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Development of multispectral optoacoustic imaging
for high resolution small animal visualization

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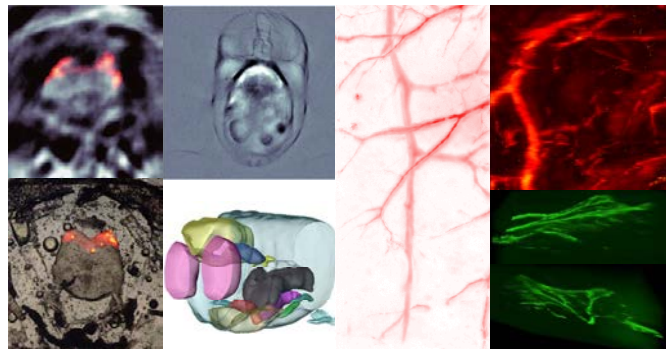
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vorgelegt von
Rui Ma
aus Pingyao, China

Meiner Familie gewidmet

I HAVE HEARD ARTICULATE SPEECH BY SUNLIGHT! I HAVE HEARD A RAY OF THE SUN
LAUGH AND COUGH AND SING!

...I HAVE BEEN ABLE TO HEAR A SHADOW AND I HAVE EVEN PERCEIVED BY EAR
THE PASSAGE OF A CLOUD ACROSS THE SUN'S DISK.

ALEXANDER GRAHAM BELL
FEBRUARY 26, 1880

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Abstract

Optoacoustic imaging is a newly developed, noninvasive, nonionizing imaging method that combines the advantages of high optical contrast and high resolution with the large penetration depth of ultrasound imaging. A multispectral optoacoustic tomography (MSOT) system developed in our lab successfully enables 3D whole-body animal imaging with high resolution. It also three-dimensionally resolves the location of fluorescent proteins in transgenic zebrafish using a multispectral imaging technique. The fast scanning speed of this *in vivo* imaging modality, as demonstrated herein in zebrafish, opens the door for the longitudinal studies of the evolution and development of diseases. A second newly developed imaging system is a real-time optoacoustic microscope providing 5 frames/s scanning speed at a 4 mm scan range; an even faster frame rate is feasible by scanning a smaller range or using a higher repetition rate laser. The system enables 3D imaging of small animals and the human palm and has the potential for clinical practices in skin cancer imaging, imaging of the nailfold capillary for the study of microcirculation, diagnosis in ophthalmology, etc.

Publications, Proceedings and Conference Talks

Publications and Proceedings

- [1] Daniel Razansky, Martin Distel, Claudio Vinegoni, [Rui Ma](#), Norbert Perrimon, Reinhard W. Köster, and Vasilis Ntziachristos, “Multispectral opto-acoustic tomography of deep-seated fluorescent proteins *in vivo*,” *Nature Photonics* **3**, p. 412, Jul. 2009.
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- [3] [Rui Ma](#), Vasilis Ntziachristos, and Daniel Razansky, “Continuous acquisition scanner for whole-body multispectral optoacoustic tomography,” *Proceedings of SPIE* 7564, p. 756429, Feb. 2010.
- [4] X. Luis Dean Ben, [Rui Ma](#), Daniel Razansky, and Vasilis Ntziachristos, “Statistical Approach for Optoacoustic Image Reconstruction in the Presence of Strong Acoustic Heterogeneities,” *IEEE Transactions on Medical Imaging* **30**, p. 401, Feb. 2011.
- [5] [Rui Ma](#), Sebastian Söntges, Shy Shoham, Vasilis Ntziachristos, and Daniel Razansky, “Fast scanning coaxial optoacoustic microscopy,” *Biomedical Optics Express* **3**, p. 1724, Jul. 2012.
- [6] [Rui Ma](#), Martin Distel, X. Luis Dean Ben, Vasilis Ntziachristos, and Daniel Razansky, “Non-invasive whole-body imaging of adult zebrafish with optoacoustic tomography,” *Physics in Medicine and Biology* **57**, p. 7227, Oct. 2012. Chosen as a Featured Article.

Talks and Posters

- [7] Talk presented at European Conferences on Biomedical Optics (ECBO), Munich, Germany, [Rui Ma](#), Vasilis Ntziachristos, and Daniel Razansky, “Multispectral optoacoustic tomography (MSOT) scanner for whole-body imaging of small animals and biomarkers”, 2009.

[8] Talk presented at SPIE BIOS, San Francisco, USA, Rui Ma, Vasilis Ntziachristos, and Daniel Razansky, “Continuous acquisition scanner for whole-body multispectral optoacoustic tomography”, 2010.

[9] Poster presented at World Molecular Imaging Congress (WMIC), San Diego, USA, Rui Ma, Martin Distel, X. Luis Dean Ben, Vasilis Ntziachristos, and Daniel Razansky, “Optoacoustic imaging - an alternative tool for noninvasive whole-body imaging of adult zebra’sh”, 2011. Selected for the Poster Session walk-through.

CHAPTER 1

Introduction

Optoacoustic imaging is a noninvasive, nonionizing imaging technique based on the photoacoustic effect - the generation of an acoustic wave by the absorption of a short pulsed electromagnetic wave. The term optoacoustic (or photoacoustic) imaging is generally used when one uses visible or near infrared light pulses [1–8], while using electromagnetic waves in the radio-frequency or microwave range is referred to as thermal acoustic imaging [9–11]. The discovery of the photoacoustic effect dates back to 1880 when it was discovered by Alexander Graham Bell. He demonstrated that sound can be generated by focusing shuttered sunlight onto a selenium cell. Due to technological limitations, it is not until the mid 1990s [12, 13] that pioneer researchers started to use the photoacoustic effect as a tool for imaging. Optoacoustic imaging combines the advantages of optical imaging and ultrasound imaging. Unlike the aforementioned imaging modalities that probe and detect biological tissue properties using the same waveform, optoacoustic imaging employs optical waves as a probe and detect ultrasound/pressure waves. In this way, optoacoustic imaging overcomes the shortcoming of ultrasound imaging, i.e. very low soft tissue contrasts, and obtains good optical contrasts; in particular, optoacoustic imaging utilizes widely developed optical probes for optical molecular imaging modalities to enhance the optical absorption contrasts. Furthermore, the scattering of ultrasound waves is approximately two orders of magnitude lower than the optical scattering in tissue, thus when detecting the generated optoacoustic wave, one can image deeper in tissue than conventional optical imaging methods. Additionally the amplitude of the generated acoustic signal is generally proportional to the absorbed energy density, which is a multiple of light fluence and tissue absorption coefficient. When assuming uniform illumination, the amplitude of the generated acoustic wave is proportional to the tissue absorption, thus, detecting acoustic waves instead of optical waves can be quantitative and can image deep-seated tissue with ultrasound resolution. With these advantages, optoacoustic imaging can visualize blood vessels, tumors in early stages, and functional cerebral hemodynamic changes due to the natural absorption contrast of blood cells, and can perform molecular imaging with the aid of absorption enhancement from optical contrast agents such as exogenously administered fluorescent probes, biomarkers, nanoparticles, and other intrinsic and exogenous chromophores.

1.1 Enabling technologies for optoacoustic imaging

The development of optoacoustic imaging relies on many technological advances. Several considerations need to be addressed in order to perform optoacoustic imaging:

- A laser with high power is essential since the amplitude of the generated acoustic signal is proportional to the illumination intensity.
- In order to get high spatial resolution, short pulses are required so that the influence of thermal diffusion and thermal expansion can be neglected and the detected

optoacoustic signal (pressure wave) is proportional to the deposited energy density.

- Sensitive ultrasound transducers are necessary. Underlaser power limitations imposed by laser safety standards, the generated pressure wave is on the order of 1 kPa on the biological tissue surface and an order of magnitude lower in deep tissue. Pressure waves originating from deep tissue amount to only a few pascals when reaching the detector.
- Transducers with a high center frequency and broad bandwidth are required for the detection of small objects with high resolution and for imaging targets over several scales.
- A high resolution data acquisition card/oscilloscope is needed. This is especially important for very small object detection in which acoustic waves with high frequency content are measured. According to the Nyquist theorem, the sampling rate has to equal at least twice the maximum frequency of the analog signal. Taking into account the transducer bandwidth, a data acquisition card with at least 150 MS/s sampling rate is necessary for high frequency transducers (> 50 MHz).

With the above mentioned technological developments, optoacoustic imaging has been drawing researchers' attention for the last two decades. Different imaging modalities have been developed for various applications, like monitoring functional changes, breast and skin tumors, and small animal physiological variations. For these developments, fast imaging performance is required. Using ultrasound array detectors is one of the solutions to accelerate data acquisition time. However, such array systems and corresponding multiple data acquisition systems are expensive, especially when applied to high resolution imaging. The price of both high frequency transducer arrays and high sampling rate data acquisition hardware are much more expensive than a system with a single-element transducer. Also, different array systems have their own bandwidths, which limit the imaging depth and resolution and can only apply to animal types with similar sizes. Furthermore, no commercial ultrasound transducer arrays are available in the market (with center frequencies exceeding 50 MHz). Using a single element transducer, on the other hand, is relatively cheap and it requires only one data acquisition channel. In addition it is very flexible to have several transducers with different center frequencies to adapt for different animal sizes. Therefore, it is cost effective to build imaging systems using a single-element transducer, which is low in cost and makes it flexible to change transducers with different penetration depths and different resolutions.

1.2 Comparison of different imaging modalities

Nowadays due to the progress of modern technologies, many different imaging modalities are developed focusing on different applications. However there is no perfect imaging

modality and each modality has its pros and cons. Here, we will compare optoacoustic imaging with other noninvasive imaging modalities in two categories. The first category lists commercialized non-optical imaging modalities for preclinical/clinical applications. The second category enumerates optical imaging modalities, which are vastly growing due to the advances of gene techniques and developments of bio-markers.

1.2.1 Optoacoustic imaging and non-optical imaging modalities

Unlike X-ray CT, single-photon emission computed tomography (SPECT) and positron emission tomography (PET) that utilize ionizing radiation with a subsequent risk of cancer, optoacoustic imaging is a nonionizing, noninvasive imaging modality that applies visible or near-infrared light for the imaging purpose. Every imaging modality has advantages to specific diseases and has limitations to other imaging applications as well. Table 1.1 compares optoacoustic imaging with commonly used commercial imaging modalities.

Modality	Optoacoustic	Ultrasound	X-ray CT	MRI
Contrast	Anatomical, functional, molecular	Anatomical, functional	Anatomical	Anatomical
Safety	Good	Good	Ionizing radiation	Good
Contrast in soft tissues	Excellent	Poor	Poor	Excellent
Resolution	0.5 - 200 μm ¹	50 μm - 3 mm ²	50 μm - 1 mm	50 μm - 1 mm ³
Maximal depth	0.5 - 5 cm	1 - 25 cm	Whole-body	Whole-body
Speckle noise	None	Strong	None	None
Cost	Medium	Low	Medium	High

Table 1.1: Performance comparison of non-optical imaging modalities with optoacoustic imaging systems.

1.2.2 Optoacoustic imaging and optical imaging methods

Optical imaging modalities are in general not expensive and a variety of contrast agents were developed for optical imaging. However, there are two challenges in the optical imaging community to overcome: the diffraction limited spatial resolution and the diffusion

¹The resolution of an optoacoustic imaging system depends on the optical beam size, the center frequency and bandwidth of ultrasound transducers.

²The resolution of an ultrasound imaging system depends on the center frequency and the bandwidth of ultrasound transducers.

³The resolution of an MRI imaging system depends on the strength of the magnetic field.

limited penetration depth with satisfying spatial resolution. To overcome the diffraction limit, stochastic optical reconstruction microscopy (STORM) [14], Stimulated Emission Depletion microscopy (STED) [15] and Structured illumination microscopy (SIM) [16], etc are developed with tens of nanometers resolution. To increase the penetration depth with high resolution, two-photon excitation microscopy [17–19] was invented with up to 1 millimeter penetration depth [18]. In optical diffusive region (< 1 mm), optical coherence tomography (OCT) [20–22] was developed with micrometer-resolution and several millimeters penetration depth. Fluorescence molecular tomography (FMT) [23–25] and diffusive optical tomography (DOT) [26] further extend the penetration depth to tens of millimeter, however the spatial resolution is not satisfying. Recently multimodality hybrid imaging systems with the combination of commercially available imaging modalities (such as X-ray CT, PET and MRI) and fluorescence molecular tomography draw researchers' attention [27–32]. These multimodality hybrid systems either enable the validation of multimodality targeted molecular probes that are promising for intra-operative fluorescence imaging for future surgical guidance or use acquired X-ray CT images as priors to improve the optical tomography reconstruction [27] with up-to-date $800\text{ }\mu\text{m}$ resolution at 20 mm depth [28].

Optoacoustic imaging, on the other hand, can perform multispectral imaging and at the same time does not have low resolution problems at large penetration depths, because the scattering of ultrasound waves is much lower than the optical scattering in tissues. Also, optoacoustic imaging has a broad range of spatial resolutions and penetration depths. The spatial resolution can vary from hundreds of nanometers (with a penetration depth of tens of microns) to ~ 3 millimeters (up to 60 mm [33]).

1.3 Thesis outline

This thesis presents two high-resolution optoacoustic imaging systems utilized for small animal and human skin imaging with fast imaging speed, representing a cost effective solution using single element transducers. The first is a multispectral optoacoustic tomography system that can image and display a 2D slice within 9 seconds with a penetration depth approaching ~ 30 mm. The 3D whole-body imaging capability further strengthen the system performance. Since fast response changes require real-time imaging, we developed a high resolution optoacoustic microscopy system that can achieve real-time data acquisition and display 5 frames/s at a scan range of 4 mm. The thesis consists of six parts.

- This chapter introduces the necessary technological advances for optoacoustic imaging, comparison with nonoptical and optical imaging modalities, and the structure of the thesis.
- The second chapter describes the theory and reconstruction principle of optoacoustic computed tomography and microscopy systems.

- The third chapter shows the design of a multispectral optoacoustic tomography (MSOT) system using a single element transducer with a continuous acquisition approach. It achieves a 2D image acquisition within 9 seconds. The reconstruction algorithms are introduced and compared using different types of transducers, namely PVDF (polyvinylidene fluoride) and PZT (lead zirconate titanate) transducers. A statistical correction approach to reduce the image distortion is introduced. The system is then calibrated using a black-dyed 10 μm sphere showing a 32 μm in-plane resolution and 155 μm vertical resolution. The performance of the system is also compared with the conventional averaged data acquisition approach using tissue mimicking phantoms and an adult zebrafish. The system is then used to image objects with different sizes ranging from drosophila pupa of 800 μm diameter to a mouse larger than 20 mm. Then we show the 3D imaging capability of the system and its ability to detect molecular probes using a multispectral imaging method.
- Chapter 4 presents small animal imaging applications using the MSOT system. The first section shows selective optoacoustic slices of a mouse *post mortem* and 3D whole-body imaging of an adult zebrafish based on the intrinsic contrasts arising from the optical absorption variation of tissue structures. The second section presents resolved fluorescent protein location in transgenic zebrafish with mCherry expressed in the brain area (zic4 zebrafish). Fluorescent histology verifies the imaging results and 3D mCherry location, and brain segmentation is presented.
- Chapter 5 illustrates the setup of a multimode (MM) fiber based real-time high resolution optoacoustic microscopy system using a single element spherically focused transducer. We performed real-time imaging of flowing ink in a polythene tube embedded in a chicken tissue, with a frame rate of 5 frames/s at a scan range of 4 mm. The system is used to image small animals such as pupa, fruit flies and mice. At the end of the chapter we further extend the system applications to human skin imaging, including the author's palm, the nailfold of the left index finger and the left opisthenar, the results of which are of interest for clinical applications such as the diagnosis of human skin cancer and the investigation of nailfold capillaries.
- Finally, a brief summary of the thesis is given in chapter 6.

CHAPTER 2

Theory and reconstruction algorithms for
optoacoustic imaging

In this chapter we will briefly describe the theoretical background and reconstruction algorithms for optoacoustic imaging. First the quantitative relationship between the generated initial optoacoustic pressure and the energy and illumination size of a short laser pulse will be introduced. Then we will introduce the forward analytical solution to optoacoustic equation, which is used to compute the propagation of optoacoustic waves through an arbitrarily shaped optoacoustic source.

Subsequently reconstruction algorithms and method for optoacoustic computed tomography and microscopy systems will be presented. We will first introduce a universal back-projection algorithm for optoacoustic computed tomography and simplified models for this algorithm. Then we will describe a statistical correction algorithm to reduce the image distortion. At the end of the chapter we will present an image reconstruction method for optoacoustic microscopy.

2.1 Initial optoacoustic pressure

For the response of a tissue upon a short laser pulse excitation, the fractional volume expansion can be expressed as [34–36],

$$\frac{dV}{V} = -\kappa p + \beta T \quad (2.1)$$

where p and T denote the changes in pressure and temperature, $\kappa = -\frac{1}{V} \left(\frac{\partial V}{\partial p} \right)_T$ is the isothermal compressibility coefficient ($\sim 5 \times 10^{-10} Pa^{-1}$ for soft tissues) and $\beta = \frac{1}{V} \left(\frac{\partial V}{\partial T} \right)_p$ is the isobaric coefficient of thermal expansion ($\sim 4 \times 10^{-4} K^{-1}$ for muscle).

In order to effectively generate optoacoustic signals and obtain high-resolution optoacoustic images, in practice nanosecond pulses are used. The excitation of optoacoustic pressure is under the stress confinement condition when the laser pulse duration (τ_p) is shorter than the time for the stress to transmit the heated area (τ_s),

$$\tau_p < \tau_s = \frac{d_c}{v_s} \quad (2.2)$$

where v_s is the speed of sound ($1.5 mm/\mu s$) and d_c is the characteristic size of the tissue being heated (namely the penetration depth of the laser pulse or the size of the imaged object). For instance, a $15 \mu m$ spatial resolution is converted as $\tau_s \sim 10 ns$, thus τ_p must be less than 10 ns.

Under the stress confinement condition, the fractional volume expansion $\frac{dV}{V}$ is insignificant and the initial local pressure rise p_0 after the short laser pulse can be determined as,

$$p_0 = \frac{\beta T}{\kappa} \quad (2.3)$$

If we assume that all the absorbed energy from a short laser pulse is converted to heat, the temperature rise can be expressed as,

$$T = \frac{H}{\rho C_V} = \frac{\mu_a U}{\rho C_V} \quad (2.4)$$

where ρ denotes the mass density and C_V the specific heat capacity at constant volume ($\sim 4 J g^{-1} ^\circ C^{-1}$). H is the deposited optical energy density (mJ/cm^3), which is a product of the optical absorption coefficient of the heating/optoacoustic source μ_a (cm^{-1}) and the optical fluence U (mJ/cm^2). Thus the initial optoacoustic pressure rise can be rewritten as,

$$p_0 = \frac{\beta \mu_a U}{\kappa \rho C_V} = \Gamma \mu_a U \quad (2.5)$$

where Γ is the Grueneisen parameter expressed by $\frac{\beta}{\kappa \rho C_V}$. Notice that the initial pressure is proportional to the absorption coefficient of the tissue and the light fluence, if a uniform light distribution is assumed, the initial optoacoustic pressure is directly a representative of the tissue absorption distribution.

For soft tissues, Γ is estimated by

$$\Gamma(T_0) = 0.0043 + 0.0053 T_0 \quad (2.6)$$

At body temperature Γ is approximately 0.20. The average optical absorption coefficient of soft tissues is 0.2 cm^{-1} , under a short laser pulse with a fluence of 20 mJ/cm^2 , which is under the laser safety standards [37], the temperature rise is approximately 1 mK and the generated optoacoustic pressure at body temperature is approximately 8 mbar .

2.2 Forward analytical solution to optoacoustic equation

In soft tissues, the generation and propagation of an optoacoustic wave is formulated by the following equation [34–36],

$$\left(\nabla^2 - \frac{1}{v_s^2} \frac{\partial^2}{\partial t^2} \right) p(\vec{r}, t) = -\frac{\beta}{\kappa v_s^2} \frac{\partial^2 T(\vec{r}, t)}{\partial t^2} \quad (2.7)$$

where $p(\vec{r}, t)$ denotes the optoacoustic pressure at location $\vec{r}$ and time t . When the laser pulse duration τ_p is shorter than the heat diffusion time τ_{th} , the thermal confinement condition is met,

$$\tau_p < \tau_{th} = \frac{d_c^2}{4\alpha_{th}} \quad (2.8)$$

here α_{th} is the thermal diffusivity ($1.4 \times 10^{-3} \text{cm}^2/\text{s}$ for soft tissues). With a $15 \mu\text{m}$ object, $\tau_{th} \sim 0.4 \text{ms}$, which is much longer than a nanosecond laser pulse. Under the thermal confinement condition the optoacoustic equation can be rewritten as,

$$\left(\nabla^2 - \frac{1}{v_s^2} \frac{\partial^2}{\partial t^2} \right) p(\vec{r}, t) = -\frac{\beta}{C_P} \frac{\partial H(\vec{r}, t)}{\partial t} \quad (2.9)$$

The forward analytical solution to equation 2.9 is described as,

$$p(\vec{r}, t) = \frac{1}{4\pi v_s^2} \frac{\partial}{\partial t} \left[\frac{1}{v_s t} \int_{S'} p_0(\vec{r}') \delta\left(t - \frac{|\vec{r} - \vec{r}'|}{v_s}\right) dS' \right] \quad (2.10)$$

where S' denotes the surface of the optoacoustic source, over which the integration is performed, $p_0(\vec{r}')$ is the initial optoacoustic pressure generated by a short laser pulse (shown in equation 2.5). We can use equation 2.10 to calculate generated optoacoustic pressure by an arbitrary optoacoustic source.

2.3 Image reconstruction for optoacoustic computed tomography systems

2.3.1 Universal back-projection algorithm

In practice, we use cylindrically focused ultrasound transducers in a closed circle to detect optoacoustic pressures ($p(\vec{r}, t)$) and the initial optoacoustic pressure ($p_0(\vec{r}')$) needs to be calculated by the detected optoacoustic pressures following an inverse solution to equation 2.10. However, there are no analytical inverse solutions available to equation 2.10. So far an approximate and fast algorithm to solve the inverse problem is the universal back-projection algorithm [38], which requires a flat frequency response of the ultrasound transducer being used.

$$p_0(\vec{r}') = \frac{1}{2\pi} \int_S \frac{p(\vec{r}, \bar{t}) - \bar{t} \partial p(\vec{r}, \bar{t}) / \partial \bar{t}}{|\vec{r}' - \vec{r}|^3} \vec{n}_0^s \cdot (\vec{r}' - \vec{r}) dS|_{\bar{t}=|\vec{r}'-\vec{r}|} \quad (2.11)$$

where $p_0(\vec{r}')$ is the initial optoacoustic pressure to be calculated, $p(\vec{r}, \bar{t})$ is the detected optoacoustic pressure at position $\vec{r}$ and time $\bar{t}$, S is the measurement surface, over which the integration is performed, $\vec{n}_0^s$ is a vector pointing outwards and normal to the closed surface S . Figure 2.1 shows how to use the universal back-projection algorithm to calculate the initial optoacoustic pressure/source. In our particular cylindrical scanning geometry, where the detector is focused in the imaging plane, we apply Eq. 2.11 in its reduced two-dimensional form, whereas the 3D imaging is achieved by scanning 2D images along the

direction perpendicular to the 2D plane.

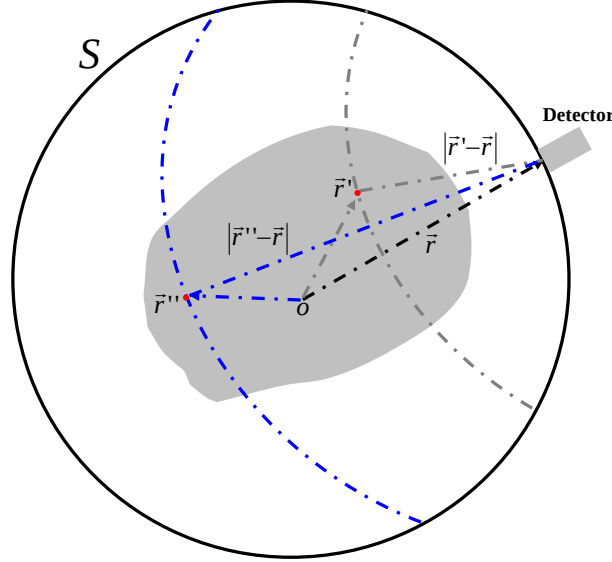


Figure 2.1: Scheme of the universal back-projection algorithm. Two red spots are optoacoustic sources located at $\vec{r}'$ and $\vec{r}''$. The detector is located at position $\vec{r}$. The detected optoacoustic pressure at time $|\vec{r}' - \vec{r}|/v_s$ will be assigned to a gray arc-shaped curve and at time $|\vec{r}'' - \vec{r}|/v_s$ to a blue arc-shaped curve. The integration is performed along the detector movement surface S .

