

High-resolution raster scan optoacoustic mesoscopy of genetically modified *Drosophila* pupae

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ABSTRACT

Optoacoustic mesoscopy aims to bridge the gap between optoacoustic microscopy and optoacoustic tomography. We have developed a setup for optoacoustic mesoscopy where we use a high frequency, high numerical aperture spherically focused ultrasound transducer, with a wide bandwidth of 25-125 MHz. The excitation is performed using a diode laser capable of >500 μ J/pulse, 1.8ns pulse width, 1.4 kHz pulse repetition rate, at 515 nm. The system is capable to penetrate more than 5 mm with a resolution of 7 μ m axially and 30 μ m transversally. Using high-speed stages and scanning the transducer in a quasi-continuous mode, a field of view of 2x2 mm² is scanned in less than 2 minutes. The system is suitable for imaging biological samples that have a diameter of 1-5 mm; zebrafish, *drosophila melanogaster*, and thin biological samples such as the mouse ear and mouse extremities. We have used our mesoscopic setup to generate 3-dimensional images of genetically modified *drosophila* fly, and *drosophila* pupae expressing GFP from the wings, high resolution images were generated in both cases, in the fly we can see the wings, the legs, the eyes, and the shape of the body. In the pupae the outline of the pupae, the spiracles at both ends and a strong signal corresponding to the location of the future wings are observed.

Keywords: Optoacoustics, high frequency, beam-forming, epi-illumination, mesoscopy, microscopy

1. INTRODUCTION

Optoacoustics is the generation of broadband, high frequency ultrasound signals from biological samples using short, high-energy, laser pulses[1, 2]. These signals could be generated using pulsed laser sources[3-5], high energy pulsed RF-signals[6], quasi-continuous wave RF excitation[7], and modulated continuous waves lasers[8]. Optoacoustics is widely used in two configurations, tomographic[4, 9, 10], and microscopic[11, 12]. The tomographic configuration is usually used to image large fields-of-view, where higher penetration depths are desired, to achieve this lower frequency detectors are used. The achieved resolutions are on the order of 100-200 μ m. Optoacoustic tomography has been shown useful in several applications such as imaging the biodistribution of targeted optical markers, examples are gold nanoparticles and fluorescent proteins, vasculature, oxygenation maps in tissue, tumor heterogeneity, tumor hypoxia, and many more[2].

As for optoacoustic microscopy it has been used to generate high-resolution images of biological samples, ranging from 1-2 μ m for optical resolution systems where focused light beams are scanned through the sample[11, 12], to 50-60 μ m for acoustic resolution systems[13, 14], where the resolution comes from the acoustic focusing, the axial resolution in both cases is determined by the bandwidth of the ultrasonic detectors used, as 50 MHz spherically detectors are usually used, the axial resolution is on the order of 15 μ m. The penetration depths achieved depends on the kind of focusing used, so it ranges from a few hundreds of microns for optical resolution systems to a few millimeters for acoustically focusing systems. Spherically focused detectors are usually used for microscopy applications, as they have high sensitivity inside the focus, and high angular coverage outside of the focus. For the reconstruction, in optical resolution optoacoustic microscopy techniques, simple concatenation of the individual measurements is enough, while for acoustic resolution; more comprehensive tomographic techniques are needed. Model-based approaches could be used as well but they are time-consuming[15].

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In this study we developed a raster-scan optoacoustic mesoscopy system (R-SOM)[16], in this system we are using a high frequency, spherically focused ultrasonic detector in order to improve the resolutions achieved in mesoscopic scale organisms without sacrificing the penetration depth of the system. This is necessary in order to monitor these organisms, where the size of the object ranges from 1-5 mm, or where the inner part of the organisms is not accessible to other techniques in a non-invasive way. Such organisms include zebrafish, drosophila melanogaster, tumor development in mice, studying the microenvironment of a disease, and many more. In the case of the zebrafish, and the drosophila melanogaster, such a system would aid in studies related to the development of these organisms on a system-level.

