it was tried, the generated images were not good enough, because of the limited aperture of the ultrasonic detectors used, hence elongated, and planar structures that are not parallel to the scanning plane, usually the xy-plane, were previously invisible[2].

Although this problem exists in optical imaigng, it is not as severe, because it is possible to use high numerical aperture (*NA*) objectives to get a more rounded focused spot, and collect energy from more directions. Additionally, some groups tried putting more than a single objective around the object in order to increase the numerical aperture in what is called a 4π-configuration. Moreover some groups working on selective plane illumination microscopy (SPIM) suggested to measure the sample from multiple directions to increase both the signal to noise ratio (*SNR*) and to increase the resolution, as a result of increasing the *NA* of the detection system[9]. In optoacoustics similar efforts have been applied based on linear arrays[10,11]. Although, this multiview collection of data improves both the *SNR* and increases the *NA* of the detection system, because it is based on linear arrays it is still limited in resolution, because of the limited *NA* parallel to the axis of rotation, as well as the limitations based on the highest manufacturable frequency of such detectors. Additionally such configurations have a limited *SNR* because they have a limited detector size. Although, some other groups have proposed the use of multiview with optical resolution optoacosutic microscopy, such a configuration is limited in its maximum achievable depth[12].

Herein based on the reflection mode implementation of RSOM[1], we introduce multi orientation raster scan optoacoustic mesoscopy (MORSOM)[13]. The new system is designed from the ground to accommodate model organisms. In this work we introduce the system at 50 MHz, show the improvements in terms of resolution, *SNR*, and *NA* compared to normal RSOM. We also showcase the system on a juvenile zebrafish, which is a model organism much larger than what is possible to image with optical microscopy.

2. METHODS

2.1 Hardware

The system has been developed based on the original RSOM, but rather than scanning the sample in the xy-plane, it is scanned in the xz-plane, see figure 1. After completing a single RSOM scan, the sample is rotated into the next angular position. This way the sample is measured from different sides, at angular steps of 20° degrees. Since the numerical aperture of the used detector is 0.5, which defines an opening angle of ~60°, hence an angular step size of 20° enables the measurement of all the generated optoacoustic waves. The main component in the system is a single element, spherically focused ultrasound detector, with a central frequency of 50 MHz, and a bandwidth of 10-90 MHz.

For a homogeneous illumination a fast diode pumped solid state laser (DPSS) (Wedge HB532, BrightsSolutions, Italy), operating at 532 nm, and pulsing at 2 kHz is coupled into a fiber bundle which is distributed into 4 arms. Each arm illuminates at 90° to the other arms. The output of the ultrasound detector is amplified with a low noise amplifier, which has a nominal amplification of 63 dB, and a bandwidth of 0.001-500 MHz (AU-1291, Miteq Inc. USA), this is subsequently connected to a high speed data acquisition card, operating at 500 MSps (1250X CompueScope, Dynamic Signals LLC, Lockport, IL, USA). The measured signal are further filtered in the 10-90 MHz band to suppress noise from outside of the detection bandwidth.

2.2 Reconstruction, weighted sum, and Wiener deconvolution

For reconstruction, the data collected from each view is reconstructed using a beamforming algorithm with a dynamic aperture in the time domain[1]. To accelerate the reconstruction, the beam forming is implemented on a graphical processing unit (GPU)[14]. After reconstruction the data from the different views is combined into a single three dimensional volume in the Fourier space. For optimum results the data are first aligned using two dimensional cross correlation of images based fiducial markers inside the image[13]. Afterwards the data is transformed into the Fourier space using, the data are then weighted using the optical transfer function (*OTF*), which is the Fourier transform of the point spread function (*PSF*). The *PSF* is measured from a point absorber. This way regions with strong amount of information are kept while regions with low information content are suppressed[13]:

$$\tilde{R}(k_x, k_y, z_i) = \sum_{\theta} \widetilde{R_\theta}(k_x, k_y, z_i) \times OTF_\theta^*(k_x, k_y)$$

Later on the images are transformed back to the spatial domain using an inverse Fourier transform, i.e. $R(x, y, z_i) = IFT_{xy}\left(\tilde{R}(k_x, k_y, z_i)\right)$. Where $\tilde{R}_\theta$ is the reconstructed volume at angle θ in the Fourier space, IFT_{xy} is the inverse Fourier transform, and $\tilde{R}$ is the weighted sum of all the views.