Empirical measurements show that $p(\vec{r}, \bar{t}) \ll \bar{t} \partial p(\vec{r}, \bar{t}) / \partial \bar{t}$ (in general 3 orders of magnitude difference), therefore the universal back-projection algorithm can be simplified as [39],

$$p_0(\vec{r}') = \frac{1}{2\pi} \int_S \frac{-\bar{t} \partial p(\vec{r}, \bar{t}) / \partial \bar{t}}{|\vec{r}' - \vec{r}|^3} \vec{n}_0^s \cdot (\vec{r}' - \vec{r}) dS|_{\bar{t}=|\vec{r}' - \vec{r}|} \quad (2.12)$$

Since in reality the ultrasound transducer is scanned in a discrete manner, for image reconstruction we use the following simplified back-projection algorithm when all the constants are dropped,

$$p_0(\vec{r}') = \sum_i -\bar{t} \partial p_i(\vec{r}, \bar{t}) / \partial \bar{t}|_{\bar{t}=|\vec{r}' - \vec{r}|} \quad (2.13)$$

where i denotes the i th ultrasound transducer position and $p_i(\vec{r}, \bar{t})$ is the detected optoacoustic pressure from the i th transducer. In practice either a transducer array is used to cover the measurement surface S or a single element transducer is used and scanned over the surface S .

It is further noted that the initial optoacoustic image represents a map of energy deposition in tissue rather than the distribution of optical absorption, which has a correlation

with the actual dimensions of optical absorbers. In other words, the image reconstructed is a product of the optical absorption coefficient and the local light fluence $U(\vec{r})$ within the tissue (see equation 2.5). Thus, targets in deep tissue may appear weaker than targets having similar optical absorption but located close to the illuminated surface. Several methods have been recently suggested for quantitative extraction of the optical absorption in non-uniform illumination configurations, including iterative approaches [40, 41], sparse signal decomposition [42], and analytical normalization [43]. The latter approach was adopted in our work mainly due to its simplicity.

2.3.2 Delay-and-sum algorithm

Another reconstruction possibility, which is convenient in practice, is to consider only the contribution of pressure in equation 2.11 [44],

$$p_0(\vec{r}') = \sum_i p_i(\vec{r}, \bar{t})|_{\bar{t}=|\vec{r}'-\vec{r}|} \quad (2.14)$$

This algorithm is also called the delay-and-sum reconstruction algorithm. Most of the reconstructed images from our tomography system are based on the delay-and-sum algorithm since the signal-to-noise ratio (SNR) is better¹.

2.3.3 Statistical correction to reduce the image distortion

Both the delay-and-sum and the universal back-projection algorithms are based on the assumption that the generated optoacoustic signals do not undergo reflection and scattering, which is not the case with heterogeneous tissues. Hence a statistical correction approach can be applied to reduce the artifacts produced by reflection and scattering. Figure 2.2 illustrates the principle of the correction method. We simplify an imaged object/optoacoustic source as a round object and assume that the optical absorption (a product of the optical absorption coefficient and the light fluence) within the object has a uniform distribution. The ultrasound transducers detect time-domain signals. When there are no acoustic scattering or reflection events, the signal generated at a point source is detected at the time instant corresponding to the time-of-flight between the source and the measuring point for a wave propagating along a straight path. However, when acoustic reflection or scattering takes place, the propagation path changes so that the time-of-flight varies accordingly. This is illustrated in figure 2.2. A wave generated at the red point on the solid curve and propagating directly to a transducer may be measured at the same instant as a wave generated inside the dashed region undergoing acoustic reflection or scattering as long as the propagation distances are the same (green lines). If a uniform acoustic medium is assumed,

¹Which algorithm should be applied depends on the frequency response of the ultrasound transducer being used. Most of our optoacoustic images were acquired using PZT (lead zirconate titanate) transducers, which do not have a flat frequency response, therefore optoacoustic images reconstructed with the delay-and-sum algorithm have better image quality. Detailed explanation can be found in section 3.3.

the reconstruction along the straight line in Figure 2.2 may contain the contribution of waves generated inside the dashed region being scattered or reflected, and then artifacts may appear in the images. For a later instant in time, such as the instant corresponding to the dashed curve in figure 2.2, it is more likely that the signal is distorted as any wave generated within the gray area having the same propagating distance (dashed blue lines) may be detected at such an instant. In reference [45] we have shown that the probability of an optoacoustic signal being distorted due to scattered or reflected acoustic waves can be approximated as the ratio of the area in which the waves can be distorted (same time-of-arrival to the transducer) to the whole area of the object. Then, the reconstruction algorithm is modified by weighting the signals with the probability that acoustic waves are not distorted due to reflection or scattering. The statistical correction method can be applied to both of the universal back-projection and the delay-and-sum algorithms.

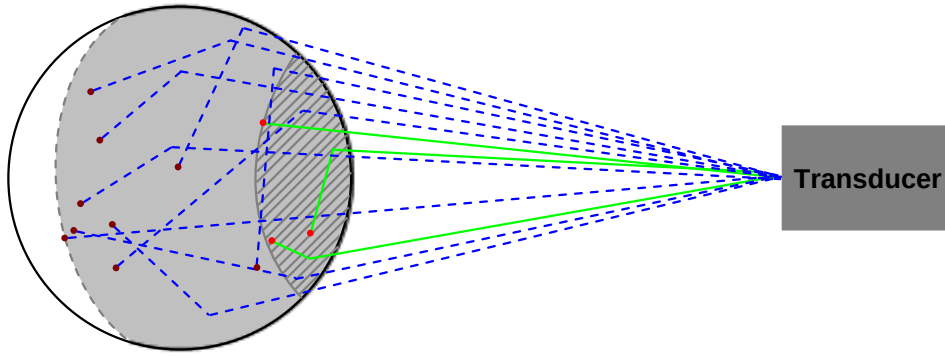


Figure 2.2: The principle of statistical correction algorithm.

2.4 Image reconstruction for optoacoustic microscopy systems

A spherically focused ultrasound transducer is commonly used for optoacoustic microscopy systems. It is a point-focus transducer (figure 2.3(a)), which is most sensitive to optoacoustic sources that are lying at the focal point and its vicinity along the transducer's central axis. Thus we assume that a detected optoacoustic signal from a spherically focused transducer contains only one-dimensional information along the central axis of the transducer. The position of optoacoustic sources are determined by the time-of-flight of the optoacoustic signal and the optical absorption distribution is approximated by the intensity of the signal. In this way, a 1D signal (A-line) shows optoacoustic sources in depth and in strength as illustrated in figure 2.3(a).

A 2D image is acquired by laterally scanning the transducer. The 2D optoacoustic

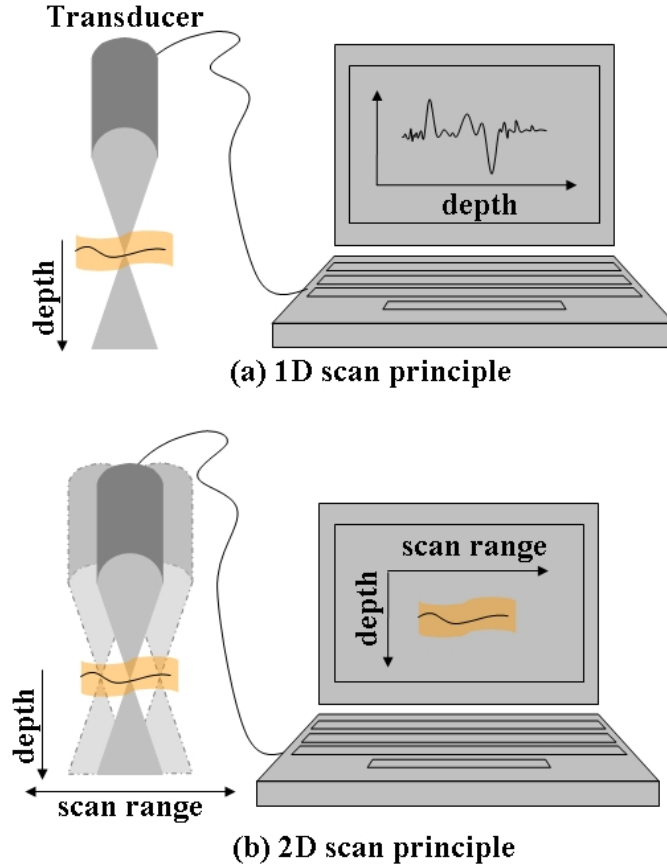


Figure 2.3: Principle of the image reconstruction using a spherically focused transducer. (a) 1D scan principle; (b) 2D reconstruction principle.

image is reconstructed with line-by-line assignment of 1D signals to the scan positions as shown in figure 2.3(b). The 3D optoacoustic image is created by scanning the transducer perpendicular to the 2D image planes.

In practice two stages are used to carry a transducer for the 3D image acquisition. Let's assume that an X-stage laterally scans 2D frames and a Y-stage moves perpendicular to the 2D planes. In order to keep the 2D frames parallel, the Y-stage stays at the same position until one 2D image-acquisition is finished, then it moves to the next position. Therefore a parallel frame-scan takes time. In order to improve the acquisition efficiency, we employ a sawtooth scan where the Y-stage moves continuously. The Y-stage must move slowly to substantially approximate the scan path as a parallel scan. The X-stage used in our imaging system is either a fast angular-scan rotor or a high-speed linear stage controlled by a piezomotor controller, both of which can fast acquire 2D frames under our current load with a frame rate exceeding 10.

CHAPTER 3

Design of a multispectral optoacoustic tomography (MSOT) system

In this chapter we present a fast-scanning, high-resolution, multispectral optoacoustic tomography (MSOT) system that has the capability of acquiring and simultaneously displaying a 2D optoacoustic image within 9 seconds and can perform whole-body small animal imaging with a $32\ \mu\text{m}$ in-plane resolution and a $155\ \mu\text{m}$ vertical resolution.¹

MSOT is a method capable of resolving chromophoric and fluorescent agents with molecular specificity through several millimeters to centimeters of tissue [2, 3]. The technique is based on the optoacoustic phenomenon, i.e. the generation of acoustic waves due to the thermoelastic expansion caused by the absorption of ultra-short optical pulses. Over the last couple of decades optoacoustics has been considered for tissue imaging, mainly for resolving vascular contrast and the corresponding physiological changes; in particular oxy- and deoxy-hemoglobin [4], superficial vascular anatomy [5], brain lesion and functional cerebral hemodynamic changes [6], cancer angiogenesis [7], blood volume and oxygen consumption changes and the associated dynamic and functional neuronal activities [8]. The introduction of endogenous or exogenous reporter agents with molecular specificity, such as fluorescent proteins and probes, further allows the propagation of this technique towards molecular imaging applications. In this case a promising approach is the use of multispectral illumination in order to differentiate specific spectral signatures of key reporter agents over the background tissue absorption [2, 3, 46–48]. In combination, imaging of physiological and molecular markers using optoacoustics has the potential to achieve high resolution optical imaging, through depths that go significantly beyond the capabilities of modern microscopy.

To perform molecular imaging of tissues and small biological organisms using MSOT, we developed an experimental prototype that is capable of multi-slice whole-body imaging. In contrast to many previous optoacoustic imaging studies that assumed plane illumination and homogeneous light distribution within appropriately selected superficial sections of tissue [5, 6, 8], this system employs multi-angle side illumination of the entire volume of interest. A major difficulty arising from optoacoustic imaging, especially when considering whole-body small animal imaging applications, is the need for a large dynamic range to capture signals generated in deep regions and the corresponding long acquisition times associated with recording signals from multiple spatial projections with sufficient signal-to-noise ratio (SNR), after averaging. This might impose severe limitations on imaging of some dynamic and longitudinal phenomena that require good temporal resolution, such as dynamic biodistribution of contrast agents, cell tracking, blood oxygenation monitoring, etc. To address this aspect we introduce herein a time-efficient continuous data acquisition method and demonstrate fast 360° data collection with sufficient SNR, without averaging optoacoustic signals. The method is showcased using a single cylindrically focused ultrasound detection element, but it is also appropriate for use with simultaneous multiple-element acquisition. The performance of the developed system is examined with tissue-mimicking phantom experiments and imaging of intact organisms of various sizes.

¹Most part of this chapter is published in Optics Express, **V** 17, p. 21414-21426, 2009 [1].

3.1 Experimental setup

Figure 3.1 is a photo of the multispectral optoacoustic tomography system. A simplified scheme of the experimental setup is displayed in figure 3.2. A tunable optical parametric oscillator (MOPO-700 series, Newport Corp., Mountain View, CA), pumped by a Q-switched Nd:YAG laser (Quanta-Ray Lab-Series 190-30 Newport), operating at its third harmonic (355nm), provides multi-wavelength illumination. The pulse duration of the laser is less than 10 ns and the repetition rate is 30Hz. The output laser beam is manipulated using an iris, slits, cylindrical/spherical lenses, and diffusers in order to improve illumination uniformity and adapt the incident beam shape to the size of the particular imaged object or region of interest. A beam splitter divides the beam into two equal-intensity parts illuminating the object's surface from opposite directions through two transparent windows in the water tank. In this way, excitation conditions close to uniform illumination are achieved. The laser pulse fluence on the surface of imaged objects is kept under 20 mJ/cm^2 in order to meet laser safety standards [37]. Optoacoustic signals are acquired using ultrasound transducers with central frequencies and focal lengths suited to the size of the imaged object and the scale of the features of interest. For smaller objects, such as the drosophila pupa and zebrafish presented herein, we use either a piezoelectric PZT (lead zirconate titanate) transducer with a 15 MHz central frequency, 19.05 mm focal length and 13 mm element diameter (V319, Panametrics-NDT, Waltam, MA) or a 200 kHz-20 MHz broadband cylindrically focused PVDF (polyvinilidene fluoride) transducer (Precision Acoustics Ltd., Dorset, U.K.) with a focal length of 40 mm and an element size of $13.2 \text{ mm} \times 3 \text{ mm}$; larger objects, such as mice and large tissue-mimicking phantoms, are imaged using a PZT transducer with a central frequency of 3.5 MHz, a focal length of 38.1 mm and an element diameter of 13 mm (V382, Panametrics-NDT, Waltam, MA). The transducers are cylindrically focused in the imaging plane to facilitate 2D image acquisition.

Two 45° tilted mirrors are used to change the beam height by moving the bottom mirror with a vertical translation stage. The beam splitter and the ultrasound transducer are translated by the same stage, thus both of the illumination and detection planes are translated simultaneously to allow for multi-slice image acquisition via vertical scanning. The sample is mounted on a rotational stage located at the bottom of the water tank while a sealed bearing (Isomag Corporation, Los Angeles, CA) is used to isolate the water-containing area from the stepper motor located underneath the tank. In-plane data acquisition is done by 360° rotation of the sample. This arrangement ensures a compact design as only a small number of necessary components are located inside the water tank. A 14-bit resolution PCI digitizer with a sampling rate of 100 MS/s (NI PCI-5122, National Instruments Corp., Austin, TX) is used to record time-resolved acoustic signals from the detector. The laser, stages, and data acquisition are controlled via a Labview-based interface (National Instruments Corp., Austin, TX). In addition, a photodiode (FDS010, 200 - 1100 nm, 1 ns rise time, Thorlabs, Newton, NJ) is positioned in the vicinity of the laser output window to record the intensity change of each pulse and normalize the detected

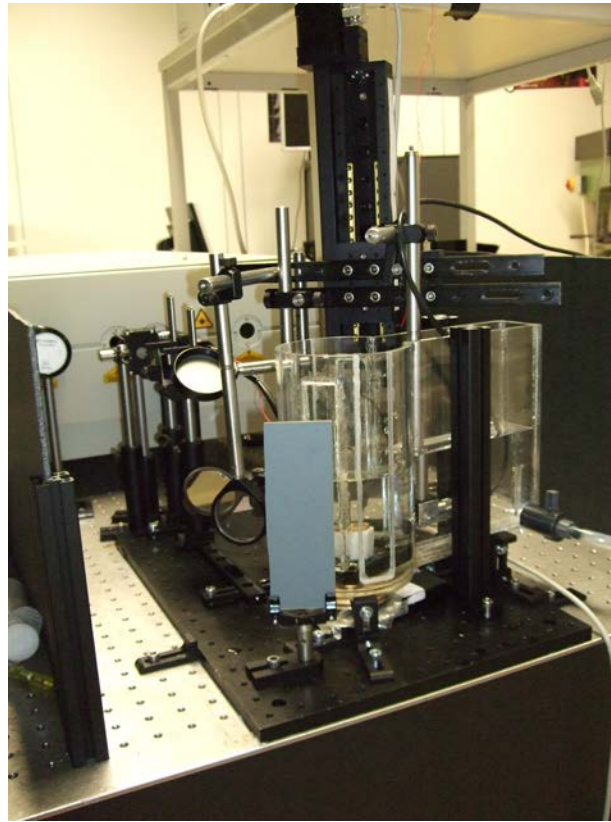


Figure 3.1: A picture of the multispectral optoacoustic tomography setup.

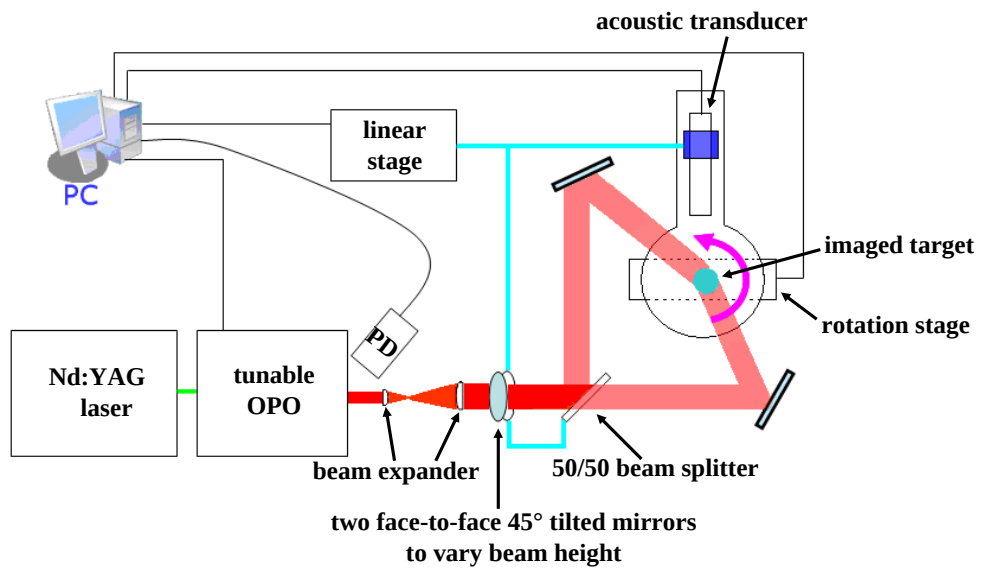


Figure 3.2: Diagram of the multispectral optoacoustic tomography setup.

signals for laser output instabilities. The readings from the photodiode are calibrated with wavelength-dependent sensitivity curves provided by the manufacturer. This continuous power monitoring is of critical importance for multispectral reconstructions since many important molecular imaging agents may present only a small variation of the optical absorption over highly absorbing background, in which case even small quantification inaccuracies may lead to uninterpretable results.

3.2 Data acquisition

The commonly applied optoacoustic acquisition loop [6, 13, 49] includes moving a stepper motor to an arranged position, detecting time-resolved signals, averaging them to attain the necessary SNR, saving the data and moving to the next angular projection or step. Here we employ instead a continuous data acquisition method, for which the rotation stage is accelerated to a predefined speed (depending on the number of projections to be acquired) and continuously moves for the entire 360° revolution. Low pulse-to-pulse jitter of the laser (< 2 ns) provides good positioning accuracy. For validation of reconstruction accuracy, exact stage positions for the initial and last angular projection are recorded. The signals are acquired and stored for every laser pulse without averaging. If more signals are required for better SNR, the rotation speed is reduced so that more projections can be acquired. Compared to averaging methods, the continuous acquisition significantly cuts on acquisition times and allows recording of a greater number of information-containing projections, which also largely reduces reconstruction artifacts due to incomplete projection data.

In a more quantitative manner, the signal-to-noise ratio of the final reconstructed image can be written as $SNR \propto S \cdot \gamma A \cdot \sqrt{P \cdot M} / N$, where S , γ , A , P , M , and N are optoacoustic signal intensity, ultrasonic detector sensitivity, its area, number of projections (or detection elements in a multi-element array solution), number of signal averages, and noise floor of the system, respectively. In order to achieve higher SNR, it is always better to increase the magnitude of the generated optoacoustic signals, detector sensitivity or its area rather than try to recover the signals via signal averaging. This also explains why it is not always possible to significantly cut on the acquisition times using multi-element detection solution, in which the signals are acquired simultaneously from multiple projections. Since the latter solution will usually employ a large number of small-area detectors, one might have to perform an extensive signal averaging to recover the SNR. However, the resulting SNR will be reduced as a function of an individual detector area while it will only increase as a square root of the number of simultaneously detecting elements or signal averages. This fact might drastically reduce or even completely diminish the time gain of simultaneous multi-element signal acquisition as compared to solution having a single mechanically-translated detector with large area.

In order to validate the suggested continuous acquisition method, it is important to

calculate the image blur resulting from moving the imaged object during data acquisition. For the simplified acquisition scheme, shown in Fig. 2, the laser repetition rate is 30 Hz, hence the time interval between two successive projections is ~ 33 ms. For the fastest possible rotation speed available in the current setup (6.7 RPM), corresponding to 270 projections over a full 360° rotation, the 2D image is acquired in 9 seconds. The object is therefore rotating with an angular speed of $40^\circ/\text{s}$. For a typical diameter of the imaged object of 2 cm and speed of sound of 1500 m/s, it will only take approximately $13 \mu\text{s}$ to record all the optoacoustic signals originating from the object. During this time, the maximal displacement of points within the object with respect to the transducer's surface can reach the value of approximately $0.1 \mu\text{m}$, which is significantly smaller than the ultrasonic resolution that can be achieved, meaning that its blurring effects on the image can be safely neglected.

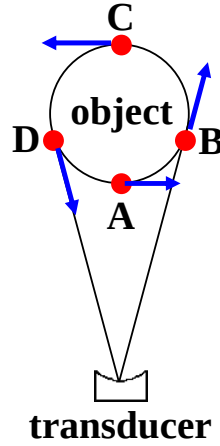


Figure 3.3: Schematic representation of the continuous acquisition method and four important points concerning Doppler effect.

Nevertheless, when the imaged object is rotating, one should also take into account the Doppler effect caused by the object's motion relative to the transducer. When the acoustic source is moving with velocity v at an angle θ relative to the direction from the transducer to the acoustic source, the relative frequency shift in the acoustic signal can be calculated by [50]

$$\frac{\Delta f}{f} = \frac{v \cdot \cos\theta}{c} \quad (3.1)$$

where in equation 3.1 v is positive when the source is moving away from the transducer and negative when it moves towards the transducer. From the previous calculation, the maximal angular speed of $40^\circ/\text{s}$ corresponds to a linear speed of $v = 6.98 \text{ mm/s}$ on the surface of a 2 cm diameter object. In figure 3.3, $\theta = 90^\circ$ at points A and C, and as a result the frequency of the detected acoustic wave has no Doppler effect. At points B and D the relative frequency shift reaches its maximum of approximately 0.0005%, or 50 Hz for 10 MHz frequency components, a value which is again negligible for the considered

object size range. We further note that typical tunable nanosecond lasers are generally unstable in terms of pulse energy, which can deviate by 20% or more between individual pulses. Clearly, when signal averaging is applied, acoustic signal fluctuations due to laser intensity instability are greatly reduced. In the continuous acquisition mode, however, the individual projections are not averaged, therefore pulse energy variations may introduce artifacts into the reconstructed optoacoustic images. To account for these effects we utilize the photodiode circuit described in the previous section for post-processing correction for pulse-to-pulse energy variations.