2. METHODS

2.1 Hardware

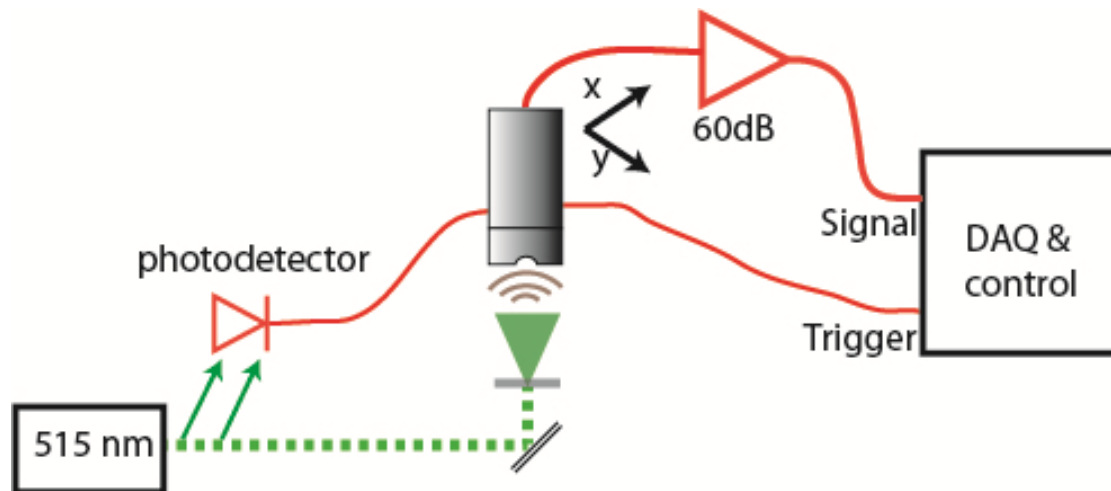


Figure 1: schematic of the system.

The center part of the system is the high frequency spherically focused detector, with center frequency of 78 MHz, and bandwidth from 25-125 MHz. The focal distance is 1.76 mm, and the f-number is approximately equal to 1 (SONAXIS, Besancon, France). This increases the sensitivity of the signals in the focus, and the angular coverage for the signals outside of the focus. We excite the sample in a trans-illumination fashion, with a broad illumination to increase the coherence between the signals generated from the same object at the different positions in the reconstruction. The detector is scanned in a continuous mode along one axis, and discrete fashion along the other axis, to increase the scanning speed. For the excitation we employed initially two lasers, an OPO with a pulse width of 8 ns, tunable in the 700-900 nm range, and works at a repetition rate of 20 Hz (Opotek, Carlsbad, CA, USA). As well as a fast 515 nm solid-state laser, with 1.8 ns pulse widths, working at a repetition rate of 1.4 kHz (Flare HP PQ, 2k-500, Innolight, Germany). Comparing the signals generated using the two lasers reveals the necessity to use the short pulse ns laser; the short pulse ns laser is capable of generating the high frequencies necessary for imaging the smaller structures. This could be attributed to the stress relaxation, where stress diffuses much faster from smaller structures, leading to a loss in resolution unless the pulse width of the laser is short enough[16, 17].

The measured signals were amplified with a low noise amplifier that has 63 dB of amplification, and a bandwidth of 500 MHz (AU-1291, Miteq Inc., USA), afterwards the signals were digitized with a fast data acquisition card, at a sampling rate of 500 MSps, and 12 bits (Gage-applied, Canada). The measured signals were transferred to a personal computer with a graphical processing unit, and filtered between 25-125 MHz to reject noise from outside of the measurement bandwidth.

2.2 Reconstruction

For the reconstruction a punctual detector was assumed in the focus of the detector, outside of the focus the reconstruction was performed using beam-forming with a dynamic aperture[18, 19]. Where the radius of the reconstruction aperture, r_{bf} , is proportional to the distance from the focal plane, z_F , to the plane where the point is being reconstructed, z_p :

$$r_{bf} = |z_F - z_p| \tan^{-1}(D/2F) \quad (1)$$

where: D is the diameter of the transducer, and F is the focal distance.

This way the signals are reconstructed from within a cone, this full 3-dimensional reconstruction has a better signal-to-noise ratio (SNR) than 2-dimensional reconstructions, where the reconstruction is performed along a single b-scan. Doing the reconstruction in this way also improves the out of plane rejection of signals, so signals at one plane do not contaminate the other planes. Finally, a full 3-dimensional reconstruction is isotropic in the plane, meaning that if you take the full-width at-half-maximum (FWHM) of a point, along the x-axis, or along the y-axis they are equal to each other.

2.3 Phantoms

Two phantoms were used; the first phantom has a single $10\text{ }\mu\text{m}$ at the focus of the detector, the microsphere is made of polystyrene, such a small microsphere is capable of producing signals with frequencies up to 150 MHz [17], these frequencies are above the center frequency of our ultrasonic detector reported by the manufacturer. The microsphere was embedded in turbid agar, where 1.3% of agar (per volume) is mixed with intralipids, this diffusive agar acts as a diffuser for the excitation, this is necessary to illuminate the sample as homogeneously as possible. Such kind of agar phantom also is similar to biological tissues in terms of optical and acoustic properties.