2.3 Wiener deconvolution

For Wiener deconvolution the OTF_θ from all the views is combined into a single, multiview OTF:

$$OTF_W(k_x, k_y) = \sum_{\theta} \left|OTF_\theta(k_x, k_y)\right|^2$$

This combined OTF_W is then used to correct for any remaining irregularities in the final image, for example reduction in the sidelobes, etc.:

$$\tilde{R}_{Wiener}(k_x, k_y, z_i) = \frac{\tilde{R}(k_x, k_y, z_i) \times OTF_W^*(k_x, k_y)}{|OTF_W|^2 + \omega^2}$$

In the actual reconstructions we empirically chose the Wiener factor (ω) to be 10^{-3}.

2.4 Alignment of the different views

First a coarse alignment is applied by co-registering two opposing views, i.e. the ones at 0° and the one at 180°. Those views basically show the same structures, albeit with 180° phase shift in the signals. The alignment happens when the maximum *SNR* of the two views is achieved. Later a fine alignment of the different views inside the sample being measured is performed. This fine alignment accounts for any imperfections in the measurement resulting from the measurement mechanics. The sample is embedded within agar which is full with small black microspheres. Since microspheres generate isotrpic optoacoustic signals, they are visible from all the side. Those microspheres are used later for alignment of the different views, i.e. as feducial markers. The alignment is done in a simple way, one or several microspheres are selected from the different views, and using two dimensional cross-correlations all the views are aligned to each another. To further improve the alignment between the different views, instead of aligning a single plane, the spheres at multiple planes are used for alignment, this way, if any tilt in the axis of rotation are accounted for by applying an interpolation between the correcting vectors.