3.3 Comparison of different reconstruction algorithms

Figure 3.4(a) and (b) show optoacoustic images of a 1-year-old wild type zebrafish (more details can be found in 4.1.2) based on the delay-and-sum and universal back-projection algorithms, respectively. We used a 15 MHz PZT transducer described in section 3.1 for this experiment. In comparison with figure 3.4(b), figure 3.4(a) shows tissue structures with higher contrast and figure 3.4(b) maintains mainly tissue boundaries.

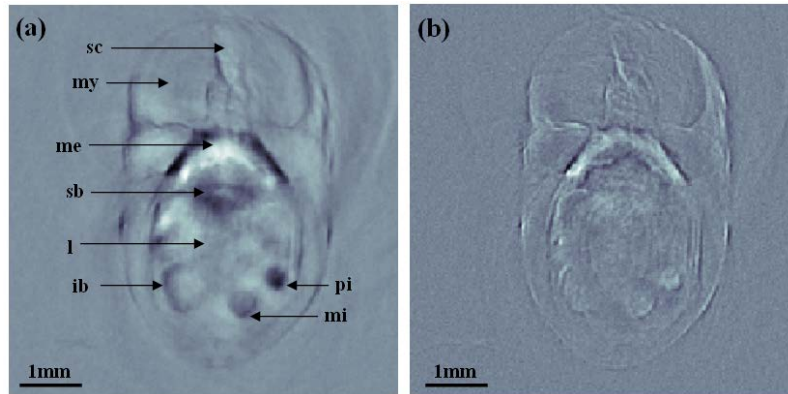


Figure 3.4: Performance comparison of the delay-and-sum and universal back-projection algorithms using a 15 MHz PZT transducer. (a) Reconstruction based on the delay-and-sum algorithm; (b) Using the universal back-projection algorithm. The field of view of the images is 6.3×6.3 mm. Annotation: spinal cord (sc), myotome (my), mesonephros (me), swim bladder (sb), liver (l), intestinal bulb (ib), posterior intestine (pi), mid intestine (mi).

Note that the universal back-projection algorithm is assumed under the transducer with a flat frequency response and in reality no transducers have a perfect match with the assumption, therefore the frequency response of a transducer can affect the performance of the universal back-projection algorithm. We made a rough measurement of the frequency response of a 15 MHz PZT transducer (the method is described in [51]) with a $10 \mu\text{m}$ microsphere. The acoustic signal was integrated to get the impulse response of the detector, followed by a Fourier transform to get the frequency response of the detector.

We can see from figure 3.5 that the frequency response of the 15 MHz PZT transducer is not flat and in the low frequency aspect of the signal, the frequency response behaves in a linear manner. The interpretation of the frequency response behavior is a semi-derivative of the optoacoustic signals in the time domain, thus performing an additional derivative in the universal back-projection algorithm is unnecessary. Therefore optoacoustic images reconstructed with the delay-and-sum algorithm represents tissue structures better. Thus we will henceforth use the delay-and-sum algorithm for optoacoustic image reconstruction with PZT transducers.

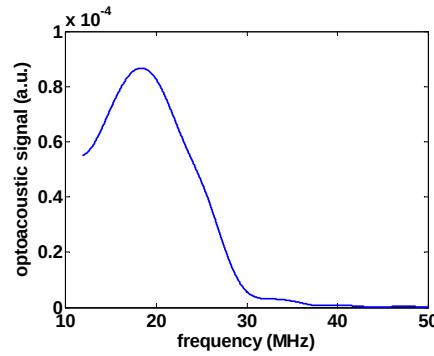


Figure 3.5: Rough estimation of frequency response of PZT 15 MHz, measured by a 10 μm microsphere.

We also compared the delay-and-sum algorithm with the universal back-projection algorithm using a PVDF transducer described in section 3.1. We imaged a pupa and a 1-year-old adult zebrafish for comparison. PVDF transducer has a relative flat frequency response [52], from figure 3.6 we can see that the universal back-projection algorithm not only enhances the boundary but also the inner structures. In addition, figure 3.6 (d) shows reduced artifacts coming from the fish surface, where the presence of negative values is suppressed. We used a bandpass filter to improve the image quality, and the pupa is filtered from 1 MHz to 10 MHz and the zebrafish from 1 MHz to 35 MHz.

We then applied the statistical correction method to the same optoacoustic image described in figure 3.4. As a comparison, figure 3.7 shows optoacoustic images with and without using the statistical correction method.

We can see that after performing the statistical correction, artifacts labeled as 1, 2 and 3 disappear, the pectoral fin labeled as 4 gets enhanced and another pectoral fin labeled as 5 pops up.

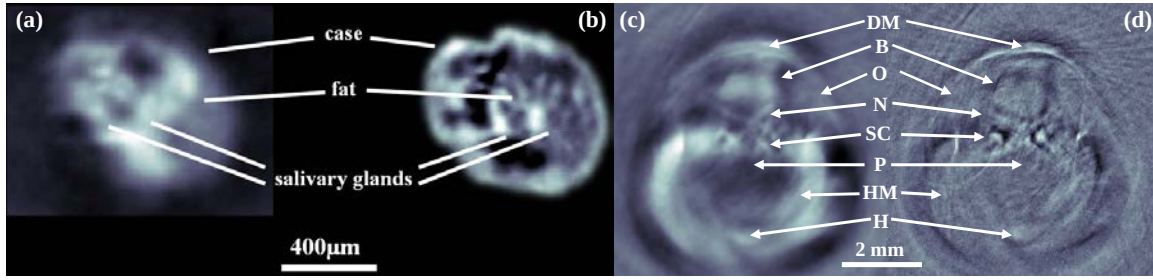


Figure 3.6: Algorithm comparison using a PVDF transducer. (a) Reconstruction of a pupa based on the delay-and-sum algorithm; (b) Using the universal back-projection algorithm; (c) Delay-and-sum reconstruction of 1-year-old adult zebrafish; (d) Universal back-projection algorithm. Main structures: dorsal fin musculature (DM), hind brain (B), operculum (O), lateral line nerve (N), semicircular canal (SC), pharynx (P), hypobranchial musculature (HM), heart (H).

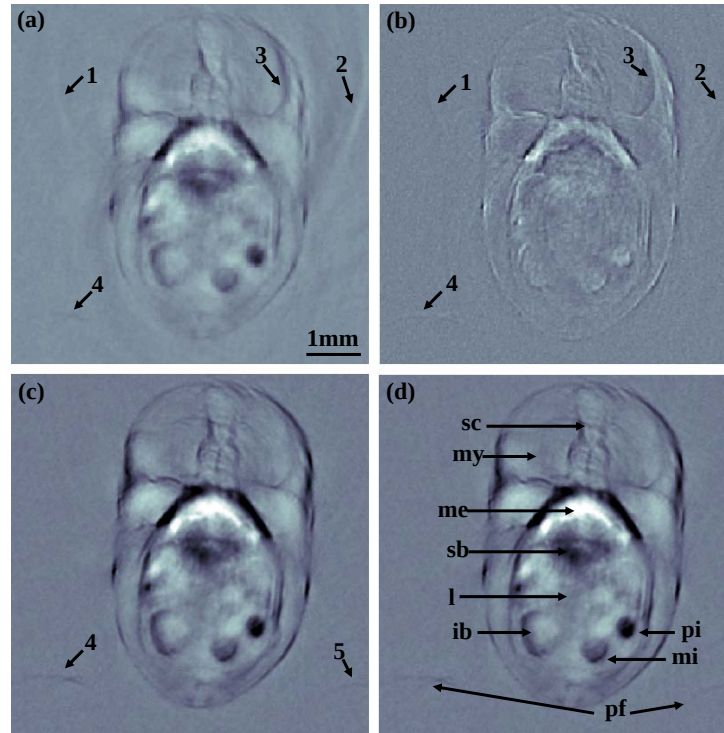


Figure 3.7: Statistical correction method applied to optoacoustic images. (a) Reconstruction based on the delay-and-sum algorithm; (b) Universal back-projection algorithm; (c) Delay-and-sum reconstruction applied with the statistical correction method; (d) The same image as (c) with main structures labeled, spinal cord (sc), myotome (my), mesonephros (me), swim bladder (sb), liver (l), intestinal bulb (ib), posterior intestine (pi), mid intestine (mi), and pectoral fins (pf). The field of view of the images is 6.3×6.3 mm.

3.4 System calibration

To determine the spatial resolution of the system, a clear agar cylinder, made of 1.5% (by weight) agar powder (Sigma-Aldrich, St. Louis, MO) mixed with water, containing 10 μm diameter black-dyed polystyrene microspheres (Polysciences, Inc., Warrington, PA) at an approximate concentration of 20 spheres per ml was prepared to determine the spatial resolution of the system. The transducer we used is a piezoelectric PZT transducer with a 15 MHz central frequency, 19.05 mm focal length and 13 mm element diameter (V319, Panametrics-NDT, Waltham, MA). The inset of figure 3.8(a) shows a reconstructed 2D image of a microsphere. In Fig. 3(a), the plotted signal intensity across the image indicates a FWHM (full width at half maximum) of 42 μm , which gives us an in-plane resolution of 32 μm when taking into account the size of the microspheres. This value corresponds well to the available detection bandwidth of the transducer (up to ~ 20 MHz).

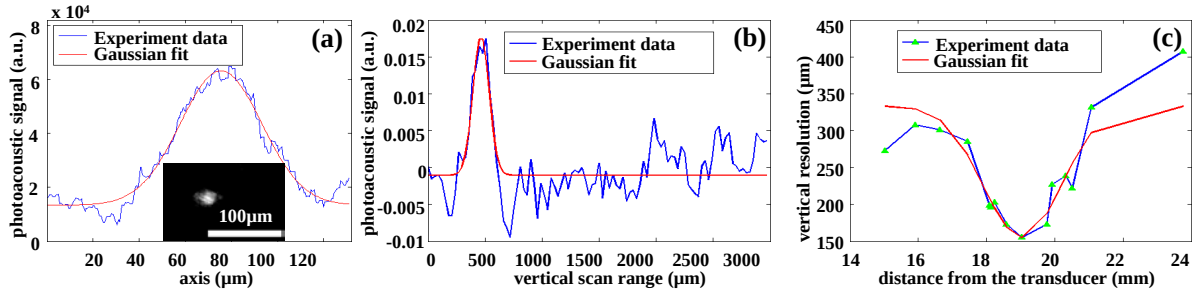


Figure 3.8: (a) In-plane resolution measurements of cylindrically-focused PZT transducer with center frequency of 15 MHz using 10 μm microsphere suspension, attaining an in-plane resolution of 32 μm , inset: optoacoustic image of a microsphere; (b) Vertical scan through a microsphere located 19.035mm in front of the transducer. Vertical resolution of ~ 155 μm is derived for this distance. The distance is calculated via the time-of-flight method, and the figure is obtained by vertically scanning the phantom and recording the acoustic signal coming from the same distance. The blue curve indicates the measured signal intensity variations and the red line is the Gaussian fit; (c) Vertical resolution dependence on the distance between the imaged object and the transducer.

To determine the vertical resolution, we scanned the transducer vertically along the cylinder embedded with microspheres for 100 steps with a 30 μm step size. Within these 100 steps, many microspheres, located at different distances from the transducer, were detected. By tracking and plotting the magnitude of optoacoustic response for each microsphere in consecutive imaging planes, calculating the corresponding distances to the transducer's surface and subsequently applying a Gaussian fit, one can derive the vertical resolution map for the cylindrically focused detector. In this way, an approximate length of the focal zone can also be determined. Figure 3(b) shows signal magnitudes obtained for a vertical scan through a sphere located close to the focus, attaining a vertical resolution of ~ 155 μm , which agrees well with the transducer specification. Figure 3.8(c) shows the vertical

resolution dependence on the distance between the imaged object and the transducer, from which we deduce an effective focal zone length of 2.7 mm, defined by the FWHM of a Gaussian fit. As can be seen from the curve, the best vertical resolution corresponds to the specified focal length of the transducer (19.05 mm).

3.5 Verification of the system performance

To compare the performance of continuous acquisition with the conventional averaged data acquisition approach, we made two cylindrical tissue-mimicking phantoms labeled as Ph1 and Ph2 with optical properties described in table 3.1. They were both made by molding 1.5% (by weight) of agar powder (Sigma-Aldrich, St. Louis, MO) mixed with 6% (by volume) of Intralipid-20% (Sigma-Aldrich, St. Louis, MO) to attain a reduced scattering coefficient of $\mu'_s = 10 \text{ cm}^{-1}$ and black india ink (Higgins, Sanford Bellwood, IL) for absorption. Hexagonal and round insertions, with optical properties as in table 3.1, were added to the phantoms.

We used a 3.5 MHz PZT transducer described in section 3.1. The background scattering and absorption properties of the phantoms were selected in the range of typical mouse tissue optical properties at the imaged wavelengths [46].

	Ph1	Ph2
Diameter (mm)	14	16
μ'_s background (cm^{-1})	10	10
μ_a background (cm^{-1})	0.2	0.6
μ'_s insertion/s (cm^{-1})	10	10
μ_a insertion/s (cm^{-1})	1	3

Table 3.1: Optical properties of the phantoms used for experimental comparison between continuous and averaged acquisition.

Figure 3.9(a) and figure 3.9(b) show optoacoustic images of Ph1 using continuous and averaged acquisition, respectively. The former uses 270 angular projections without averaging and the latter 90 projections with 32 averages per projection. The quality of the reconstructed images for both acquisition methods look similar; however, in continuous acquisition mode it took approximately 9 seconds to acquire the 2D image while the acquisition time with averaging was 200 seconds. In this case, with similar image quality, continuous acquisition took only 1/22 of the time required for averaging mode. Figure 3.9(c) and 3.9(d) show the resulting images from the two acquisition methods for Ph2. Here 960 projections were used for the continuous acquisition and 20 projections with 32 averages each for the averaging method so that both methods have a similar acquisition time (about 35 seconds per 2D slice). However, the continuous acquisition image has significantly better quality and is free of the arc-like artifacts resulting from the averaged acquisition with too few projections.

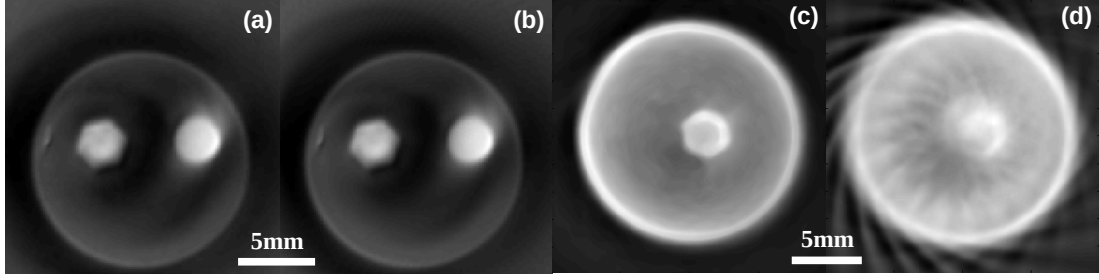


Figure 3.9: Cross-sectional optoacoustic image of Ph1 acquired in continuous acquisition mode with 270 angular projections; (b) Reconstruction of Ph1 in averaged acquisition mode with 90 projections and 32 averages per projection; (c) Reconstruction of Ph2 in continuous acquisition mode with 960 projections; (d) Reconstruction of Ph2 in averaged acquisition mode with 20 projections and 32 averages per projection.

To further verify the performance of continuous and averaging acquisition we imaged an adult zebrafish *in vivo* (anesthetized in 5% tricaine) using a 20 MHz PVDF transducer described in section 3.1. Figure 3.10(a) is a 2D optoacoustic image using continuous acquisition method acquired with 400 projections and figure 3.10(b) using averaging method, with 120 projections and 64 averages for each projection. There is no obvious difference between these two acquisition methods and the time taken by the averaging method is nearly 20 times longer than the continuous method. Hence continuous acquisition brings the advantage of fast acquisition and enables *in vivo* imaging or imaging phenomena that need to be monitored as fast as possible. In our case we need to image the zebrafish in a short time, since the zebrafish would stop breathing when we put it in 5% tricaine for a long time.

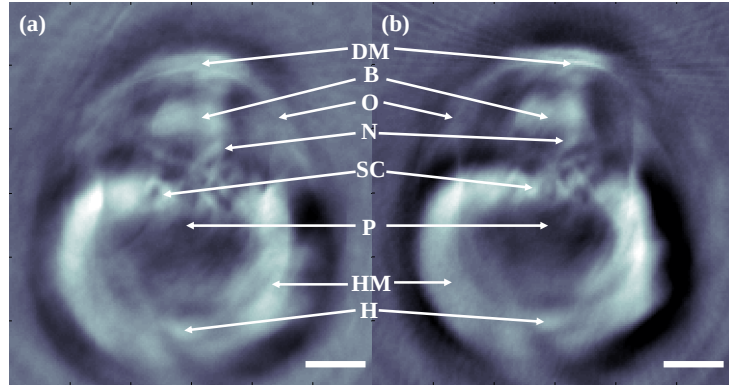


Figure 3.10: Cross-sectional optoacoustic image of an adult zebrafish *in vivo*. (a) Continuous acquisition mode with 400 angular projections; (b) Reconstruction of zebrafish in averaged acquisition mode with 120 projections and 64 averages per projection. Abbreviations: dorsal fin musculature (DM), hind brain (B), operculum (O), lateral line nerve (N), semicircular canal (SC), pharynx (P), hypobranchial musculature (HM), heart (H). Scale bar: 1mm

3.6 2D optoacoustic imaging of objects with various sizes

We used different sizes of imaging targets, ranging from $800\ \mu\text{m}$ to $28.5\ \text{mm}$, to demonstrate that the system is capable of imaging objects with various sizes. The image of the drosophila pupa obtained at $498\ \text{nm}$, shown in figure 3.11(a), clearly reveals the anatomical details of the animal, i.e. the absorbing case, two low absorbing salivary glands and the rest of the volume filled with fatty structures. The transducer is a $15\ \text{MHz}$ PZT transducer described in section 3.1. Since the case of the pupa is very thin (typically less than $10\ \mu\text{m}$), it can be used to verify the spatial resolution of the system (with the particular PZT detector used) in a real biological imaging scenario. As can be seen in figure 3.11(b), the case thickness is translated into $\sim 43\ \mu\text{m}$ (FWHM) in the optoacoustic image, which, assuming an actual case thickness of approximately $10\ \mu\text{m}$, gives an in-plane resolution of $\sim 33\ \mu\text{m}$, in good agreement with the value obtained in section 3.4.

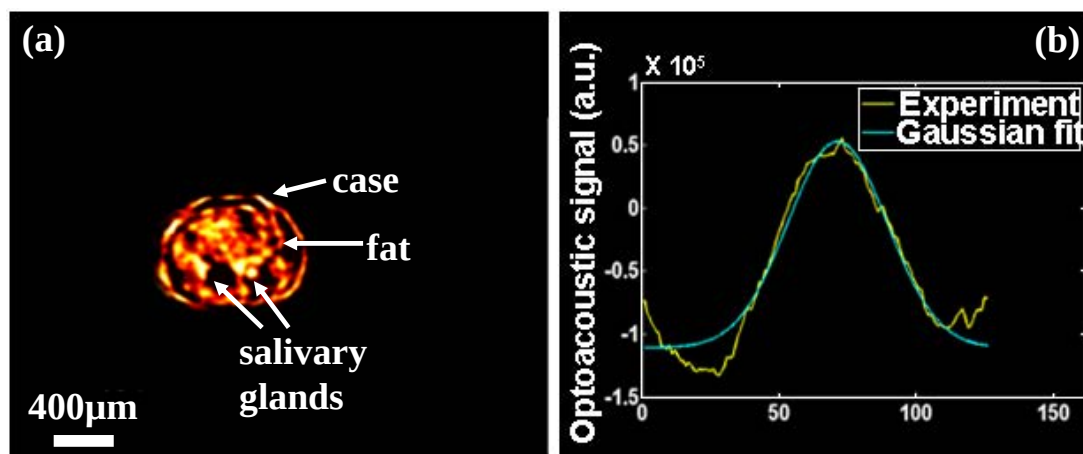


Figure 3.11: (a) Optoacoustic image of intact drosophila pupa using a $15\ \text{MHz}$ PZT transducer; (b) One-dimensional cut through the reconstructed image in (a) for verification of in-plane resolution.

We also used a $20\ \text{MHz}$ PVDF transducer (see section 3.1) to image the pupa at $498\ \text{nm}$. Compared with the optoacoustic image obtained with a PZT transducer shown in figure 3.11, figure 3.12 reveals similar histological structures.

Similarly, the cross-sectional optoacoustic image of a 1-year-old adult zebrafish obtained at $585\ \text{nm}$ with a $15\ \text{MHz}$ PZT transducer in figure 3.13(a) further demonstrates the high-contrast and high-resolution feature of the system and correlates well with the corresponding histological slice shown in figure 3.13(b).

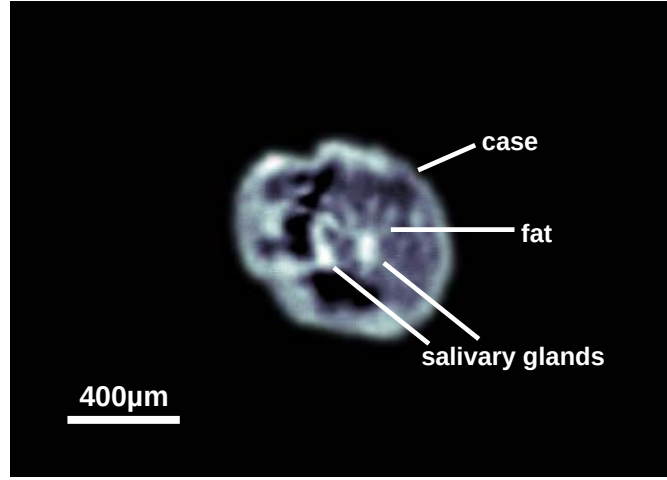


Figure 3.12: Optoacoustic image of intact drosophila pupa imaged at 498 nm with a PVDF transducer.

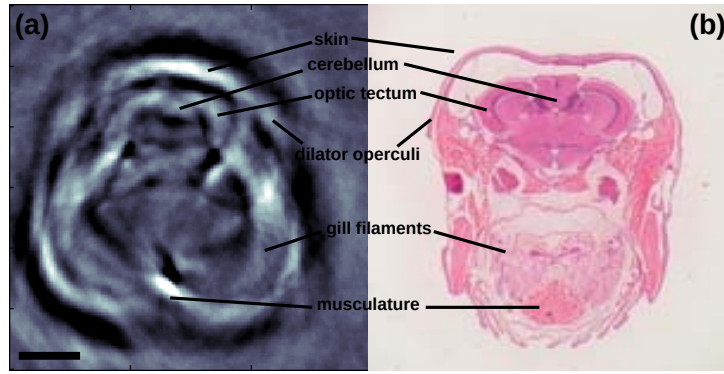


Figure 3.13: (a) A cross-sectional optoacoustic image of an intact 1-year-old adult zebrafish; (b) Corresponding histological slice made through a sacrificed zebrafish specimen. Scale bar: 1mm.

To simulate imaging of deep-seated targets in a very absorbing tissue-like medium, we made a cylindrical phantom with a diameter of 28.5 mm [figure 3.14(a)]. The materials used to make the phantom is the same as in section 3.5. It was made with a background optical absorption coefficient of $\mu_a = 0.8 \text{ cm}^{-1}$, which is approximately four times higher than average mouse tissue absorption at the imaging wavelength of 650 nm. The optical absorption coefficient of insertion is 8 cm^{-1} and the reduced scattering coefficient of the whole phantom is 10 cm^{-1} . Figure 3.14(b) shows optoacoustic reconstructions of the phantom acquired at 650nm containing a hexagonal insertion at a depth of more than 1 cm, which is clearly visible in the images with high resolution and SNR. Figure 3.14(c) shows a magnified view of the insertion from the image in figure 3.14(b). The calculated SNR of the image in figure 3.14(c) is 22.25 dB, where the green and magenta labeled regions represent signal and background respectively. The system has therefore been clearly demonstrated

to have the capability of imaging deep tissue contrast within large objects with relatively high background absorption.