In phantom 2, many microspheres ($10\text{ }\mu\text{m}$), were embedded in turbid agar, the phantom was raster-scanned in the xy-plane. This phantom is used to characterize both the resolution and the penetration depth of the system. The phantom is excited in a trans-illumination mode through a turbid medium; this is to homogenize the illumination.

2.4 Biological samples

To showcase the raster-scan optoacoustic mesoscopy (R-SOM)[16], we imaged a genetically modified drosophila melanogaster fly, and a genetically modified drosophila melanogaster pupae, both of them were genetically modified in to express GFP from the wings in the fly, and from the future location of the wings in the pupae.

The drosophila melanogaster is a standard organism used in developmental biology, some of the reasons for this is the small size of the drosophila, its extremely fast lifecycle, where a new generation could be generated within two weeks, all of the genes are known, and could be easily mapped, this way any organ could be engineered in such a way that it expresses a certain fluorescent protein. Usually it is imaged with optical microscopy techniques at the initial stage, such as the embryonic stages, at later stages, when the drosophila melanogaster goes into the pupal stage it becomes very opaque, and the traditional optical microscopy techniques, such as two photon laser scanning microscopy, confocal microscopy, and selective plane illumination microscopy, cannot image the drosophila melanogaster at high-resolution. The reason for this is the high diffusion inside the pupae.

3. RESULTS

3.1 System characterization

For characterizing the bandwidth of the transducer we measured a single microsphere in the focus of the ultrasonic detector, this was done twice, once with an 8 ns laser, and once with a 1.8 ns laser. Theoretically the 8 ns laser should not be enough to generate the high frequencies necessary to image the small structures with enough resolution. From the measurement of the microsphere in the focus the Fourier transform is taken. The same is repeated with the measurement from the microsphere using the 1.8 ns laser.

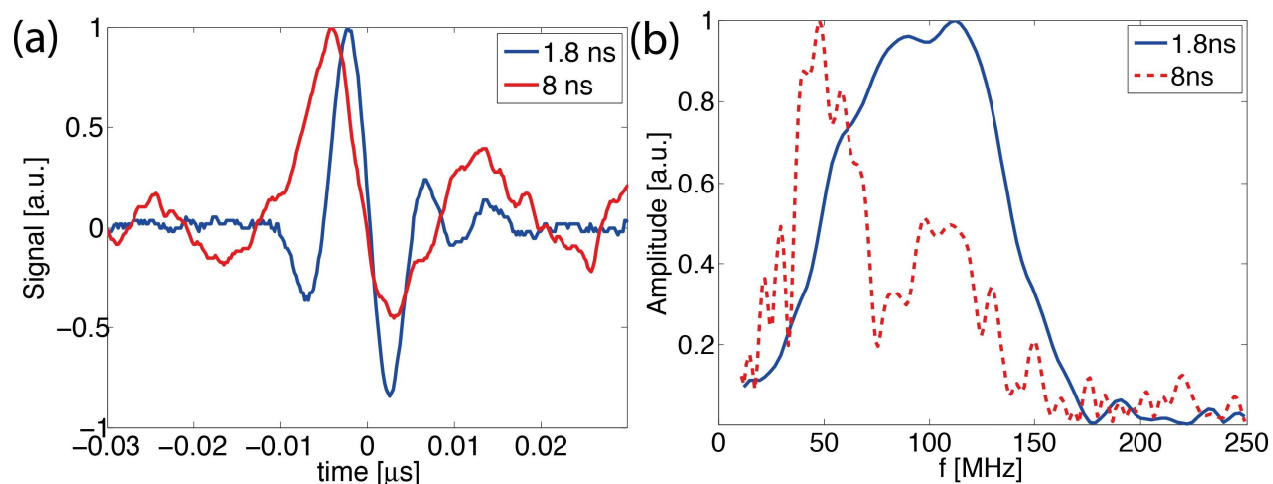


Figure 2: comparison of the signal generated from a 10 μm black microsphere in the focus of the ultrasonic detector using 8 ns and 1.8 ns laser pulses (a), amplitude of the Fourier transform of the time-domain shown in (a) (b)

From Fig.2, in order to be able to properly generate, and measure the high frequency components necessary for generating high-resolution images we have decided to use the 1.8 ns laser.