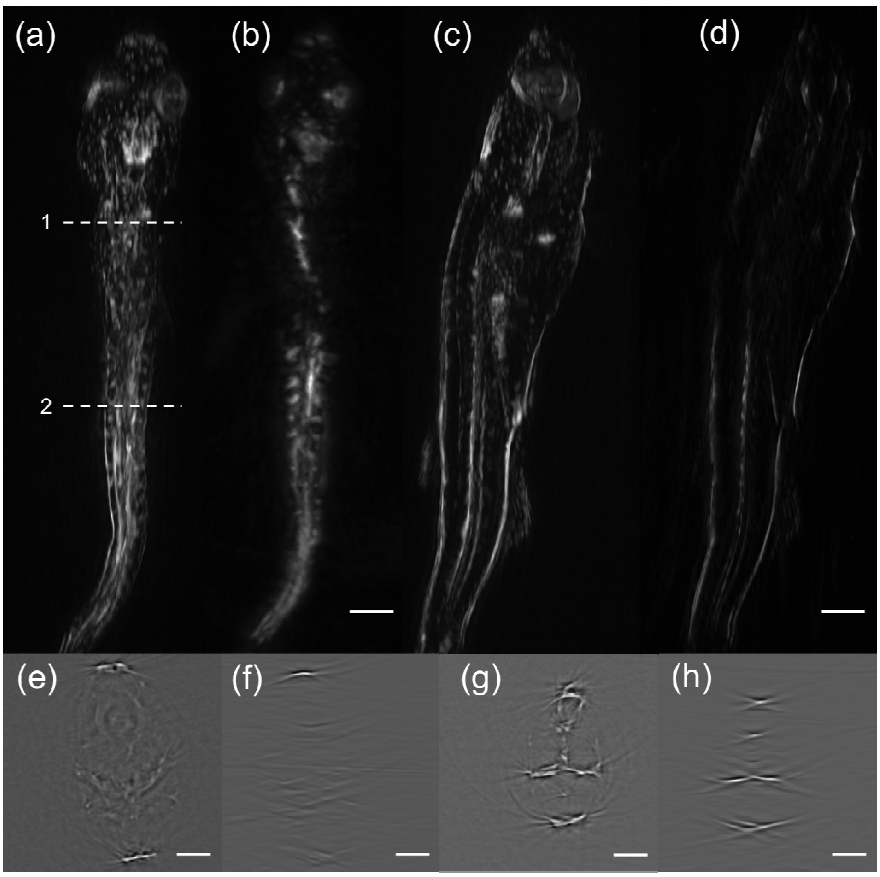

Figure 2: top view from MORSOM (a), top view from RSOM (b), side view from MORSOM (c), side view from RSOM (d), cross section from position 1 from MORSOM (e), same cross section from RSOM (f), cross section from position 2 from MORSOM (g), same cross section from RSOM (h).

3. RESULTS

3.1 System characterization and measurement of the point spread function (PSF)

To characterize the performance of the system we imaged a sample full of 10 µm microspheres, those microspheres served to characterize the system on the one hand, and to measure the *PSF* of individual views on the other. Using single view RSOM, the resolution has been aronud 60 µm laterally, i.e. parallel to the scan direction, and 15 µm axially. After measuring the sample from multiple views, and combining all the views into a single volume the resolution improves by a factor of 3 into ~18 µm along the x-direction. The single view *PSF* has been measured from a single view image of a microsphere, several single view *PSF*s have been averaged to improve the *SNR* of the *PSF* measurement. By applying the weighted sum of the different views, and later on

applying a Wiener deconvolution to the data improves the *SNR* by ~15 dB.

3.2 Imaging of Juvenile Zebrafish

As previously mentioned, microscopic techniques such as multiphoton microscopy[15], and selective plane illumination microscopy[16] are capable of generating high resolution images of biological tissue, albeit down to a depth of several hundreds micrometers only[3]. This means it is possible to image zebrafish, which is a standard model organism only up to a certain age, usually 6-7 dpf. Herein, we imaged a juvenile zebrafish at an age of 21 dpf, such zebrafish have a diameter usually of 1-2 mm, hence it is the kind suitable for MORSOM.

Compared to single view RSOM, MORSOM shows an improvement. Several features inside the zebrafish which are invisble in RSOM become visible, additionally on the cross sections instead of seeing unclear structures, boundaries of organs in the intestines of the fish become visible, several vessels become also visible. Moreover, the *SNR* of the image significantly improves. See figure 2.

4. SUMMARY & DISCUSSION

Herein we introduced MORSOM, a multiview version of RSOM specifically designed for high resolution imaging of model organisms. Using MORSOM we imaged juvenile zebrafish, which is much larger than what is possible with optical microscopic techniques, and hence shows the advantage of using MORSOM. Using MORSOM it was possible to see the boundaries of the inner organs, the fins, the lateral line and other parts of the zebrafish with high resolution. The system operating at 50 MHz achieved a resolution of 15 μm in-plane and ~40 μm along the axis of rotation. This means an isotropic resolution in-plane was achieved. Moreover, an improvement of ~15 dB in *SNR* has been achieved; part of this improvement is related to the increased number of views, while an additional contribution comes from the weighted sum and the Wiener filter. Currently a MORSOM scan takes around 20 min, this time could be reduced by appropriate combination of the number of raster points and the number of rotational angles. We moreover believe that by adding the capability for multicolor imaging MORSOM will become a strong modality for imaging juvenile and adult model organisms. Finally, by combining MORSOM with other techniques such as SPIM[17], it will be possible to add contrast from multiple modalities such as absorption and fluorescent into a single multi-contrast image, multiscale image.

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