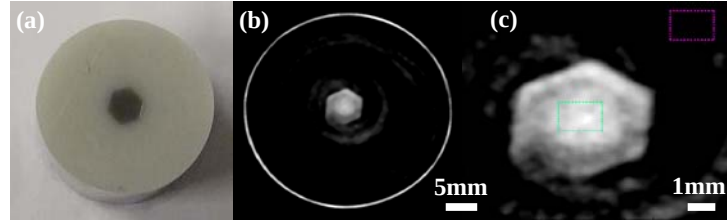


Figure 3.14: (a) Photograph of a phantom with diameter of 28.5 mm; (b) Optoacoustic reconstruction of the phantom acquired at 650nm; (c) Magnified image around the insertion. SNR is calculated from the green and magenta labeled regions, representing signal and background, respectively.

3.7 3D imaging capability

We imaged a complex hair structure in a pure agar phantom to demonstrate the 3D imaging capability of the MSOT system. We used a 15 MHz PZT transducer (see section 3.1) to image the phantom at 635 nm with 60 slices and 100 μm distance between adjacent slices. The 3D visualization was obtained by stacking all 60 slices together using Amira (Visage Imaging GmbH).

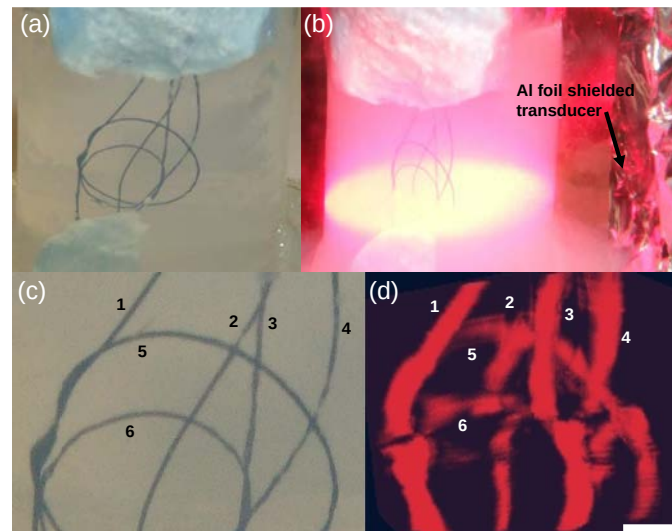


Figure 3.15: (a) A photo of a complex hair phantom; (b) A picture of the illuminated hair phantom with transducer during measurement; (c) An enlargement of the hair phantom picture; (d) Corresponding reconstructed 3D optoacoustic image. Scale bar: 1mm.

Figure 3.15(a) shows a photograph of the complex hair phantom and figure 3.15(b) is a photograph of the illuminated phantom together with the transducer during the imaging experiment. The phantom was illuminated through a slit to optimize the vertical resolution and the transducer was shielded with an aluminum foil to prevent direct illumination of the laser beam. Figure 3.15(c) shows an enlargement of the hair phantom picture and figure 3.15(d) is the corresponding 3D optoacoustic image. We can see that the main structures (labels from 1 to 6) in the optoacoustic image agree well with the picture. The hairs in the optoacoustic imaging are thicker than the photograph, due to the poor ultrasound resolution outside the focal zone of the PZT transducer. Since our phantom is placed outside the center of rotation and as can be seen from section 3.4 that the vertical resolution of the transducer degrades dramatically outside the focal zone, this would affect the image quality especially when the imaged structures are not placed perpendicular to the image plane. Besides structures labeled as 2 and 4 are distorted when there are other hairs in the vicinity. This is because the optoacoustic signals contain both positive and negative values, when several objects lie close to each other, the negative value coming from one object could interfere with the positive value of another object, which smears signals containing the true position information. Figure 3.16 shows the 3D visualization of the hair phantom from 8 different angles.

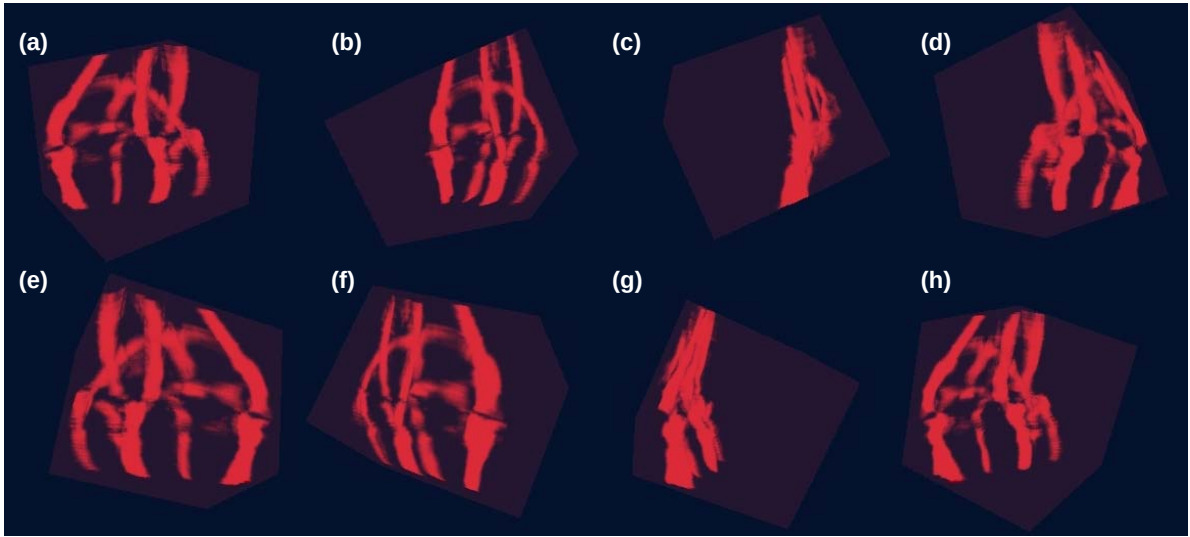


Figure 3.16: Reconstructed 3D hair phantom from 8 different angles.

3.8 Molecular probe detection

In order to demonstrate the system's sensitivity towards molecular probe detection, we made a phantom of Polyvinyl chloride-plastisol (PVCP) with Titanium dioxide (TiO₂)

powder and black plastic color (BPC) to introduce the necessary background scattering and absorption [46] as 20 cm^{-1} and 0.15 cm^{-1} , respectively. Hydrophobic PVCP was used in order to hold solutions of fluorescent dyes, which tend to diffuse through agar phantoms. The phantom has two insertions, one was embedded with a solution containing a common fluorescent dye, Texas Red (peak excitation 596 nm; peak emission 615 nm) at $5 \text{ }\mu\text{M}$ concentration. The dye was in an already highly absorbing solution of ink mixed with intralipid having $\mu_a = 2 \text{ cm}^{-1}$ and $\mu'_s = 20 \text{ cm}^{-1}$, which were selected in order to simulate the realistic case of a fluorescent imaging agent located in whole blood. Another insertion is the same concentration of ink and intralipid as a control. We employed continuous data acquisition to obtain optoacoustic images at the peak excitation wavelength of Texas Red (596nm) and at 612nm where its absorption drops significantly [figure 3.17(a)-3.17(b)]. By subtracting optoacoustic images at these two wavelengths one can efficiently suppress the slowly varying background absorption and reveal the correct location of the Texas Red dye, as shown in figure 3.17(c).

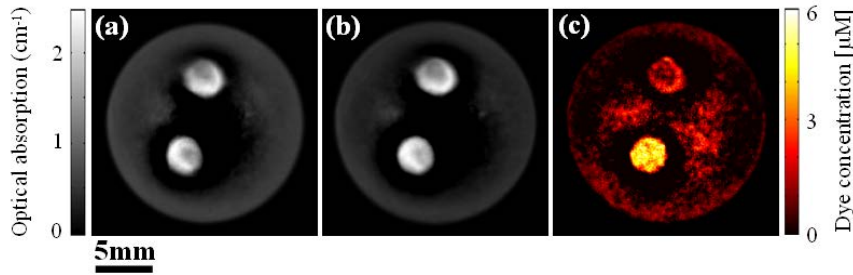


Figure 3.17: (a) Optoacoustic image of Ph4 acquired at 596 nm; (b) 612nm; (c) Subtraction between 596nm and 612nm images revealing location of the Texas Red dye.

As can be seen, the subtraction process reduces background signals coming from both insertions containing the highly absorbing ink solution and from the background of the phantom as well. We calculated the Contrast-to-noise ratio (CNR) of the multispectral image using the following formula:

$$CNR = \frac{S_{Dye} - S_B}{\sigma} \quad (3.2)$$

where S_{Dye} is the averaged value of dye signal obtained after wavelength subtraction [figure 3.17(c)], S_B is the averaged value of the background after wavelength subtraction, and σ is the standard deviation of S_B . The calculated SNR and CNR in figure 3.17(c) were 25.17 and 16.87, respectively, which translates to a noise floor equivalent dye concentration of $\sim 300 \text{ nM}$. This translates to an amount of 12 femtomoles of the dye that was detected in each resolution-limited voxel of the 3.5 MHz PZT transducer ($\sim 0.2 \times 0.2 \times 1 \text{ mm}$). However, in our phantom experiment, the actual optoacoustic signals were recorded from a large amount of the dye contained in the insertion. As we have shown in [46], the detection sensitivity limits cannot therefore be linearly extrapolated using measurements performed on larger amounts of the probe. A more accurate analysis based on the

methodology described in [46], which takes into account optical properties, size and depth of the target as well as light and acoustic attenuation, will lead to detection limits on the order of several hundreds of femtomoles for the current experimental setup and phantom properties. Clearly, in realistic animal experiments, additional factors, such as absorption inhomogeneities, acoustic mismatches, movement artifacts and other issues related to *in vivo* imaging, will adversely affect image quantification and sensitivity limits. There we expect for sensitivity limits in the few picomoles range, similar to what is achievable with state-of-the-art fluorescent molecular tomography systems [23]. Finally, it has to be noted that, besides the particular optical properties of the imaged object and characteristics of the target probe, the absolute sensitivity limits also greatly depend on a large number of other experimental and instrumentation related parameters, such as laser energy, detector sensitivity, number of averages/projections, etc. With improvement of system performance, the practical sensitivity limits are expected to be well in the sub-picomole range, also for *in vivo* experiments.

3.9 Conclusions

One difficulty arising from whole-body optoacoustic imaging is the long acquisition times associated with recording signals from multiple spatial projections. The acquired signals are also generally weak and the SNR is low, problems that are often solved by multiple averaging but complicates the measurement and makes *in vivo* imaging challenging. To have a rough estimate on the order of magnitude of a typical optoacoustic disturbance, one can consider a simplified one-dimensional case of a short pulsed beam impinging on an absorbing half-space [53]. In a typical imaging scenario, illumination of tissue with maximal permissible fluence of $U = 20 \text{ mJ/cm}^2$ [37] will only result in optoacoustic disturbances on the order of 1 kPa on the tissue surface light is incident upon, which will translate to an order of magnitude lower detectable pressure variations on the surface of the detector. If deep tissue imaging is of interest, the pressure variations will be further affected by optical and acoustic attenuations, which will bring the signals down by another order of magnitude and more, so that only few Pascals are available for detection. Finally, when considering multispectral optoacoustic tomography (MSOT) data acquisition [2], in which the same tomographic data is recorded at several different wavelengths, whole-body image acquisition times might become unrealistic. Additional considerations arise from the requirement for high resolution and quality optoacoustic tomographic reconstructions that directly correspond to the detection bandwidth, which should ideally be as large as possible. For this, one could use ultrawideband ultrasound detection technologies, such as PVDF film detectors [43] or Fabry-Perot interferometric approaches that have already proven to be a potential tool for high-resolution optoacoustic imaging *in vivo* [5, 48]. However, compared to these broadband approaches, PZT and other piezocomposite technologies can normally provide higher SNR and robustness, at the cost of narrower bandwidth.

In tomographic applications, in order to attain the best quality quantified reconstructions, the optoacoustic responses have to be collected from as many locations as possible around the imaged object or region of interest. To reduce acquisition times, one could possibly use multiple detectors (ultrasound array) to simultaneously record the outgoing signals, as in [10, 54, 55]. Having multiple simultaneous detectors is, however, a costly and technologically challenging option. Furthermore, multi-element detection solution will usually force one to use small area elements. Therefore, due to signal-to-noise ratio considerations (as discussed in Section 3.2), it might fail to achieve a significant improvement in the acquisition times. Our single element solution allows to use highly sensitive (large-area) interchangeable transducers and offers therefore a good compromise between acquisition speed, complexity and noise performance.

The presented fast-acquisition multispectral optoacoustic tomography system for whole-body imaging of small animals enables compact tomographic design by using sealed bearing that effectively isolates liquid area from the rotation driving parts. The scanner provides a variety of optical means for optimal adjustment of sample excitation. This offers the flexibility required for imaging at different object dimension scales, from organisms like *Drosophila* pupa (diameter below 1 mm) up to larger animals and samples with characteristic sizes of few centimeters and more. The two-way illumination configuration is adopted for attaining approximate uniformity in object's illumination, which is crucial for high quality quantitative image reconstruction. To speed up the acquisition process, we employ a continuous acquisition methodology, for which the object is rotating constantly during data acquisition, a method that was shown to save up to one order of magnitude in typical acquisition times.

In its current implementation, the scanner is capable of acquiring a single wavelength 2D image in approximately 9 seconds using a single cylindrically-focused ultrasonic detector, which corresponds to acquisition times of about 20 minutes for typical whole-body multiwavelength data acquisition using 40 vertical slices and 3 wavelengths. A cylindrically focused element is used here in order to increase detection area and sensitivity, in exchange for compromising image quality and resolution along dimensions where focusing is performed. The best in-plane and vertical spatial resolutions demonstrated in this study were $32\ \mu\text{m}$ and $155\ \mu\text{m}$, respectively.

Furthermore, multi-wavelength illumination offers the possibility to resolve exogenously administered fluorescent probes or other chromophores. The simplest image subtraction approach, demonstrated here, works optimally with absorbers that offer a steep absorption (extinction) change, characteristic of fluorochromes. However, measurements at multiple wavelengths can provide further ability to simultaneously resolve multiple biomarkers, dyes and other intrinsic and exogenous chromophores.

CHAPTER 4

Small animal imaging using the MSOT system

In chapter 3 we demonstrated the MSOT system is capable of imaging objects with various sizes. In this chapter we perform an intensive study of small animal imaging based on the intrinsic optical absorption of tissues, as well as the optical absorption enhanced by molecular contrast agents.

4.1 Optoacoustic imaging based on intrinsic contrast

The advantage of optoacoustic imaging is due to its high sensitivity towards optical absorption in soft tissue structures, the value of which varies by two to three orders of magnitude in the visible range [34, 56]. Accordingly this leads to a vast change of optoacoustic signals upon different tissue structures, hence optoacoustic imaging produces images with excellent optical contrast. Figure 4.1 compares the micro-CT, ultrasound and optoacoustic images of an adult (1-year-old) wild type zebrafish.¹ Without the aid of contrast agents optoacoustic imaging resolves the inner structures of the zebrafish with high contrast and high resolution. Micro-CT imaging, on the other hand, is good at distinguishing bones and other hard structures; however, it is limited to identify soft tissues such as the brain, the cardiac ventricle, intestines, etc, due to the small variation in tissue densities.² Ultrasound imaging is successfully used to image fetuses and breast cancers; however, the slight variation of the reflection coefficient in soft tissues (varying at most 256% from fat to muscles [50]) leads to low contrast in soft-tissue imaging. Additionally speckle noises further reduce the quality of ultrasound images.

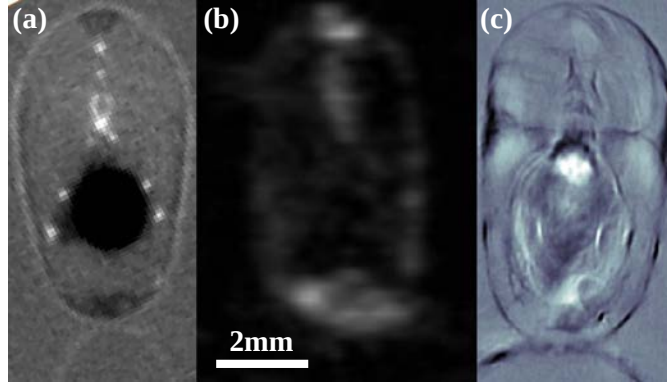


Figure 4.1: Comparison between micro-CT, ultrasound and optoacoustic imaging of an adult (1-year-old) wild type zebrafish.

¹The micro-CT image was acquired by Dr. Ralf Schulz from a commercial micro-CT (eXplore Locus, General Electric HealthCare, London, Ontario, Canada), and the ultrasound image was acquired by Dr. Jérôme Gâteau from a commercial ultrasound system (Terason 2000+, Terason Ultrasound, Division of Teratech Corporation) with a central frequency of 7.5 MHz.

²Recently a newly developed imaging method called *phase-contrast x-ray imaging* can conquer the weak soft-tissue contrast [57].

4.1.1 Nude mouse imaging *post mortem*

We imaged a nude mouse *post mortem* to demonstrate the good intrinsic contrast between different tissue structures. The transducer is a 3.5 MHz PZT transducer described in 3.1. Figure 4.2 shows transverse optoacoustic images taken at three heights through the thorax and neck of a nude mouse, with corresponding CT images using a blood contrast agent (eXIA 160, Binitio Biomedical, Ottawa, Canada).³

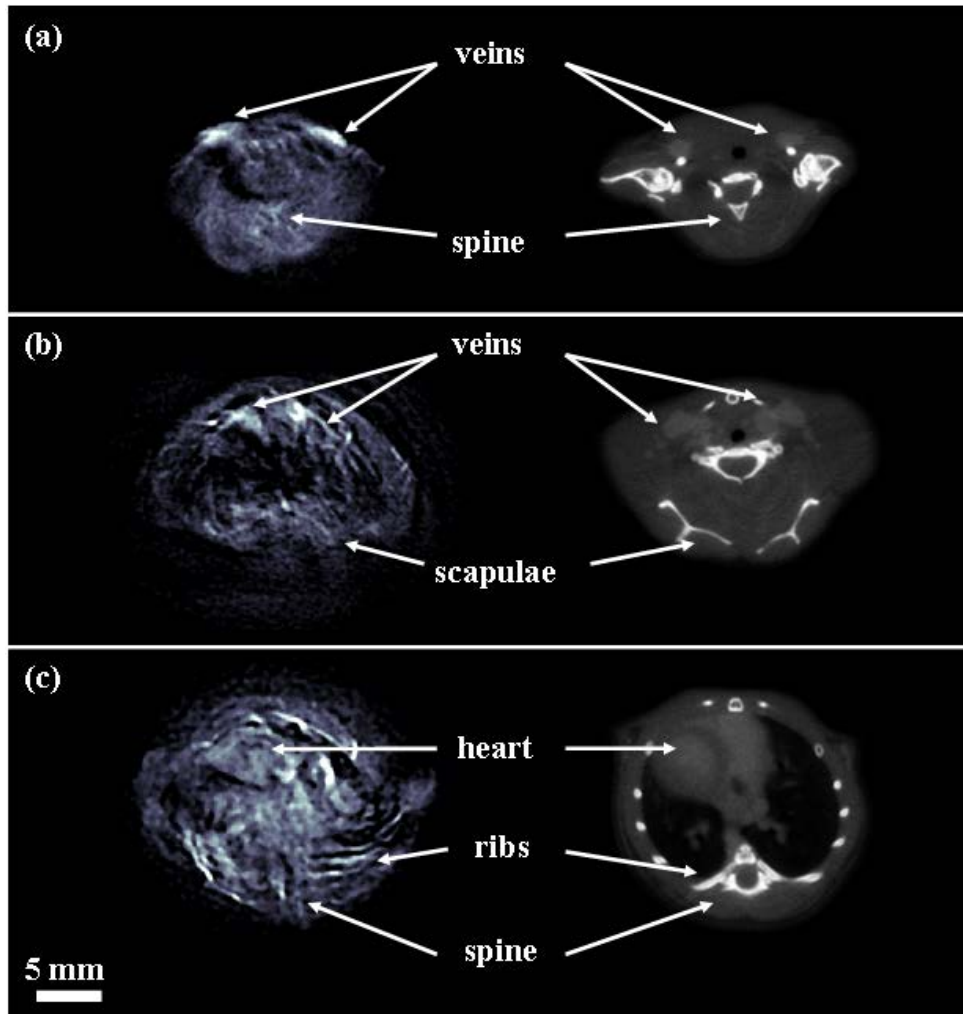


Figure 4.2: Transverse optoacoustic images (left) from the thorax and neck regions of a nude mouse with corresponding CT images (right) using a blood contrast agent. The images were acquired at three different heights on the mouse: (a) was acquired at the top of the shoulders, (b) through the scapulae, and (c) at the height of the heart. Prominent features visible on both the optoacoustic and CT images are labeled.

³Published in Optics Express, **V** 17, p. 21414-21426, 2009 [1].

In figure 4.2 features such as blood vessels, spinal structures, the heart, and ribs can be found in both the optoacoustic and CT images. Differences in the images can be explained by the differing contrast mechanisms of the two modalities, the significant difference in vertical resolution between 2D optoacoustic tomography using a cylindrically focused transducer and CT, and effects on the shape of the mouse caused by different positioning in the optoacoustic system compared to the CT machine. As can be qualitatively assessed from the optoacoustic images, high resolution is maintained deep inside the mouse.

4.1.2 Three dimensional whole-body imaging of an adult zebrafish

We already showed 2D optoacoustic images of adult zebrafish in chapter 3. In this section we demonstrate the 3D whole-body optoacoustic image of an adult zebrafish.

An adult female zebrafish was sacrificed and embedded in an agarose phantom. After the agarose solidified, the zebrafish was mounted onto the rotation stage and the tank was filled with water to ensure transmission of the ultrasound signal to the transducer. We used our nanosecond laser at 587nm to illuminate the zebrafish. 2D optoacoustic slices with a field of view (FOV) of 6.3 mm X 6.3 mm were reconstructed. For 3D visualization we recorded a stack of 68 slices starting at the posterior end of eyes, moving posterior at a step size of 150 μm .

Selected transverse optoacoustic slices

Figure 4.3 presents selected optoacoustic slices starting from the fifth slice with 600 μm distance between adjacent slices. The optoacoustic images were filtered between 1 and 35 MHz.

In order to evaluate the optoacoustic images, histological sections (thickness 10 μm) of an adult female zebrafish stained with hematoxylin and eosin were made. Comparison of the different sections revealed good concurrence between optoacoustic and histological sections. Representative sections are shown in figure 4.4. Organs and tissues like the brain and eyes (figure 4.4 (a,c)), myotome, swim bladder, pronephros, intestines, and pectoral and pelvic fins (figure 4.4 (e,g)) can be identified in the optoacoustic images.