3.2 Resolution and depth of penetration

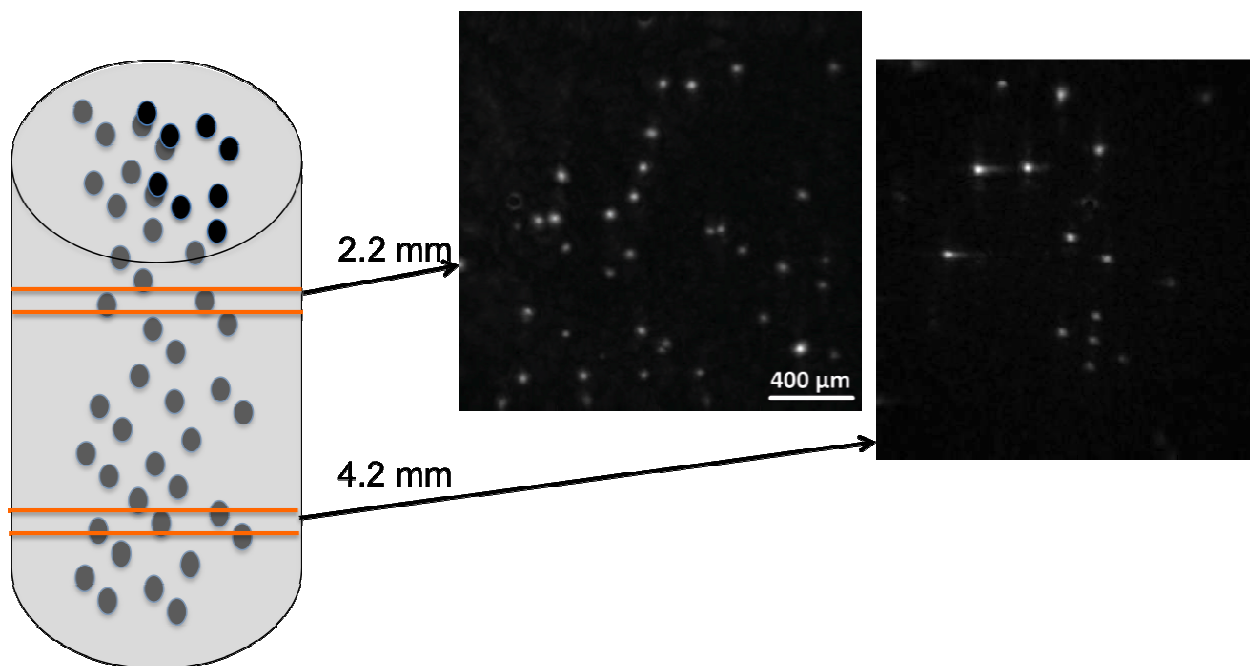


Figure 3: Characterization of the resolution and the penetration depth of the system; The in-plane resolution is homogeneous and isotropic, and the full width at half maximum of the 10 μm spheres is around 30 μm . This resolution is also achievable at depths reaching 4-5 mm.

We have characterized the resolution by using 10 μm black spheres embedded in diffusive agar, the resolution was the full width at half maximum, from the different depths we took 100 μm slices, and then took the maximum intensity projection of it along the z-axis, for all the depths the maximum intensity projection was around 30 μm , and it was

isotropic in all the direction, Fig. 3, also the axial resolution was measured as the full width at half maximum of the maximum intensity projection along the x-axis. The axial resolution was around $7\text{ }\mu\text{m}$, and homogenous at the different depths. The axial resolution is also consistent with the bandwidth of the transducer 25-125 MHz.