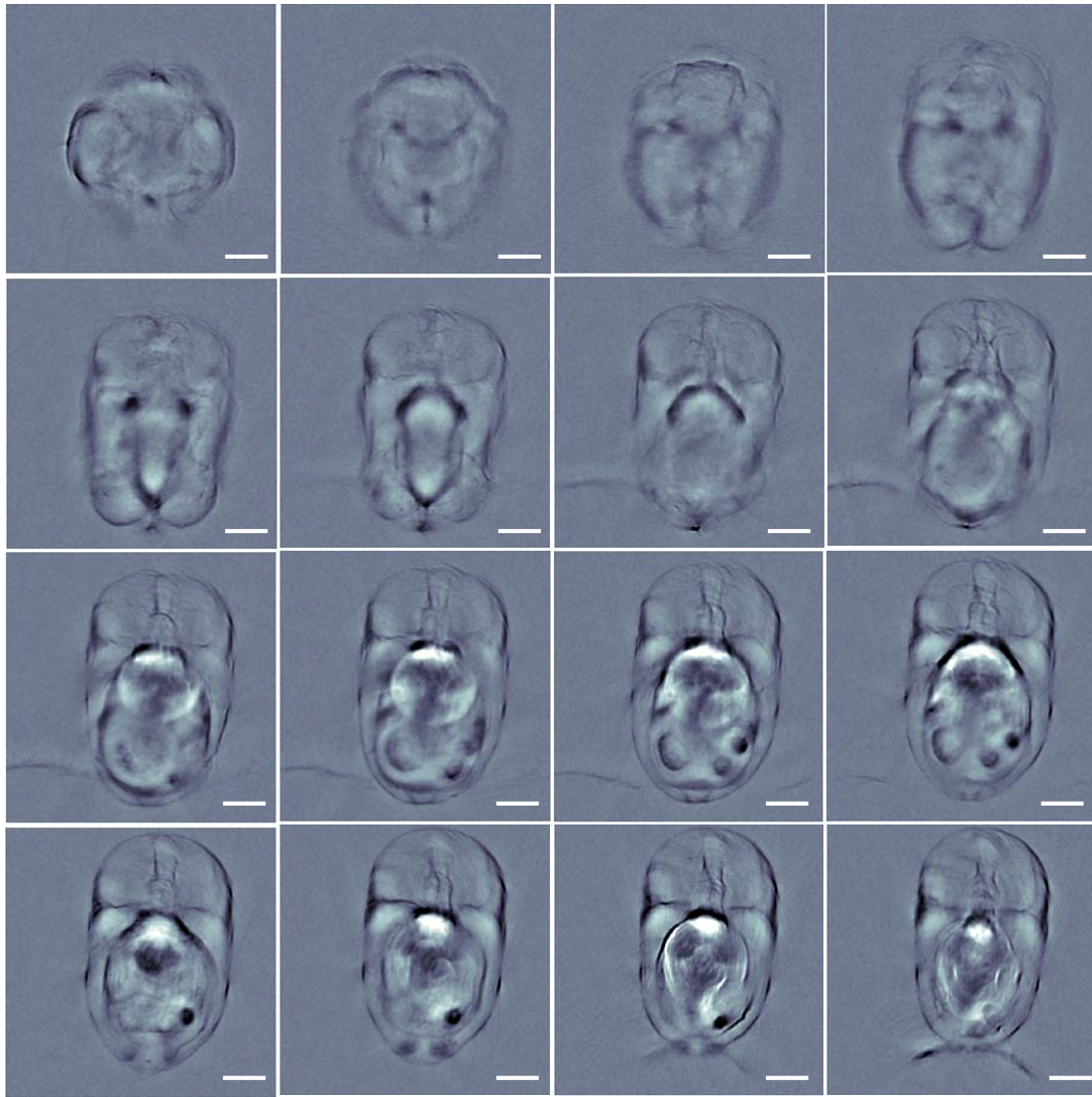


Figure 4.3: Selected slices of high resolution optoacoustic imaging of an adult female zebrafish starting from the fifth slice, with $600\ \mu\text{m}$ distance between adjacent slices. Scale bar: 1mm.

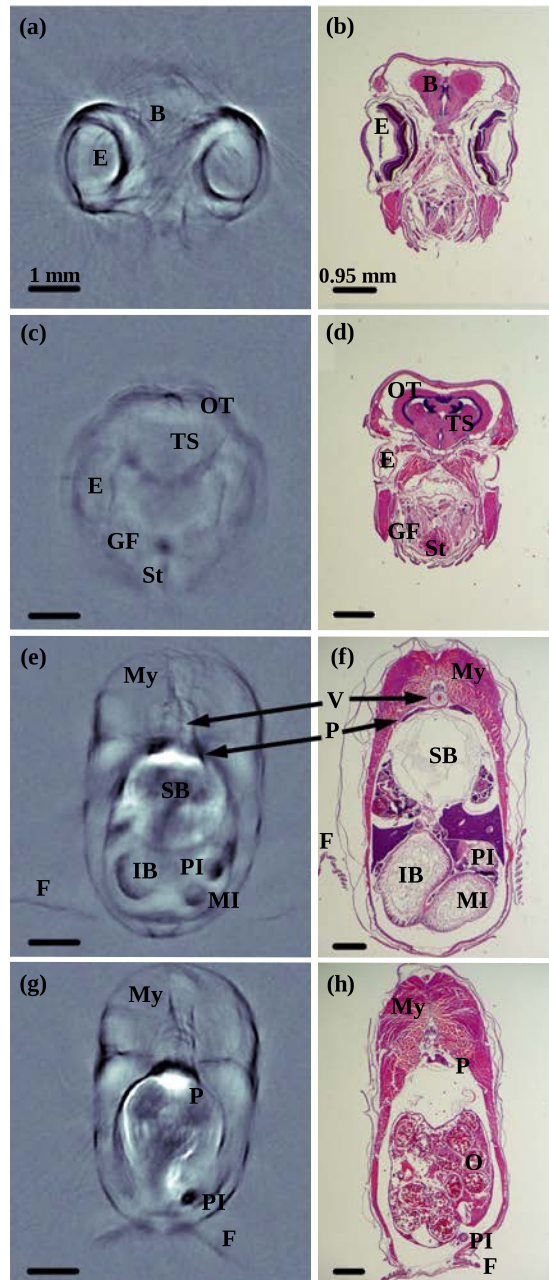


Figure 4.4: High resolution optoacoustic imaging of an adult female zebrafish with corresponding paraffin sections of an adult female zebrafish (a,c,e,g) optoacoustic images. (b,d,f,h) corresponding paraffin sections stained with hematoxylin and eosin. Abbreviations: brain (B), eye (E), optic tectum (OT), torus semicircularis (TS), gill filaments (GF), sternohyoid (St), myotome (My), vertebrae (V), pronephros (P), swim bladder (SB), intestinal bulb (IB), posterior intestine (PI), mid intestine (MI), fin (F), ovaries (O).

Selected sagittal and coronal optoacoustic slices

Figure 4.5 shows selected sagittal and coronal optoacoustic slices. We could clearly see from the images that the main anatomical structures differentiate from each other and from the background.

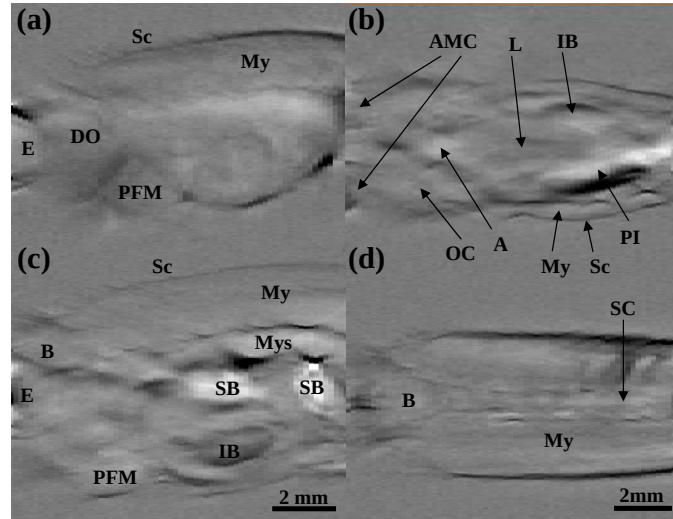


Figure 4.5: (a,c) Selected sagittal slices of a 1-year-old adult zebrafish obtained by optoacoustic imaging; (b,d) Coronal optoacoustic slices. Abbreviations: eye (E), dilator operculi (DO), scale (Sc), pectoral fin musculature (PFM), myotome (My), brain (B), myoseptum (Mys), swim bladder (SB), intestinal bulb (IB), adductor mandibulae complex (AMC), liver (L), opercular cavity (OC), atrium (A), posterior intestine (PI), spinal cord (SC).

3D segmentation

We performed manual segmentation of organs using Amira (Visage Imaging GmbH) based on the 68 optoacoustic slices. Figure 4.6 demonstrates a 3D segmented image of the zebrafish section ranging from the anterior end of eyes to the pelvic fin with main anatomical structures labeled. These are eyes (magenta), brain (yellow), surface (light blue), dilator operculi (blue), pronephros (yellow), swim bladder (white), liver (brown), cardiac ventricle (red), pectoral fin musculature (light yellow), intestinal bulb (purple), posterior intestine (light green), mid intestine (green), and fin (blue).

Figure 4.7 shows the 3D segmentation over 360° rotation providing a thorough view of the structure locations.

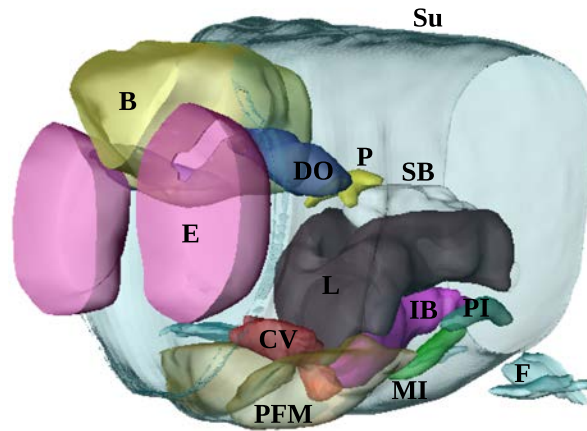


Figure 4.6: 3D segmentation of a zebrafish with several main anatomical structures from the optoacoustic imaging ($150\ \mu\text{m}$ slice thickness). The segmentation was produced in Amira (Visage Imaging GmbH). Abbreviations: brain (B), eye (E), dilator operculi (DO), liver (L), pronephros (P), swim bladder (SB), surface (Su), cardiac ventricle (CV), intestinal bulb (IB), mid intestine (MI), posterior intestine (PI), pectoral fin musculature (PFM), fin (F).

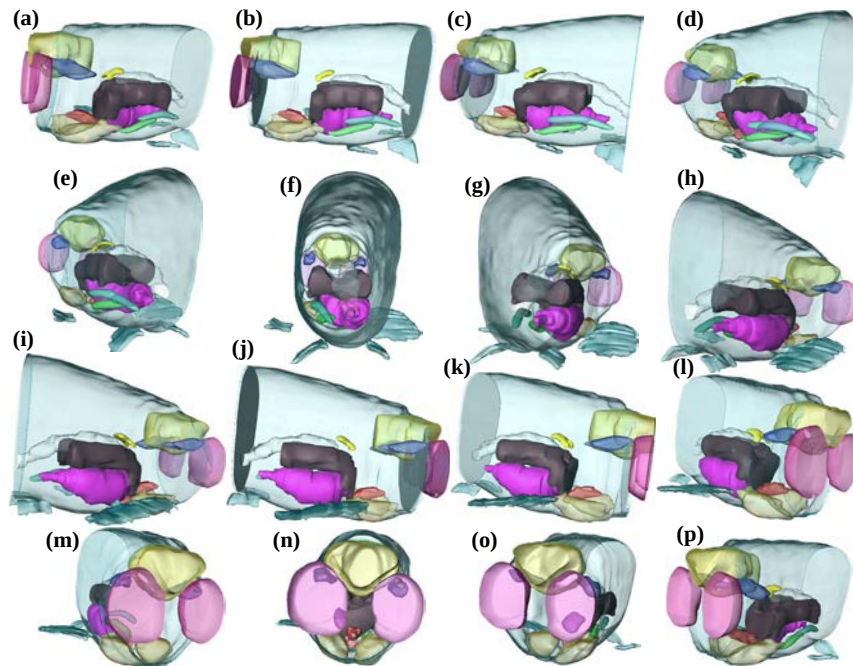


Figure 4.7: Rotational view of the 3D zebrafish segmentation.

4.2 Multispectral molecular imaging

We demonstrated in section 4.1 that the MSOT system can resolve tissue structures with the help of their intrinsic optical absorption distribution. In this section we image transgenic zebrafish with mCherry expressed on the brain [58] both *post mortem* and *in vivo* (short: zic4 zebrafish) to demonstrate that the MSOT system is capable of performing molecular imaging.

4.2.1 Imaging of a transgenic zebrafish (zic4) *post mortem*

In this experiment, a 1-year-old zic4 zebrafish was imaged *post mortem*. Figure 4.8(a) and figure 4.8(b) present optoacoustic images obtained at 585 nm (the excitation wavelength of mCherry) and at 615 nm (at this wavelength the absorption of mCherry drops to 10% of the peak absorption), respectively. Then we subtracted these two images to take off the contribution of the background absorption, which changes slightly within a wavelength range of 30 nm. The subtracted image, which corresponds to the mCherry expression, is superimposed onto the optoacoustic image obtained at 585 nm (Figure 4.8(c)). Figure 4.8(d) and 4.8(e) show the fluorescent histological sections of a zic4 zebrafish with the same age. The image plane of the optoacoustic images lies approximately between these two histological slices, where the size of the inferior lobe decreases and the cerebellum moves to the top of the brain.⁴

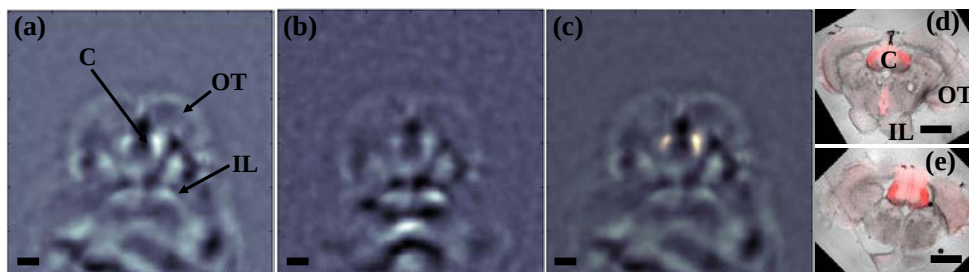


Figure 4.8: (a) An MSOT image obtained at 585 nm; (b) An MSOT image obtained at 615 nm; (c) Fluorescent protein distribution superimposed on the optoacoustic image obtained at 585 nm; (d,e) Fluorescent histological slices of a similar adult zic4 zebrafish. The image plane of the optoacoustic images lies approximately between these two histological slices, where the size of the inferior lobe decreases and the cerebellum moves to the top of the brain. Abbreviations: cerebellum (C), optic tectum (OT), inferior lobe (IL). Scale bar: 500 μm .

We also imaged the zic4 zebrafish three-dimensionally. Figure 4.9(a-d) show MSOT images that were taken posterior to the slice shown in figure 4.8. The step size between adjacent slices is 300 μm . Fluorescent histologies of a similar adult zic4 zebrafish that were taken at approximately the same region are shown in figure 4.9(e-h). Since we used a

⁴This result is published in Proceedings of SPIE 7564, p.756429, Feb. 2010 [59].

cylindrically focused transducer for the MSOT images, the further the imaged object is off the focal zone of the transducer, the more out-of-plane information the MSOT obtains (see figure 3.8(c) in section 3.4). Consequently the MSOT images show all anatomical structures over a certain thickness ($> 155 \mu\text{m}$ in our case). On the other hand, the histological sections were made much thinner than the MSOT slices, thus the resolved mCherry expression from the MSOT images does not correspond exactly with the fluorescent histological sections. This can be improved by placing the fish brain in the focal zone of the transducer or by using a set of optics to reduce the beam height. However, both fluorescent histologies and MSOT images show the same trend: the mCherry fluorescent protein expresses at the cerebellum moving from the middle of the brain to the exterior of the brain.

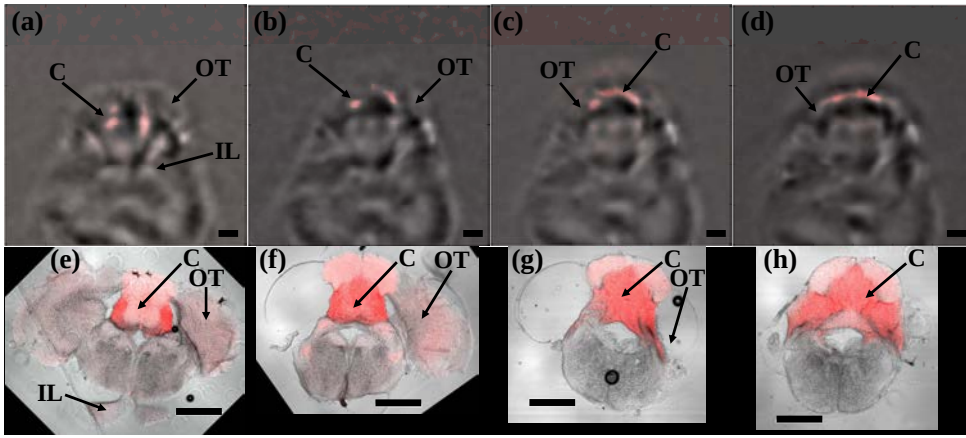


Figure 4.9: (a,b,c,d) Resolved mCherry expression superimposed on optoacoustic images obtained at 585 nm with a step size of $300 \mu\text{m}$; (e,f,g,h) Fluorescent histologies made at approximately the same region of a similar adult *zic4* zebrafish. Abbreviations: cerebellum (C), optic tectum (OT), inferior lobe (IL). Scale bar: $500 \mu\text{m}$.

4.2.2 *In vivo* imaging of a *zic4* zebrafish

Since the MSOT system is a fast image acquisition system, it is advantageous to image small animals *in vivo*, which enables longitudinal studies of physiological changes, developmental biology and a variety of disease models. To further showcase the MSOT capability of performing three-dimensional *in vivo* scans we also imaged the head of an adult (six-month-old) mCherry-expressing *zic4* zebrafish with a cross-sectional diameter of $\sim 6 \text{ mm}$. To guarantee survival, the fish was attached to the rotation stage by partially embedding it into modeling clay so that the upper parts including the head and gills were exposed to the anaesthetic solution (Tricaine) in the imaging tank. The fish fully recovered from the *in vivo* imaging sessions. The imaging results, shown in figure 4.10, demonstrate the ability of the process to reveal many morphological features, as is evident figure 4.10a,b, supported by the corresponding histology (figure 4.10c). Moreover, multispectral reconstructions accurately resolved FP expression in the hindbrain of an intact living animal

(figure 4.10d), in high congruence with the corresponding epifluorescence images of the dissected brain (figure 4.10e)⁵.

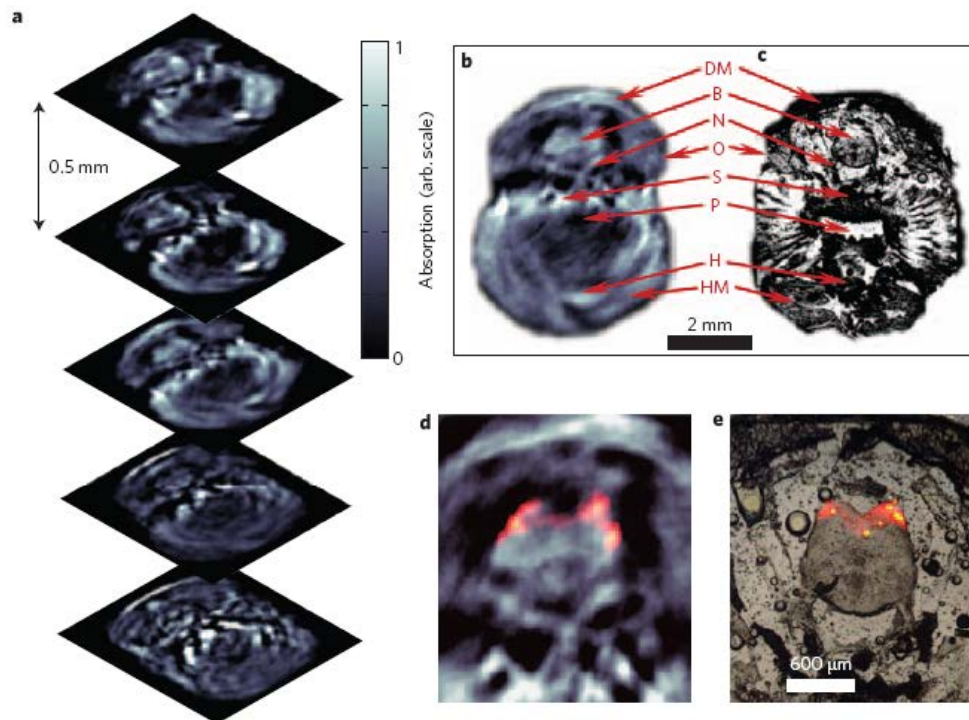


Figure 4.10: Three-dimensional *in vivo* imaging through the brain of an adult (six-month-old) mCherry-expressing *zic4* zebrafish. (a) Five transverse optoacoustic imaging slices through the hind brain area at the level of crista cerebellaris of a living zebrafish taken at 585 nm; (b,c) Example of an imaged slice (b) and its corresponding histological section in inverted colors (c). dorsal fin musculature (DM), hind brain (B), lateral line nerve (N), operculum (O) skull bones (S), pharynx (P), heart (H), hypobranchial musculature (HM); (d) MSOT image of the brain (enlarged) with mCherry expression shown in color. (e) Corresponding fluorescent histology of a dissected fish at the hind brain level.

Figure 4.11 (a) shows the fluorescent imaging merged with the corresponding white light microscopic image before performing the optoacoustic imaging. Figure 4.11 (b) shows a microscopic image of the *zic4* zebrafish after optoacoustic imaging merged with the corresponding fluorescent image. Figure 4.11 (c) shows purely the fluorescent image. We can see that no obvious bleaching occurred and the fish was fully recovered after an imaging process for more than 1 hour (we need only a few minutes to acquire 5 slices with 3 wavelengths, here it took longer since we used both of the continuous and averaging methods to compare their performances).

⁵This work is published in Nature Photonics, V 3, p. 412-417, 2009 [2].

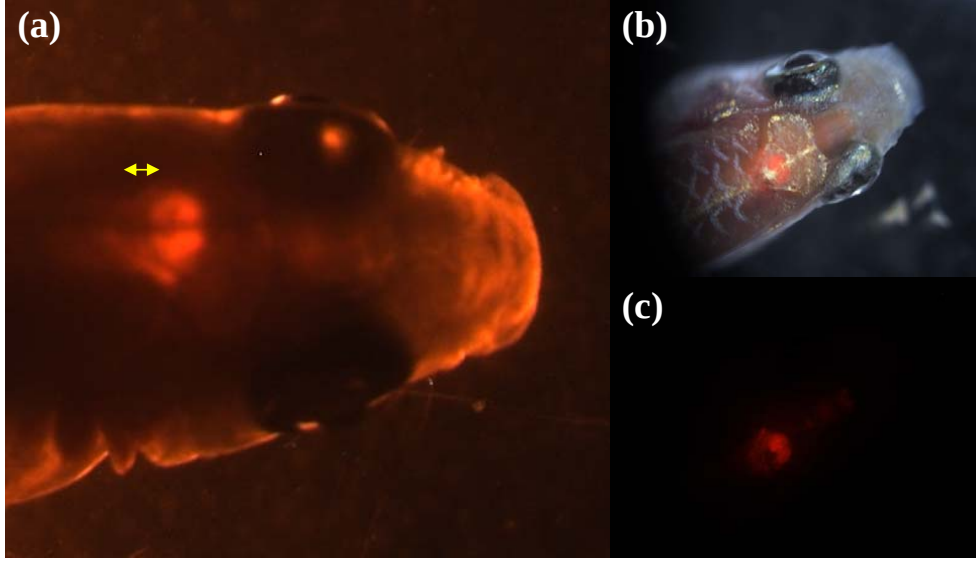


Figure 4.11: Fluorescence image of the corresponding *zic4* zebrafish. (a) A microscopic image merged with the corresponding fluorescent image before optoacoustic imaging; (b) A microscopic image merged with the corresponding fluorescent image after optoacoustic imaging; (c) Purely fluorescent image after optoacoustic imaging. Arrow: scan range.

4.2.3 Multispectral 3D imaging of a *zic4* zebrafish *post mortem*

We performed 3D MSOT imaging of a six-month-old *zic4* zebrafish around the brain area. Unlike the method presented in subsection 4.2.1 and 4.2.2 that subtracts two optoacoustic images acquired at two wavelengths, we used five wavelengths for this measurement. The disadvantage of using only two wavelengths is that there are other tissue structures such as oxygenated hemoglobin (HbO₂) and deoxygenated hemoglobin (Hb) that have the same absorption trend at these two wavelengths as mCherry. Even the absorption of these tissue structures does not drop as fast as mCherry after 587 nm (in terms of the percentage change), the absolute absorption difference of these tissues at these two wavelengths can be as high as of mCherry. Thus after an image subtraction the absorption difference can be the contribution of both mCherry and other tissues. Here we employed a blind source unmixing algorithm [60] based on independent component analysis (ICA) [61] developed by Glatz and Dr. Deliolanis. With this method we can randomly select several wavelengths and identify different spectral changes due to different tissue absorptions.

We scanned the fish in the brain area for 20 slices with a step size of 0.1 mm. Figure 4.12 shows 2D MSOT images in one image plane obtained at 5 wavelengths (531 nm, 544 nm, 565 nm, 601 nm and 616 nm) and the corresponding mCherry expression sought by the blind source mixing algorithm is superimposed on the optoacoustic image acquired at 565 nm. The image plane is similar to figure 4.10, therefore the mCherry expression takes place at the same location.

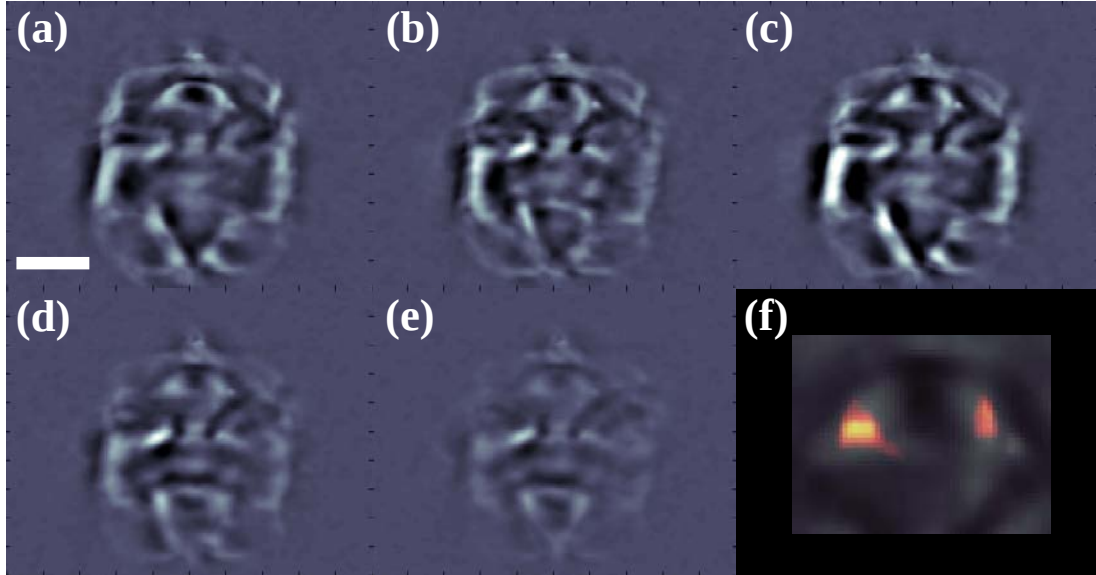


Figure 4.12: 2D MSOT images obtained at 5 wavelengths. (a) 531 nm; (b) 544 nm; (c) 565 nm; (d) 601 nm; (e) 616 nm; (f) Enlargement of the brain area with mCherry expression, obtained from the blind source unmixing algorithm, superimposed on the optoacoustic image obtained at 565 nm. Scale bar: 1mm

Figure 4.13 shows the 3D segmented fish skin and brain superimposed with mCherry expression resolved by the blind source unmixing algorithm.

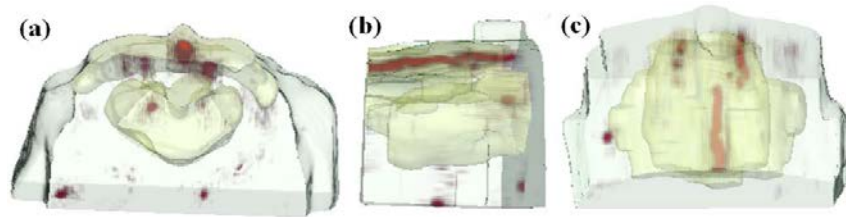


Figure 4.13: 3D segmentation of the fish skin and brain superimposed with mCherry expression. (a) Front view; (b) Side view; (c) Top view.

4.3 Conclusions and outlook

We have shown in this chapter firstly optoacoustic imaging of small animals based on the intrinsic contrast of tissue structures, which arises from a broad change of the optical absorption. The MSOT system is able to resolve inner structures of a nude mouse *post mortem*, the size of which exceeds 20 mm. Furthermore we performed 3D whole-body

imaging of an adult zebrafish. The optoacoustic images of both a mouse and a zebrafish correspond well with X-ray CT images or histological sections.

Then multispectral imaging was applied to image transgenic zebrafish with mCherry expressed in the brain area (zic4 zebrafish). We performed *post mortem* and *in vivo* measurements, and both resolved the mCherry expression in the brain. In addition 3D rendering of the mCherry fluorescent protein location together with the segmentation of the fish skin and brain was conducted to demonstrate that the system can not only present histological views of biological tissues, but can also perform 3D volumetric imaging of contrast agents.

We have to emphasize the *in vivo* capability of the system due to its fast acquisition speed. Figure 4.14 shows optoacoustic imaging of zebrafish in different ages ranging from 2 weeks old to 1 year old, where white circles illustrate the brain area. Figure 4.14(a) does not show much contrast since a 2-week-old fish is quite translucent and there are not much absorption changes within the body. These images were taken at approximately the same developmental area, where the cerebellum is nearly detached from the brain and tends to disappear. Although we did not perform these images *in vivo*, the experiment described in subsection 4.2.2 demonstrates that the MSOT system has the capability of *in vivo* imaging. Thus we deliver a method that has the potential for longitudinal studies of development, tumorigenesis, angiogenesis and regeneration in the adult developmental stage and so on.

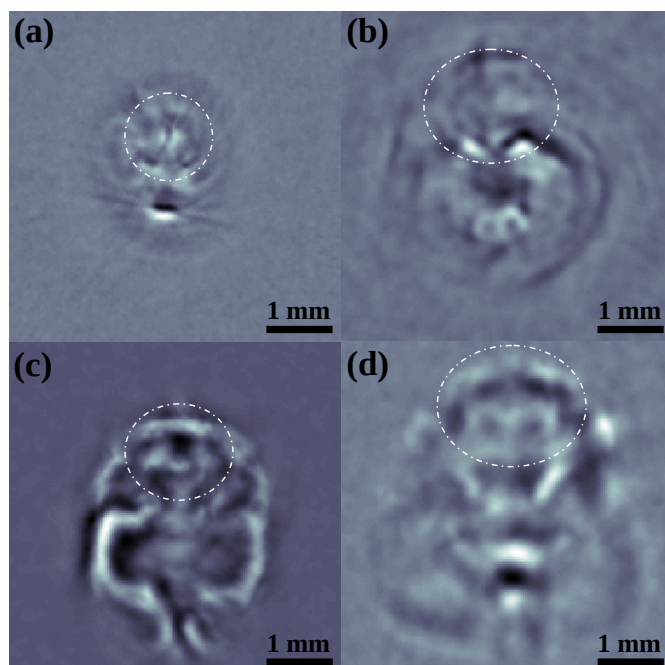


Figure 4.14: Optoacoustic images of zebrafish in different ages ranging from 2 weeks old to 1 year old. White circles indicate the brain.

CHAPTER 5

Design of a high resolution real-time
optoacoustic microscopy system

5.1 Motivation

Chapter 3 demonstrates a fast tomographical optoacoustic imaging technique based on a continuous acquisition method. Although it achieved ~ 30 mm imaging size, there are two major problems that limit the application of this technique. First, the tomographic system uses a low repetition rate laser (30 Hz) that can image one frame within 9 seconds, thus it is not able to trace fast functional changes that occur within one second. Second, a cylindrically focused transducer is used for the detection of optoacoustic signals, and the vertical resolution (proportional to the focal length of the transducer) degrades dramatically when it is off the focal zone of the transducer (see figure 3.8(c)). Thus in this chapter we introduce a high-resolution real-time optoacoustic microscopy system, which utilizes a high repetition rate laser and a high-resolution spherically focused transducer, with a compromise of half the imaging size in comparison with the tomography system.

High-resolution optoacoustic microscopy is a newly developed imaging modality. There are two types of such systems developed in recent years. One type is relying purely on the acoustic resolution and uses a dark-field confocal illumination. It can achieve an axial resolution of $15\text{ }\mu\text{m}$ and a lateral resolution of $45\text{ }\mu\text{m}$ [8]. With a virtual detector focusing technique (VDFT) the depth-of-focus of the system can be expanded extensively [62]. This type of system has many applications including human palm imaging [63, 64] and noninvasive imaging of mouse brains [65]. However it is not a real-time imaging system since the design is robust and heavy. The other type of systems is focusing the optical beam into optical subwavelength range by a high numerical aperture (NA) optical objective and detecting the optoacoustic signal with an unfocused transducer. It can achieve a 220 nm resolution with a imaging depth of a few tens of microns [66, 67]. However the system is relying purely on the optical focus. When an imaging target is off the focus of the transducer, the resolution degrades dramatically (0.1 mm off the focus leads to an optical beam size of $486\text{ }\mu\text{m}$). Thus scans at different depths are necessary, which slow down the imaging speed. Also with a 10 nJ illumination and 220 nm beam size the fluence is 1000 times higher than laser safety standards [37]. Recently the imaging system has been adapted to be real-time and has been improved by introducing an acoustic lens to focus the optoacoustic wave [68]. It achieves a lateral resolution of $3.4\text{ }\mu\text{m}$ and an axial resolution of $15\text{ }\mu\text{m}$. The imaging depth is reaching $1.2 - 1.4\text{ mm}$ in a mouse leg. Again the fluence is 55 times higher than laser safety standards [37] even the lowest energy is used, i.e. 100 nJ [68], which is not suitable for *in vivo* imaging. Also the skin of a mouse is much less absorbing than the human skin (see figure 5.1), the imaging depth in refrence [68] would decrease dramatically for human skin imaging, thus for clinical applications this method is not well suited.

Therefore high resolution systems with real-time imaging capability that can penetrate deeper and meet safety standards are required. In the optoacoustic community there are two ways of speeding up the image acquisition: One is to use a transducer array that can

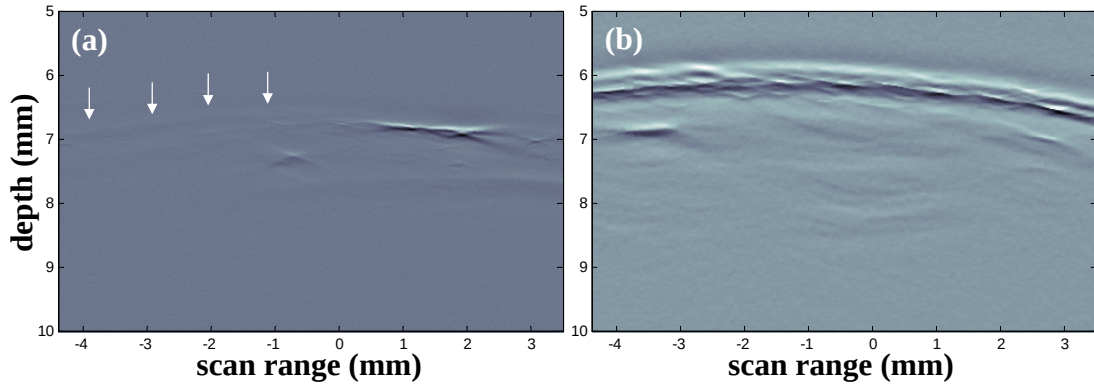


Figure 5.1: Comparison of optoacoustic images of the mouse skin and the skin of the author’s palm. (a) A 2D optoacoustic image of the mouse skin, where white arrows indicate the skin location; (b) A 2D optoacoustic image of the human skin.

detect optoacoustic signals in many close-by projections/positions with one single pulse; the other alternative is to use a single element transducer, which requires fast mechanical scanning and a high repetition rate laser. The costs of transducer arrays and corresponding data acquisition cards, however, are high. Considering that a certain transducer array is only applicable for a limited range of imaging resolution and depth, the costs increase with several sets of transducer arrays for imaging various animal sizes. Most importantly, no transducer arrays with frequencies larger than 40 MHz are commercially available, leaving the only choice to a single element transducer for high-resolution, real-time imaging.

In this chapter, we will describe a compact, multimode (MM) fiber based, high resolution, real-time, optoacoustic microscopy system. It consists of a fast back-and-forth scanning machine and a slow scanning linear stage to form a sawtooth scan. We use either an open-loop controlled angular scan rotor (section 5.2) or a closed-loop controlled piezo linear stage (section 5.3) to perform the fast scan, which carries a spherically focused transducer with a hole and a MM fiber that is inserted in the hole. An optional part attached to the fast scanning rotor/piezo stage is a fine adjustable lens tube to control the illumination size relative to the tissue surface. The system therefore abandons any optical constructions and reduces the load of the fast scanning device, which is necessary for high-speed scanning. The real-time microscopy system is capable of 5 frames/s with a scan range of 4 mm. The frame rate is limited by the current laser repetition rate (~ 1.5 kHz) and can be further increased by scanning a shorter range or by using a laser with faster repetition rate.

The high-resolution, real-time optoacoustic microscopy system can be applied to monitor haemodynamic response [6], mouse intracerebral hemorrhage imaging for the study of pathophysiology and corresponding treatment of hemorrhagic stroke [69], dermatology [8] and ophthalmology applications [70, 71], etc. It can also be used for clinical applications such as human skin cancer [63, 64] and nailfold capillary imaging [72, 73].

5.2 A microscopy system based on a limited angle torque rotor

5.2.1 Experimental setup and data acquisition

In this section we present a high-resolution, real-time microscopy system based on a customized limited angle torque rotor (H2W Technologies, Santa Clarita, USA) for fast 2D image acquisition and an additional motorized linear stage (LTM 60F, OWIS GmbH, Staufen, Germany) for the 3D scan. The limited angle torque rotor is open-loop controlled and can scan 14 frames per second with a load of 250 g. The rotor is driven by a linear current amplifier module (Quanser Inc., Markham, Canada), which is controlled by a function generator (Agilent 33210A, Meilhaus Electronic GmbH, Puchheim, Germany) giving required scan frequency and amplitude.

Figure 5.2 shows a picture of the real-time optoacoustic imaging system and figure 5.3 shows main parts of the system. Figure 5.4 gives a simplified sketch. We use a passively Q-switched solid state diode pumped laser (Flare PQ HP GR, InnoLight GmbH, Hannover, Germany) for the generation of optoacoustic signals. The power of the laser is 800 mW. It has a pulse length < 3 ns and a repetition rate of ~ 1.5 kHz. We use an uncoated broadband precision window (WG10530, Thorlabs, Dachau, Germany) to split $< 10\%$ of the laser energy to a photodiode (FDS010, 200-1100 nm, 1 ns rise time, Thorlabs, Dachau, Germany) serving as trigger signals. The rest of the output energy is coupled to a multimode (MM) fiber (M29L05, 600 μm core diameter, 0.39 NA, Thorlabs, Dachau, Germany).

One side of the MM fiber is cleaved and inserted through a hole in a customized PZT ultrasound transducer with a central frequency of 50 MHz, a focal length of 7.15 mm and an element diameter of 6 mm (InSensor, Denmark). The hole is located at the center of the transducer with a 1 mm diameter. The fiber is fixed to a lens tube containing one fine and one coarse adjustable tuning, which allows adjusting the delivered light fluence at the targeted tissue surface.

Since the laser is passively Q-switched, the time interval between adjacent pulses varies significantly. The laser output was calibrated using acquired signals from the aforementioned photodiode. The peak-to-peak instability is 10.4% and the standard deviation of pulse-to-pulse jitter is 41.4 μs with an average pulse period of 630.9 μs , which indicates 6.6% of timing jitter instability. The pulse-to-pulse jitter can reach up to 100 μs , which is $\sim 16\%$ of the pulse period.

The large laser jitter and sinusoidal movement of the rotor (which means that at the border the rotor scans slower than in the middle) indicate that the time at which the laser pulses appear is unpredictable and at a fixed time interval the rotor's travel range varies. These two problems bring uncertainty of scan positions for each trigger event. In

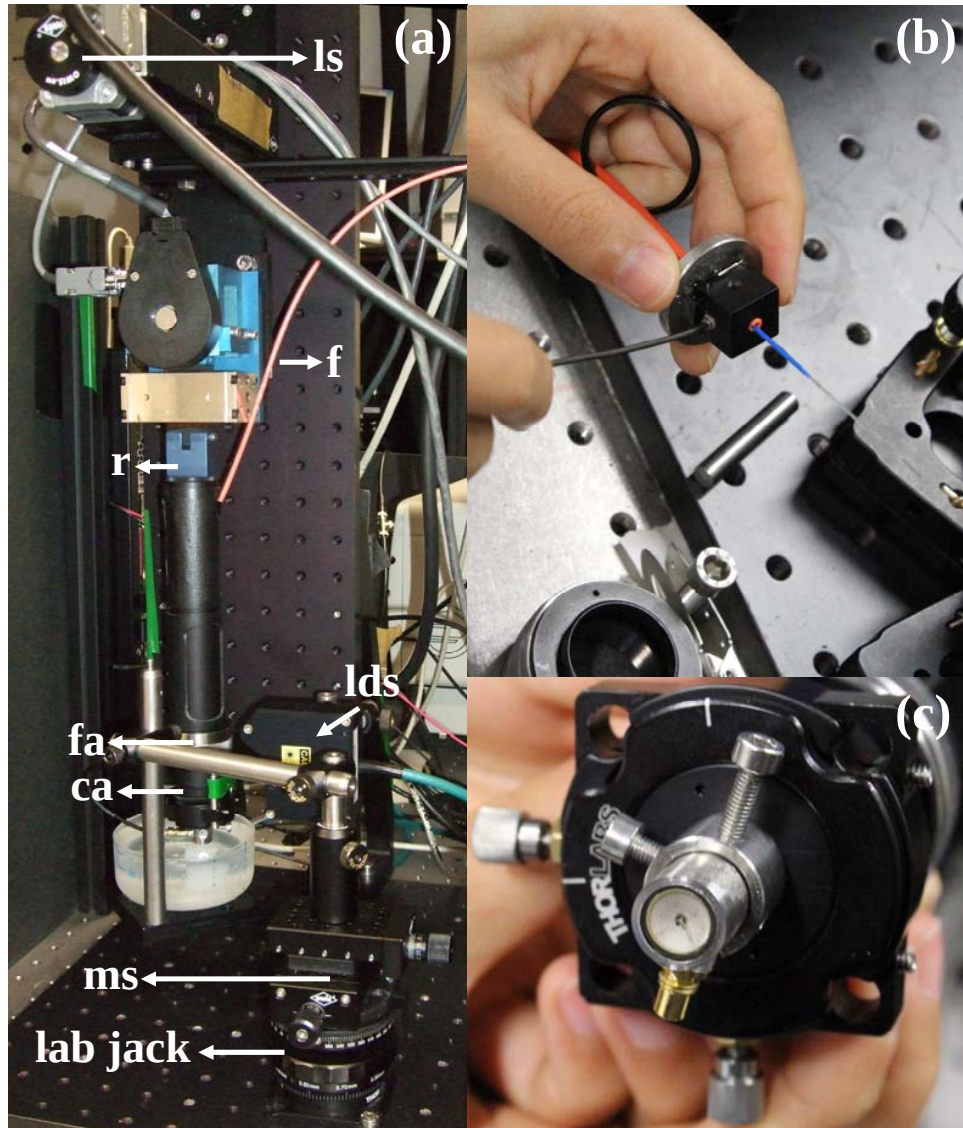


Figure 5.2: A picture of the real-time optoacoustic microscopy system. (a) Full view of the system. Abbreviations: linear stage (ls), fiber (f), rotor (r), fine adjustable lens tube (fa), coarse adjustable lens tube (ca), XY manual stage (ms), lab jack (lj), laser distance sensor (lds); (b) Inside the fine adjustable lens tube; (c) Close view of the transducer holder.

order to solve the problem, we need to real-time record the true position of the transducer when a trigger event occurs. Here we choose a laser distance sensor M11L/10-10B (MEL Mikroelektronik GmbH, Eching, Germany) to measure the position of the transducer. The laser distance sensor provides an analog output representing the scan position. The sensor has a total measure range of 10 mm with $2.5 \mu\text{m}$ resolution and up to a sampling rate of 10 kHz.

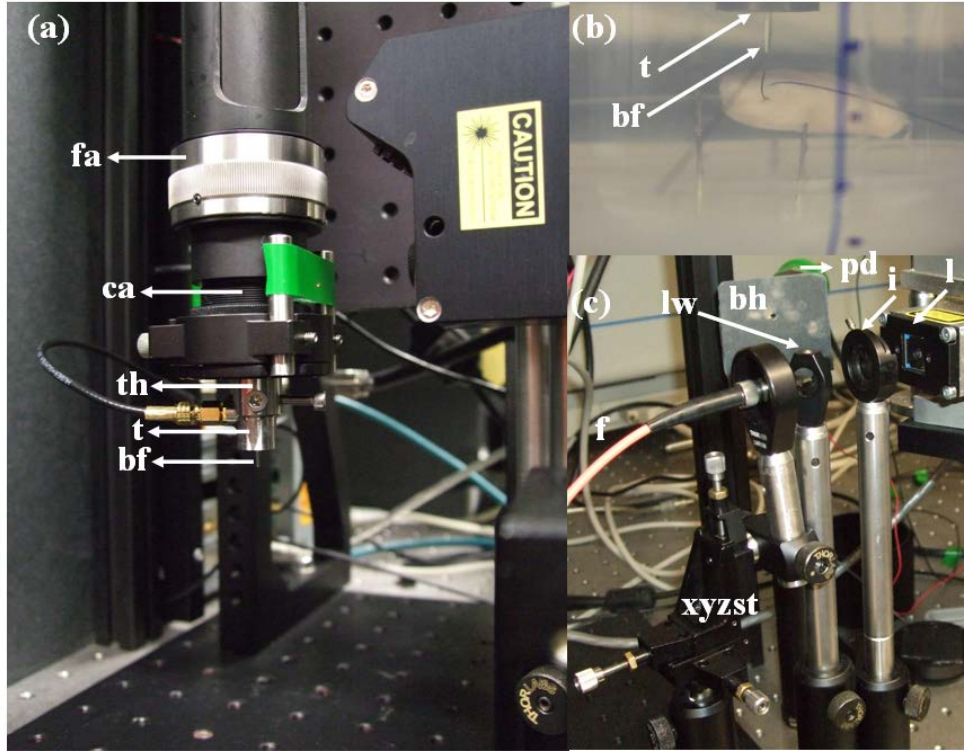


Figure 5.3: (a) Enlargement of the illumination and acoustic wave detection part for the real-time optoacoustic microscopy system; (b) Enlargement of a bar fiber in the transducer during imaging; (c) A picture of the free beam getting splitted and coupled to a MM fiber. Abbreviations: fine adjustable lens tube (fa), coarse adjustable lens tube (ca), transducer holder (th), transducer (t), bare fiber (bf), fiber (f), XYZ manual stage (xyzst), laser winder (lw), black hardboard (bh), home made photodiode (pd), iris (i), laser (l).