3.3 Imaging of Biological samples

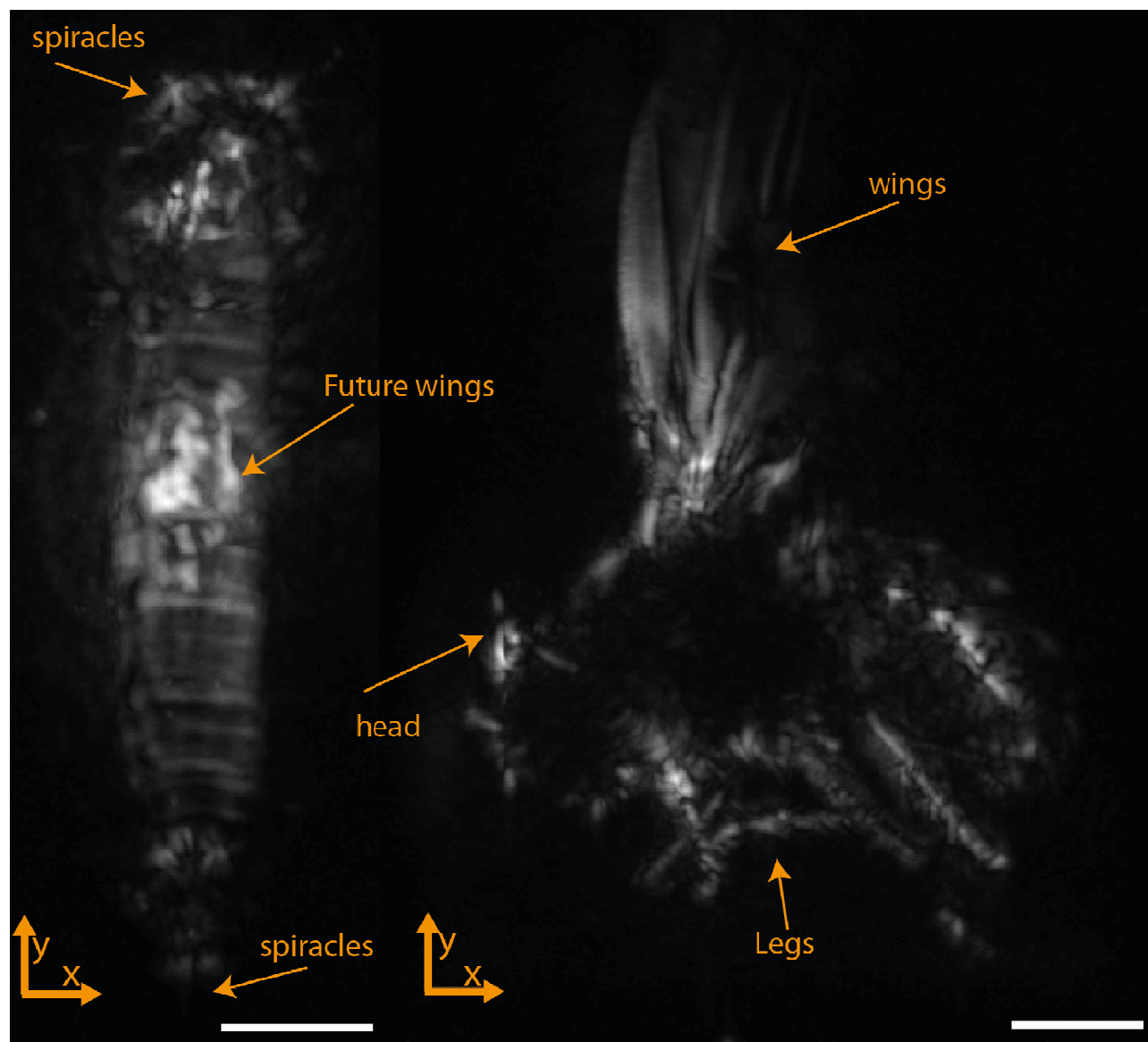


Figure 4: imaging of a genetically modified *Drosophila melanogaster* reveals both anatomical and molecular details, in the pupae the shape of the pupae and the spiracles could be observed, as well as the GFP in the future location of the wings. In the fly, the head, the legs, the body, and the wings, which express GFP strongly, are all visible.

From the measurements of the genetically modified *Drosophila melanogaster* in the raster scan optoacoustic mesoscopy several details could be observed, both anatomical and molecular. Anatomical details appear such as the shape, the spiracles in the pupae, the legs, the head, and the body in the fly. And Molecular details such as the future location of the wings in the pupae, and the wings in the fly, which express GFP.

4. CONCLUSIONS

We have developed a raster-scan optoacoustic mesoscopy (R-SOM) system, capable of generating 3-dimensional images of biological specimen with resolutions reaching 30 μm in-plane, and 7 μm axially. The resolution is moreover isotropic in-plane and homogeneous at the different depths. The system is capable of imaging to depths reaching up to 5 mm. The resolution and the penetration depths were characterized using 10 μm black microspheres embedded in diffusive agar at many different depths.

Imaging in *drosophila melanogaster* reveals the suitability of the system in imaging both anatomical and molecular features, such as imaging of the GFP expression in the wings in the fly, and in the future location of the wings in case of the pupae.

The presented system should be capable of imaging the development in biological organisms in *drosophila melanogaster*, and in the zebrafish among others. Combining the system with tunable fast lasers should enable higher sensitivity and multispectral imaging of biological samples of interest, enabling the imaging and the separation of multiple biomarkers and molecular markers simultaneously.

Further improvement of the limited view of the system should improve the images, this could be done by using a combination of rotation and translation[20], point detectors based on silicon bragg gratings[21], and the use of speckle properties of the illumination light[22].

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