The ultrasound signals detected by the transducer and the corresponding positions of the rotor are acquired by a UF3-4142 PCI Express card (Strategic Test AB, Sweden). The digitizer has a 128 MSample (256 Mbyte) memory and a 14-bit resolution. The sampling rate of the digitizer is 400 MS/s ($3.75 \mu\text{m}$ resolution) using one channel and 250 MS/s ($6 \mu\text{m}$ resolution) using 2 channels simultaneously. We set the input range of the transducer channel and distance sensor channel as 200 mV and 10 V, respectively. This in turn translate to the smallest measurement unit as $24.4 \mu\text{V}$ and $0.6 \mu\text{m}$. Since we have more than 1500 trigger events within one seconds and more than 1000 sample per trigger, it is a large amount of data to be stored, we operate the digitizer in FIFO mode using multiple recording option. The multiple recording option allows thousands of trigger events recorded without resetting and restarting the board. When the number of samples is predefined for each trigger event (always a fixed value), the onboard memory is divided into many segments, the length of which is the predefined number of samples. When the first trigger event is detected (in our case an external analog trigger coming from the photodiode), the

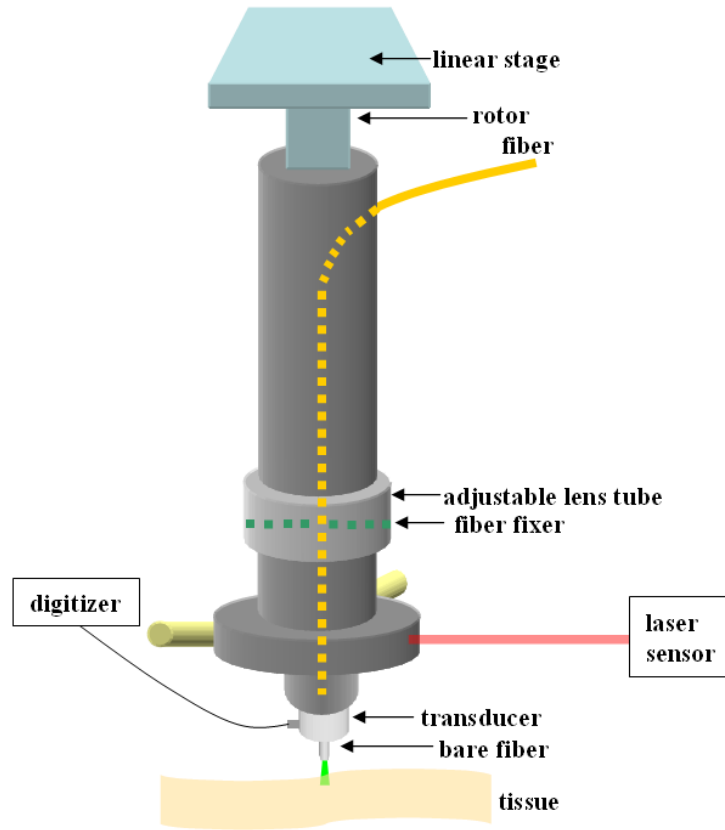


Figure 5.4: Illustration of the real-time optoacoustic microscopy system.

card starts to record signals and the data is automatically transferred to the next available segment. Since we record more than 1000 samples per trigger per channel, this translates to a data stream of $1500 \cdot 1000 \cdot 2 \cdot 4 = 12$ Mbytes/s, if we image objects in 3D it can record ~ 21.3 seconds but not longer. Thus we also apply FIFO mode that continuously transfers the recorded data from the board to the PC hard disk. Since we are streaming to a single hard disk, the transfer rate is not very high ($\sim 25 - 50$ Mbytes/s), which is the limitation of our system. Faster transfer rate can be further improved by using advanced disk controller technologies to stream at up to 200 Mbytes/s.

The real-time acquisition software has been implemented by Sebastian Söntges in Lab-view and C++ programme. The programme enables real-time image display with correctly interpolated scan positions and simultaneously shows the maximum amplitude projection of the acquired 3D optoacoustic image, which is essential to show the current imaged location and to guide us moving the object to the desired region of interest (ROI). The basic idea of the programme is the following. The signals triggered by the photodiode is continuously captured. The program searches the local maximum and minimum of the rotor readout position to define where a new frame starts and stops. Within each frame the distance between adjacent measurements is not a constant number due to the

large laser jitter and a sinusoidal movement of the rotor, thus the image is distorted if we only use the raw scan positions. To avoid this, the image is interpolated so that the new positions are equally spaced and the acoustic signals are assigned to the new positions by nearest neighbor approach. The new interpolated image is then displayed on the screen.

Since the transducer is scanned angularly and we reconstruct the image linearly, the actual image is pressed from an angular image to a rectangular one, which introduces image distortion and errors. Suppose we have a scan range of (denoted as s) 6 mm, we will calculate the maximum lateral and axial errors. The length between the center of rotation and the surface of the imaged object (L) is 320 mm, the depth of the ROI (d) is 3mm. Figure 5.5 illustrates an exaggerated scan angle to show the lateral and axial errors more clearly. The maximum lateral error $= d \cdot \sin\theta = d \cdot \sin(\arctan(\frac{s}{2L})) = 3\text{mm} \cdot \sin(\arctan(3/320)) = 28.1 \mu\text{m}$, which is 0.94% error. The maximum axial error $= d - d \cdot \cos\theta = 3\text{mm} - 3\text{mm} \cdot \cos(\arctan(3/320)) = 0.13 \mu\text{m}$, comparing with $6 \mu\text{m}$ card resolution the axial error can be neglected.

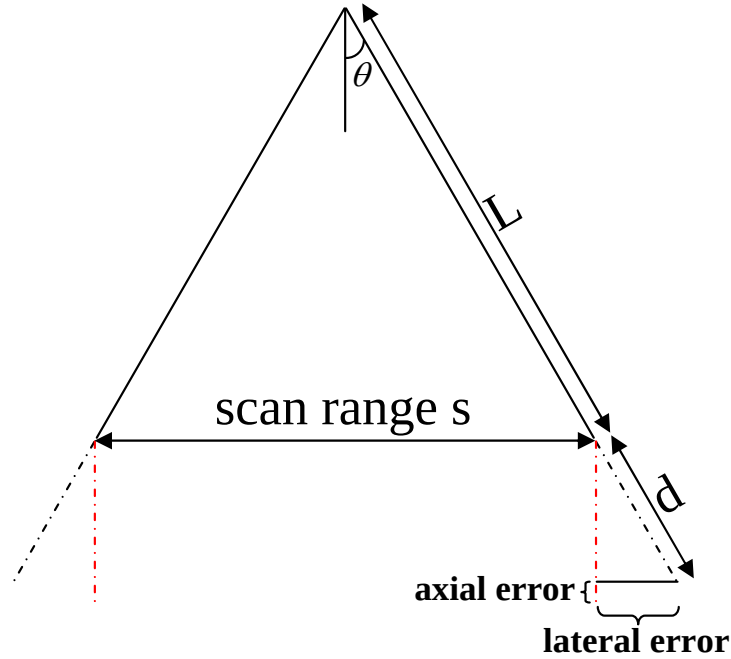


Figure 5.5: Illustration of rotor scan errors. The scan angle $2 \cdot \theta$ is exaggerated to show the errors more clearly.

We have to point out that the cable length is very important for high-resolution optoacoustic imaging in order to preserve the original optoacoustic signals without reflection, since we need ultrasound transducers with high center frequencies. Figure 5.6 shows the influence of optoacoustic images using different cable lengths by imaging a $10 \mu\text{m}$ black-dyed microsphere. The input impedance of the digitizer for both images is set to 50Ω .

Figure 5.6(a) uses a 3 m coaxial cable and figure 5.6(b) a 35 cm coaxial cable. We can see that by shortening the cable the image quality improves with the reduction of the false resonance.

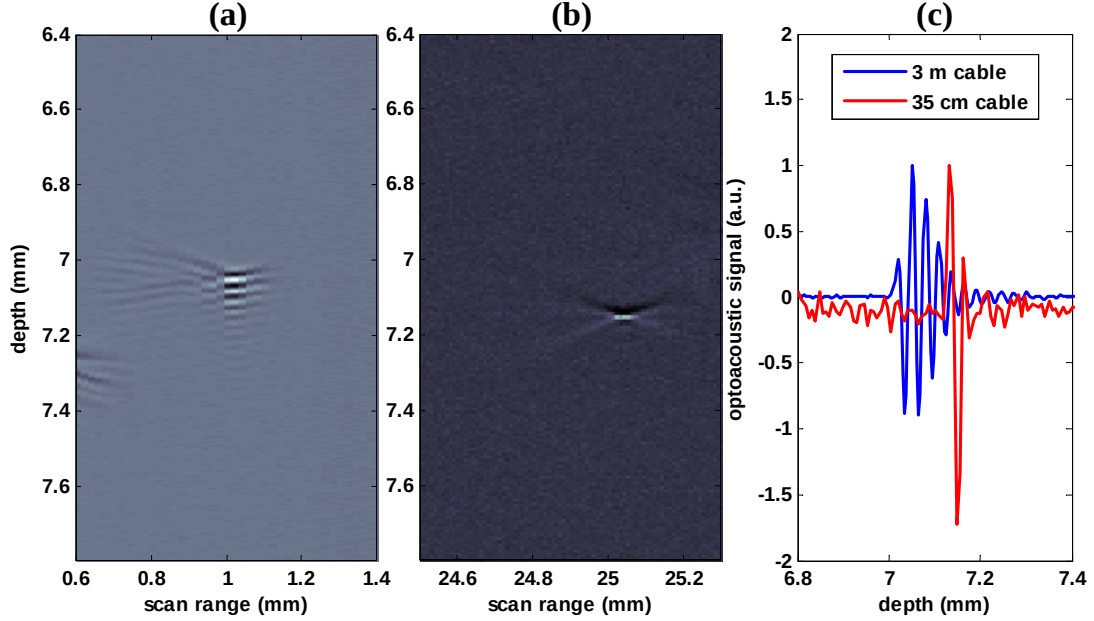


Figure 5.6: Influence of optoacoustic images using different cable lengths. (a) An optoacoustic image of a $10\ \mu\text{m}$ black-dyed microsphere using a 3 m coaxial cable; (b) An optoacoustic image of a $10\ \mu\text{m}$ black-dyed microsphere using a 35 cm coaxial cable; (c) 1D optoacoustic signal of the microsphere.

5.2.2 The virtual detector focusing technique (VDFT)

Since we are using a spherically focused transducer that is tightly focused, the optoacoustic signal degrades dramatically if it is outside the focal point. Figure 5.7 shows simulated optoacoustic signals generated from a $10\ \mu\text{m}$ sphere at different image positions. The focal length and diameter of active transducer area used for simulation are 7 mm and 6.5 mm respectively.¹ The signal collected by the ultrasound transducer corresponds to the integral of the acoustic pressure in its active area. In order to simulate such signal, we discretized the transducer surface as a set of equally spaced points, so that the signal is estimated as the summation of the pressure corresponding to such set of points. For each given point detector, the optoacoustic pressure is computed using equation 2.10. We assume that the deposited energy density is a parabolic heating profile in our model, then the signal collected by the transducer is obtained by adding up the signals corresponding to all the point detectors in which the transducer is discretized. If the number of point detectors is large

¹The 3D simulation codes were provided by Daniel Queirós.

enough, the simulated signals faithfully represent the actual ones.

Figure 5.7 shows the simulated optoacoustic signals at different locations relative to the transducer and without filtering. The x-axis represents depth in millimeter and the y-axis indicates the optoacoustic signal with an arbitrary unit scale. The focal point of the transducer is set as (0,0,0) in the Cartesian coordinate system (shown in the inset of figure 5.7(i)). A transducer with a 1 mm diameter hole (shown in blue) and one without a hole (shown in red) are both simulated. From the simulation we can see that the optoacoustic signals get broadened when the sphere lies off the focal point of the transducer, due to the fact that the generated optoacoustic signals of the sphere arrive at the transducer surface at slightly different times, which leads to the signal distortion. We could notice that when the sphere is horizontally off the focal point (5.7(e,f)) the middle of the optoacoustic signals get strongly influenced. The difference between these signals can be explained as the following: The missing 1mm hole is approximately a point transducer and detects the 10 μm sphere with an N-shaped optoacoustic signal similar to figure 5.7(d), thus the signal coming from a transducer with a hole is the signal from a normal transducer subtracted by the signal from the 1 mm point-like transducer. Figure 5.7(a) and (g) indicate that when the optoacoustic source is below the focal point ($x = -1\text{mm}$) the negative value of the optoacoustic signal determines the source position and when the source is above the focal point ($x = 1\text{mm}$) the positive value counts. This is because different time arrivals at different point detectors distort the perfect N-shaped optoacoustic signal. Above the focal point the optoacoustic signal arrives at the transducer center the earliest, while below the focal point the latest.

Also, we did a simulation to see whether the sphere location would influence its axial detection size. The y and z positions of the sphere are kept as 0 in the simulation. Since the sources of simulated optoacoustic signals locate at different depths and have different intensities, we shift the signals to one position and normalize them for an easy comparison. Figure 5.8 shows that at the focal point the detected size is the smallest (red line marked with triangles), the further the sphere locates from the focal point, the bigger the detected size. This can also be explained by the fact that different time of arrival at the transducer surface leads to a broadening of the optoacoustic signal.

Figure 5.9 shows 2D reconstructed images based on a simulation when we scan the transducer along a 10 μm sphere. The sphere is lying 500 μm above, at the focal point and 500 μm below the focal point for the three simulations. We could see that when the sphere lies below the focal point, the negative values correspond to the correct position of the sphere, which is indicated in figure 5.7(a,b,c) as well.

In order to reduce the out-of-focus artifacts of a tightly spherically focused transducer, a virtual detector focusing technique (VDFT) is applied.² The VDFT uses a synthetic

²Sebastian Söntges and Miguel Ángel Araque Caballero programmed softwares employing the virtual

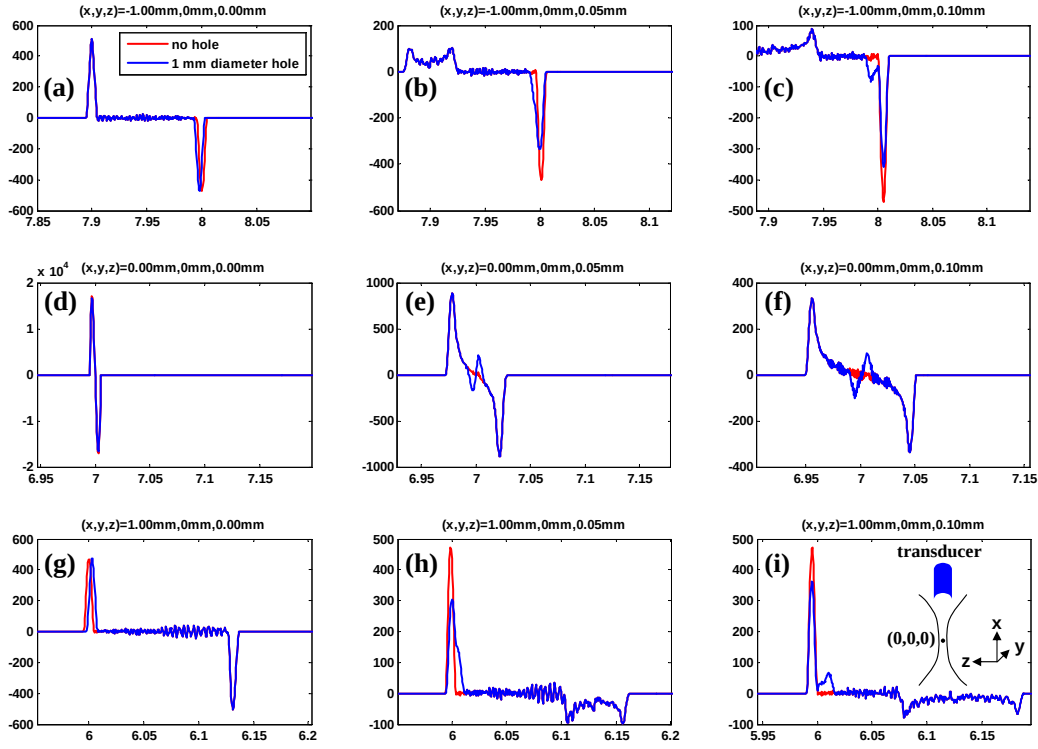


Figure 5.7: Simulated optoacoustic signals coming from a $10\ \mu\text{m}$ sphere at different positions without filtering. The x-axis represents depth in millimeter and the y-axis indicates the optoacoustic signal with an arbitrary unit scale. Red curves: simulated signals detected by a spherically focused detector. Blue curves: simulated signals detected by a spherically focused detector with a 1 mm diameter hole in the middle.

aperture focusing technique (SAFT) and a coherence factor (CF) technique to enlarge the depth of focus and to increase the SNR of optoacoustic signals [62]. Figure 5.10 shows the explanation of the VDFT. The concept assumes that the focal point of the transducer is a virtual point detector and the virtual detector has its fixed detection angle determined together by the transducer's focal length and active element diameter. When we linearly scan the transducer, an optoacoustic point-source lying within the detection angle but outside the focal point gets out-of-focus artifacts. Blue lines show the travel distance from the initial source to the the virtual detector at different scan positions. A longer travel distance can be interpreted as a longer travel time. Due to the image reconstruction principle of a linearly scanned transducer using the time-of-flight method, the final optoacoustic image is shown in the bottom left of the figure 5.10, where an arc-shaped curve in the figure represents the reconstructed image of the single point-source. Since the generation of an arc-shaped curve is due to the different time arrivals from the one and only optoacoustic source to the virtual point detector that is being scanned, we can apply mathematical models such as SAFT and CF to narrow down the arc to a point as shown at the bottom

detector focusing technique using C and Matlab, respectively.

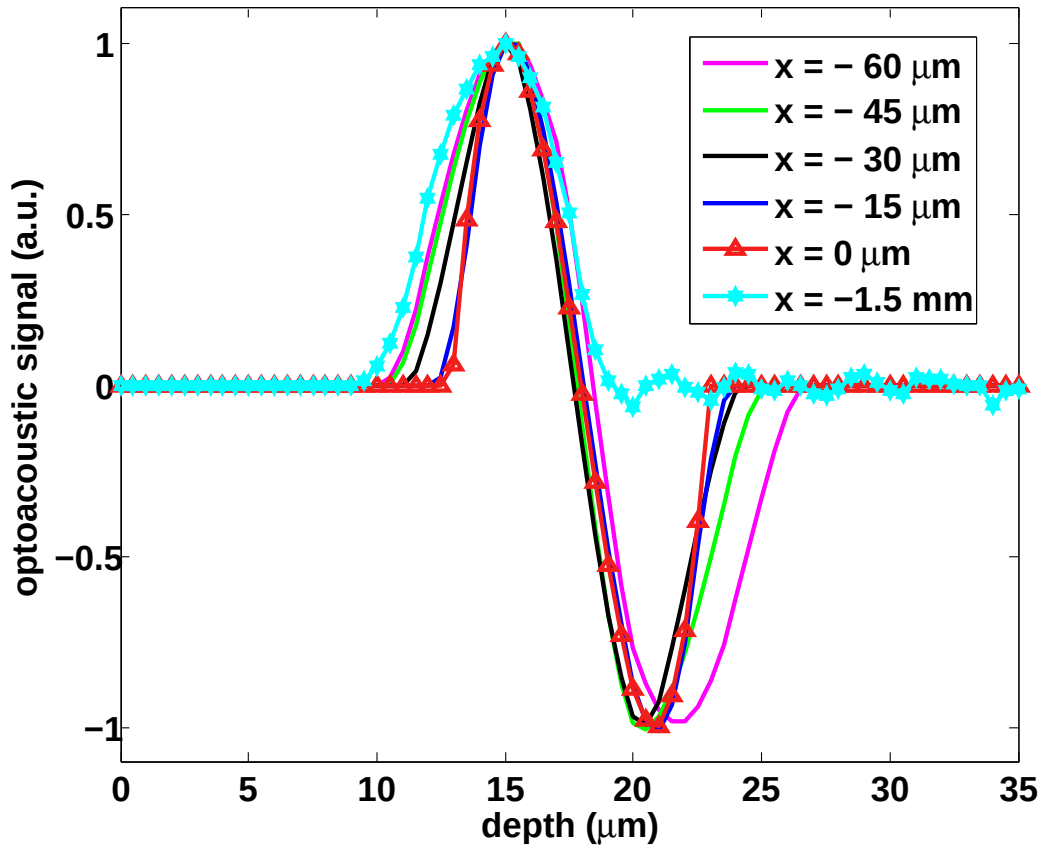


Figure 5.8: Simulated optoacoustic signals coming from a $10\ \mu\text{m}$ sphere at different x positions.

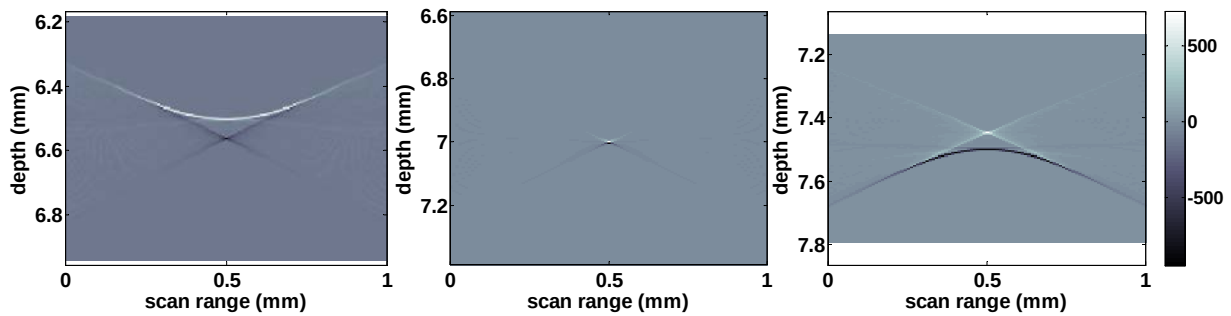


Figure 5.9: Simulation of optoacoustic images of a $10\ \mu\text{m}$ sphere at different positions. (a) $500\ \mu\text{m}$ above the focal point; (b) At the focal point; (c) $500\ \mu\text{m}$ below the focal point.

right of figure 5.10..

For one scan position, SAFT calculates the arc-shaped curve for each point along one scan line (a depth-resolved optoacoustic signal) and constructively sums up the values along the arc-shaped curve. The new summed value is assigned back to each point. SAFT

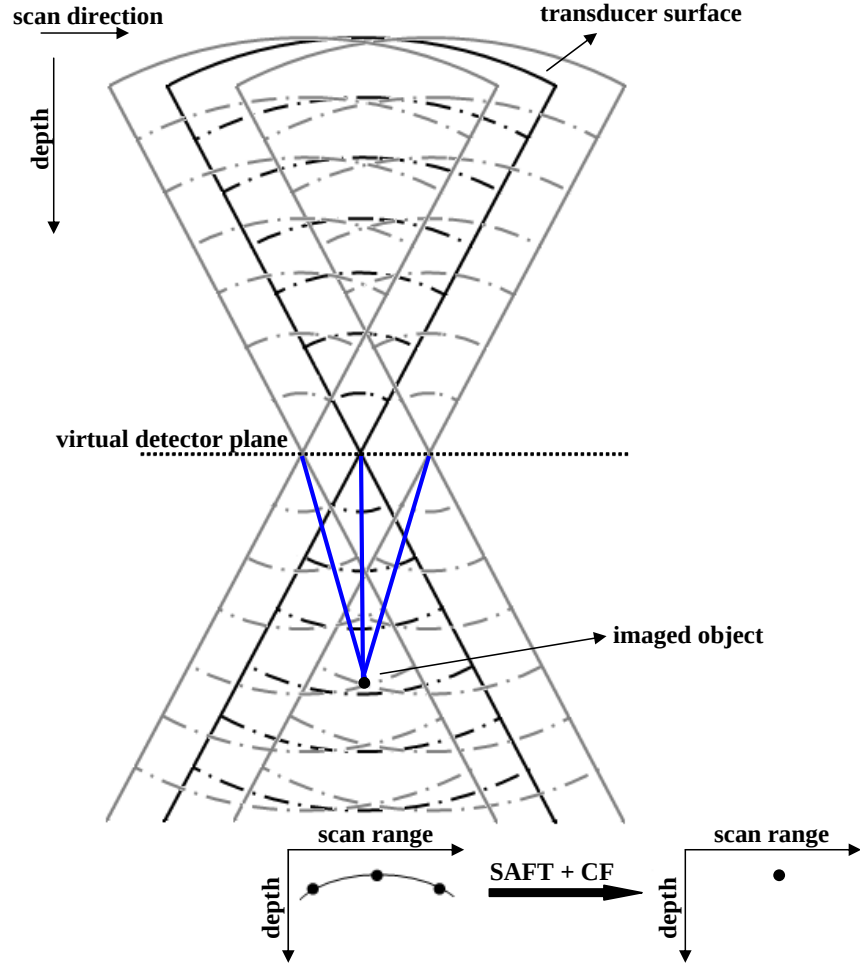


Figure 5.10: Scheme of the virtual detector focusing technique (VDFT).

employs the following expression [62, 74, 75],

$$S_{SAFT}(t) = \sum_{i=0}^{N-1} S(i, t - \Delta t_i) \quad (5.1)$$

$S_{SAFT}(t)$ is the new value for the scan position considered. $S(i, t)$ is the optoacoustic signal from the i th scan position. Δt_i is the difference of time-of-arrival between the i th scan position and the scan position being considered, the sign of Δt_i is negative when $t < \text{focal length}/\text{speed of sound}$, i.e. the point being calculated is above the focal point of the transducer. N is the number of scan positions for the summation, determined by the numerical aperture of the transducer and the step size between adjacent scan positions.

CF can further reduces the side lobes of the artifacts with the following expression [62, 76],

$$CF(t) = \left| \sum_{i=0}^{N-1} S(i, t - \Delta t_i) \right|^2 / \left[N \sum_{i=0}^{N-1} |S(i, t - \Delta t_i)|^2 \right] \quad (5.2)$$

The CF at the main lobe is high because the signals are coherent, which leads to a constructive summation. The signals at the side lobes contains a lot of noise and the denominator of the CF expression will be larger than the numerator, which leads to a low CF value. In this way the CF further reduces the out-of-focus artifacts.

Figure 5.11(a) shows a simulated optoacoustic image from a 10 μm sphere, the transducer has a focal length of 7 mm, an active diameter of 6.5 mm with a 1 mm hole in the center. Figure 5.11(b) shows improved image resolution after applying SAFT with improved SNR as well. Figure 5.11(e) is the enlargement of the figure 5.11(b), comparing with the enlargement of the original image in figure 5.11(d), we can see that the out-of-focus artifacts are greatly decreased. Figure 5.11(c) shows that by further applying CF the side lobes are indeed reduced comparing with figure 5.11(b). Figure 5.11(f) is the enlargement of figure 5.11(c) and gives more obvious comparison between the SAFT and SAFT+CF.

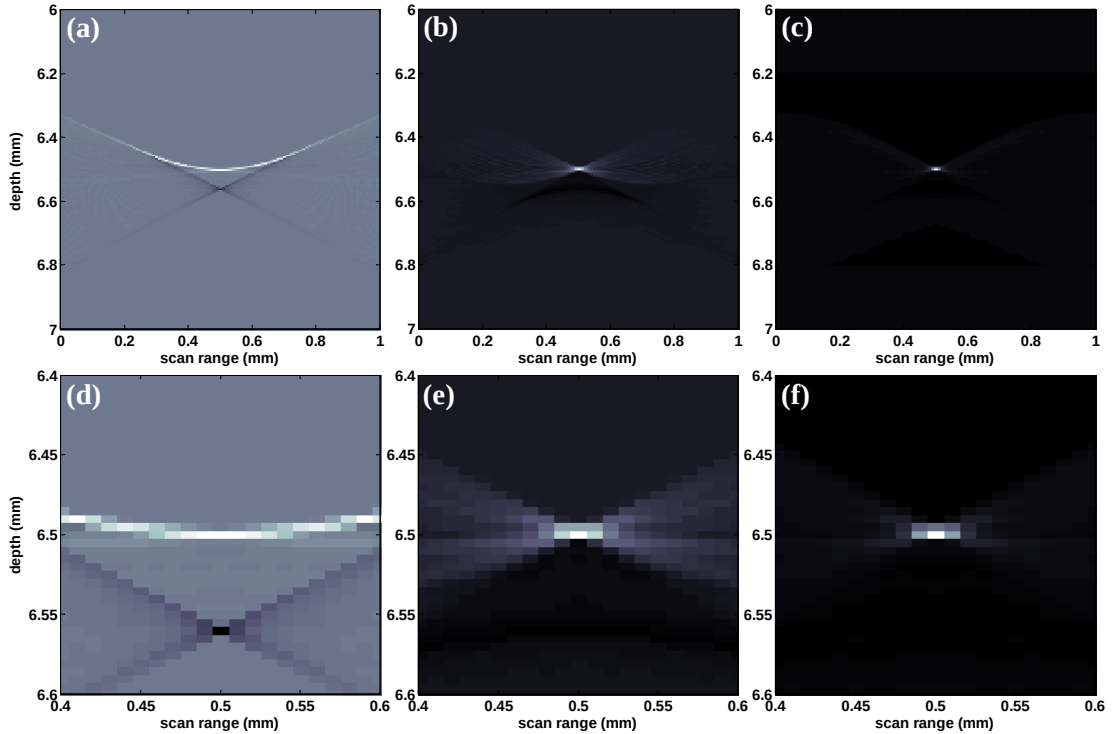


Figure 5.11: Applying SAFT and CF to the simulated optoacoustic images in figure 5.9(a). (a) Originally simulated optoacoustic image; (b) Applying SAFT; (c) Applying SAFT and CF; (d,e,f) are enlargements of (a,b,c), respectively.

Figure 5.12 shows applying the VDFT on the simulated 2D images in figure 5.9. The red arcs in the figure demonstrate the mask of time delays. Taking figure 5.12(a) as an example, the red arc demonstrates the time delays around $(x, \text{depth}) = (0.5 \text{ mm}, 6.5 \text{ mm})$, where values along the mask will be summed up and create a new value to the point $(x, \text{depth}) = (0.5 \text{ mm}, 6.5 \text{ mm})$. Since below the focal point the negative part shows the correct object size and position. We will henceforth invert the values below the focal point for certain optoacoustic images, in order to present reasonable visual images.

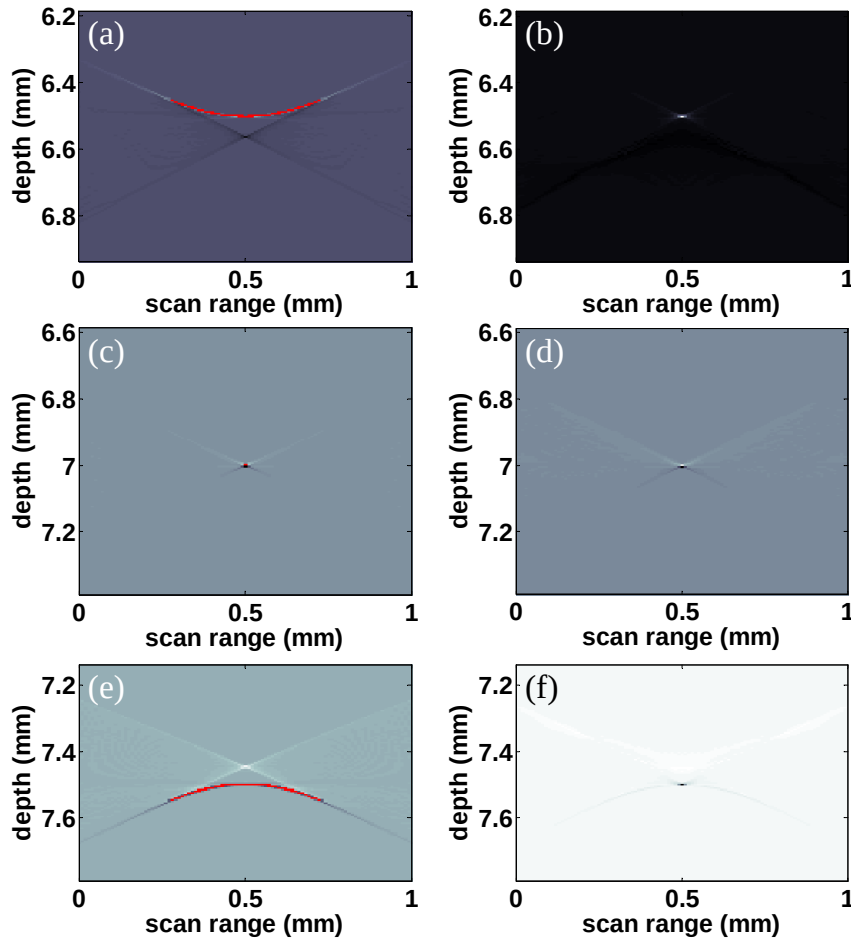


Figure 5.12: Applying the VDFT to the simulated optoacoustic images in figure 5.9. (a,c,e) Simulated optoacoustic images before applying the VDFT; (b,d,f) Simulated optoacoustic images after applying the VDFT. In each image a red curved mask indicates where the signals are delayed. Taking (a) as an example, the optoacoustic signals around neighboring scan lines of point $(x, \text{depth}) = (0.5 \text{ mm}, 6.5 \text{ mm})$ (labeled by the red curve) will be constructively summed up by SAFT and denoised by CF. The new value will be given to the point $(x, \text{depth}) = (0.5 \text{ mm}, 6.5 \text{ mm})$.

5.2.3 System calibration

In order to calibrate the system we prepare a pure agar phantom embedded with $10\ \mu\text{m}$ black-dyed polystyrene microspheres (Polysciences, Inc., Warrington, PA). Since the microspheres are very tiny and cannot be seen by the naked eye, we put the phantom under a stereo microscope to label a sphere for orientation purpose. Figure 5.13(c) shows a picture of a labeled microsphere with two $150\ \mu\text{m}$ black sutures. The phantom is then put into a water tank under the optoacoustic microscope. We imaged the sphere at different depths to calibrate the system also outside its focal zone. Figure 5.13(a) is the optoacoustic image of a $10\ \mu\text{m}$ sphere before applying the VDFT and 5.13(b) shows improved system resolution after applying the VDFT. The system resolution upon imaging depth is shown in table 5.1. The lateral and axial resolutions are calculated by the full width at half maximum (FWHM) for a Gaussian fit of the optoacoustic signal minus the diameter of the microsphere.

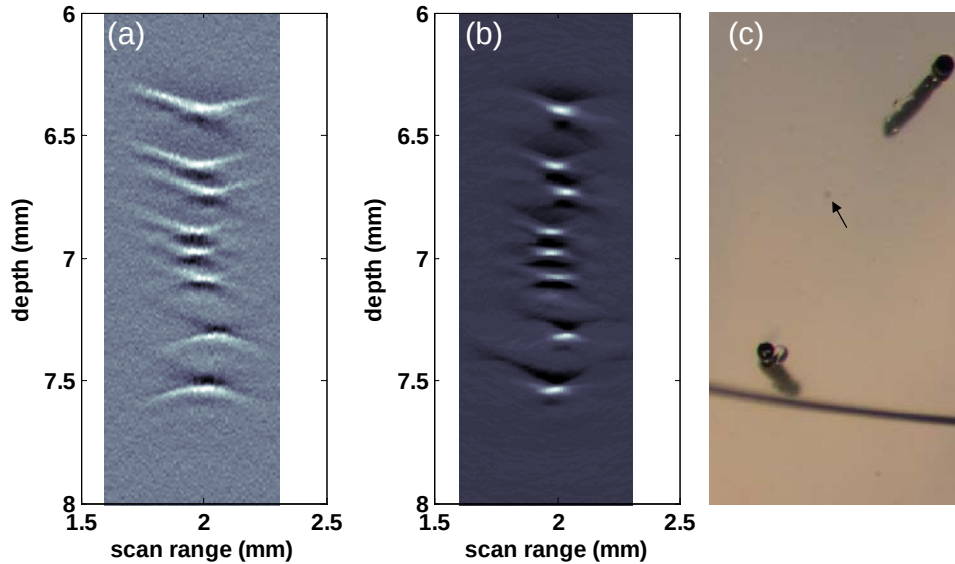


Figure 5.13: System calibration using a $10\ \mu\text{m}$ sphere. (a) Before applying the VDFT; (b) After applying the VDFT; (c) A photo of a $10\ \mu\text{m}$ sphere for imaging pointed by an arrow.

distance (mm)	6.39	6.66	6.73	6.89	6.97	7.08	7.31	7.54
lateral resolution (μm)	69.09	67.62	59.83	72.21	68.28	84.27	63.31	78.19
axial resolution (μm)	18.91	15.09	13.22	10.48	10.66	7.36	11.00	14.03

Table 5.1: System resolution upon imaging depth after applying the VDFT.

Figure 5.14 shows an imaging example above the focal point of the spherically focused transducer. Figure 5.14(a) and (c) are optoacoustic images of $10\ \mu\text{m}$ sphere before and

after applying the VDFT. Figure 5.14(b) and (d) are the horizontal cuts at the center of the sphere. We could see that the VDFT not only improves the lateral resolution, but also smoothes the image and increases the SNR because the noise is incoherent.

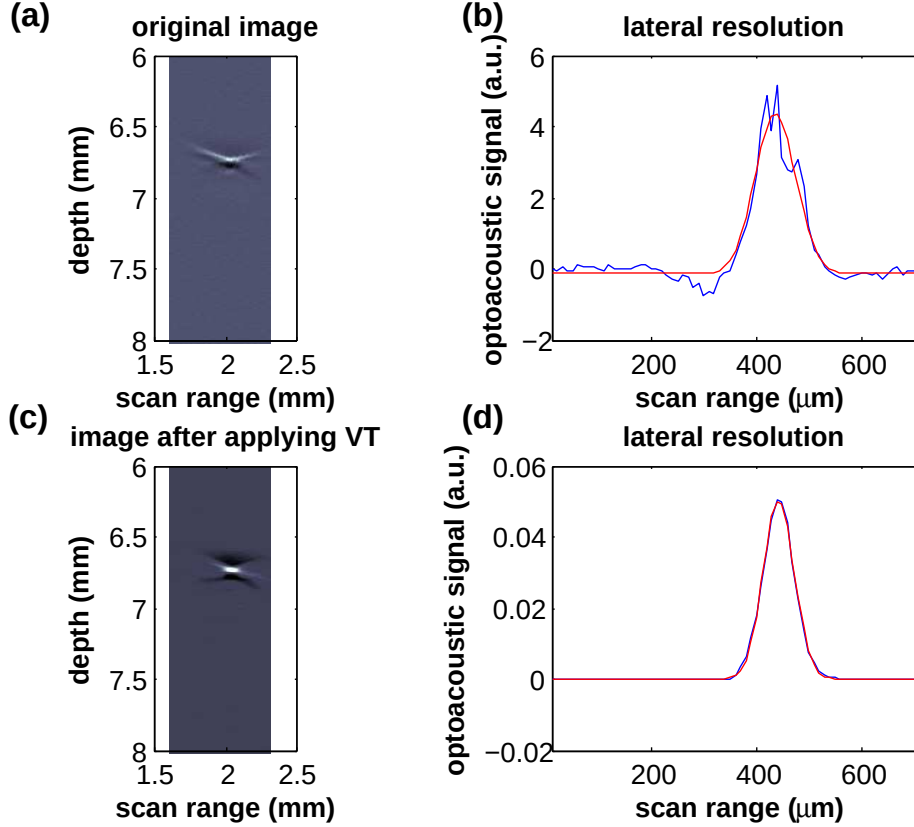


Figure 5.14: An imaging example above the focal point of the spherically focused transducer. (a,c) Optoacoustic images of 10 μm sphere before and after applying the VDFT; (b,d) Horizontal cuts around the center of the sphere.

From table 5.1 we can find out that at the focal point the axial resolution is the smallest and the further the object is away from the focal point, the larger the axial resolution, which matches well with the simulation shown in figure 5.8. The axial resolution is worse than expected. The reason is that the original images are noisy, by applying the VDFT the added noises tend to smear the real signal. Also the lateral resolution is not as high as expected due to several reasons. Firstly, the hole inside the transducer might influence its focusing. Secondly, the images were acquired using an angular scanned rotor and reconstructed under a linear-scan assumption, which leads to an unequally spaced image compression. Third, during imaging the fiber tip was extruding 5 mm outside the transducer (see figure 5.15) and can lead to the reflection and scattering of acoustic waves, this in turn will affect the system resolution.

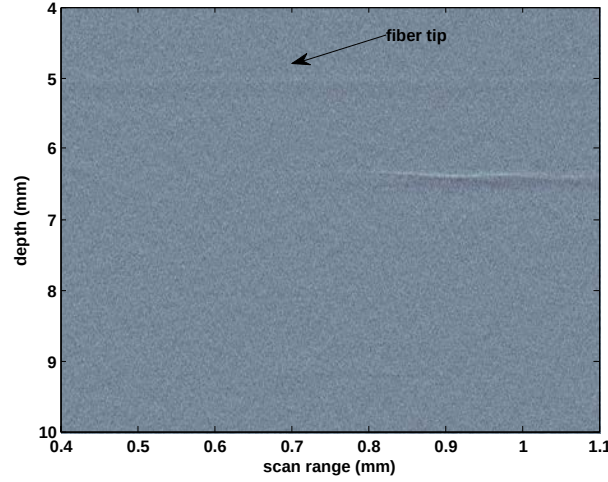


Figure 5.15: Fiber tip presents in optoacoustic imaging. Black arrow points at where the fiber tip locates.

In order to see the influence of the hole to the lateral resolution, we used Field II to simulate the sensitivity of the hole-transducer in comparison with a normal transducer with the same configurations. Field II calculates the spatial impulse response (SIR) of arbitrary transducer geometries using an efficient quantization of the surface of the transducer and the sensitivity field is the maximum amplitude of the SIR. Figure 5.16 compares the sensitivity pattern of a normal transducer without a hole with a transducer having a 1 mm hole in the middle simulated by Field II toolbox [77–79].³ We can see that there is no obvious change in the sensitivity field, which proves that the influence of a 1 mm hole over the lateral resolution is insignificant.

Since Field II is not able to simulate the influence of the fiber tip, we use the k-Wave simulation toolbox [80, 81] in addition to quantitatively verify the influence of a fiber tip. The k-Wave is a simulation toolbox for the propagation of sound waves in heterogeneous media, based on a time/frequency and space/k-space iterative solution of the wave equation. Figure 5.17 and figure 5.18 explain why during the system calibration, with the presence of a 5 mm long fiber tip, the resolution of the system is not as high as expected. The simulated transducer contains only a frequency response at 50 MHz. We can see that a transducer with a 1 mm hole has two stronger side lobes than a transducer without a hole, but the effect is not dominant. However, when the fiber tip extrudes longer, the intensity of two side lobes increase dramatically. Figure 5.18 shows a direct comparison of the beam profile at the focal point.⁴ In reality, transducers has a broad bandwidth, thus the branch-like sensitivity pattern of a 50 MHz transducer in figure 5.17 becomes smeared and looks similar to the pattern in figure 5.16. Also the side lobes in figure 5.17 and figure 5.18 get smoothed out and exhibit a continuous pattern.

³The code for a 50 MHz transducer was modified by Miguel Ángel Araque Caballero.

⁴The k-Wave simulation code for a 50 MHz transducer was modified by Erwin Bay.

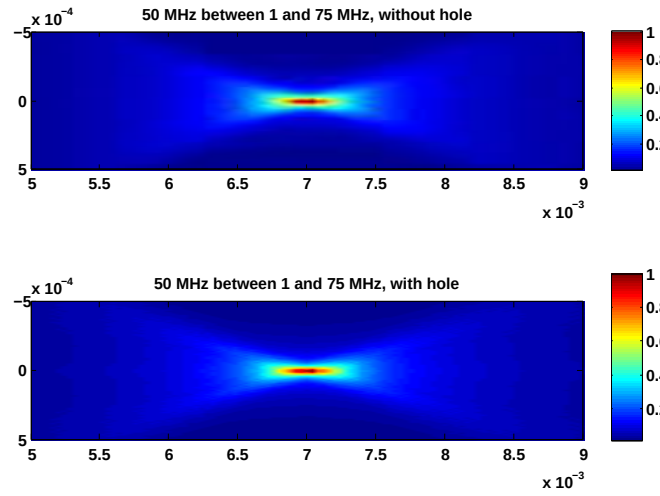


Figure 5.16: Comparison of the sensitivity pattern between a normal transducer without a hole and with a transducer having a 1 mm hole in the middle simulated by Field II toolbox. The x-axis represents depth in meter (along the central axis of the transducer) and the y-axis indicates the lateral axis in meter.

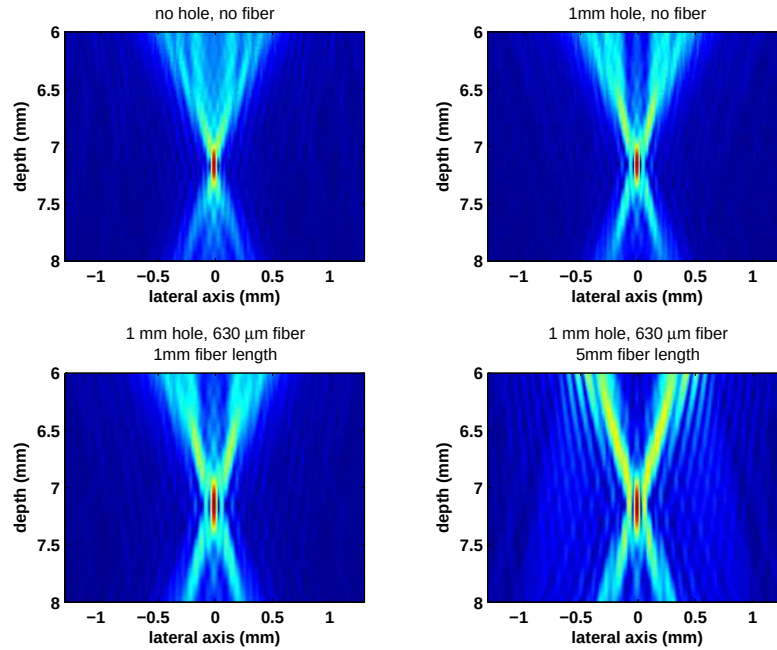


Figure 5.17: Influence of a hole and a fiber tip on the sensitivity field of a 50 MHz transducer using k-Wave simulation toolbox.

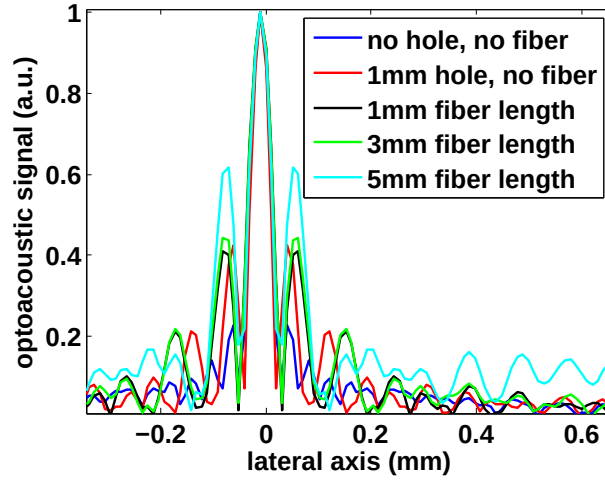


Figure 5.18: Direct comparison of the beam profile at the focal point.

5.2.4 Real-time optoacoustic imaging

We demonstrate in this subsection the real-time imaging capability of the optoacoustic microscopy system using a chicken fillet, in which a polythene tube (ID 0.28 mm, OD 0.61mm, Smiths Medical Deutschland GmbH, Grasbrunn, Germany) was inserted. The tube was filled with ink and air bubbles and wrapped around inside the chicken. At one end of the tube a syringe filled with ink was connected to push the ink with air bubbles further. We imaged the ink flow in the polythene tube with a frame rate of 5 frames/s and a scan range of 4 mm. A faster frame rate scan is feasible when we reduce the scan range, however, due to the limitation of the system resolution ($\sim 50 \mu\text{m}$) there are only 20 mesh grids within one millimeter scan range. In general the scan speed of current system is limited by the repetition rate of 1.5 kHz. Since the step size of each scan line should be smaller than the lateral resolution of the transducer preventing information losses, we assume a step size of $15 \mu\text{m}$, which is about $1/4$ of the lateral resolution. In this way the total scan range within one second is $15 \mu\text{m} \cdot 1500 = 22.5 \text{ mm}$. For a 2D scan range of 4 mm, the system cannot afford more than 6 frames/s. Figure 5.19 shows the tube filled with ink before the real-time measurement, where two cross sections of the tube are indicated by black arrows. The contrast of the ink is low in this measurement. The reason is that the chicken was placed in the distilled water for more than one hour before this measurement and became whitish and more optically diffusive, thus less light is penetrated in deeper tissue. Using isotonic saline instead of distilled water can decrease water diffusing into the chicken and improve the SNR of the image.

Figure 5.20 shows selected 9 frames where air bubbles in ink are present and the images are distorted due to strong acoustic mismatch between the air, the polythene tube and the chicken tissue.

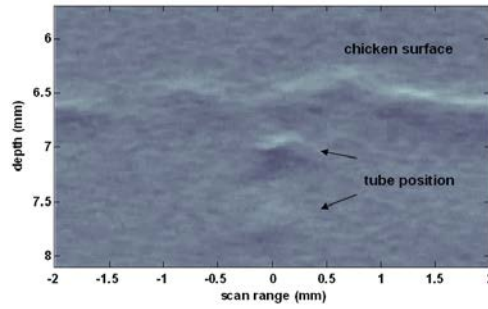


Figure 5.19: Frame where the real-time imaging was taking place.

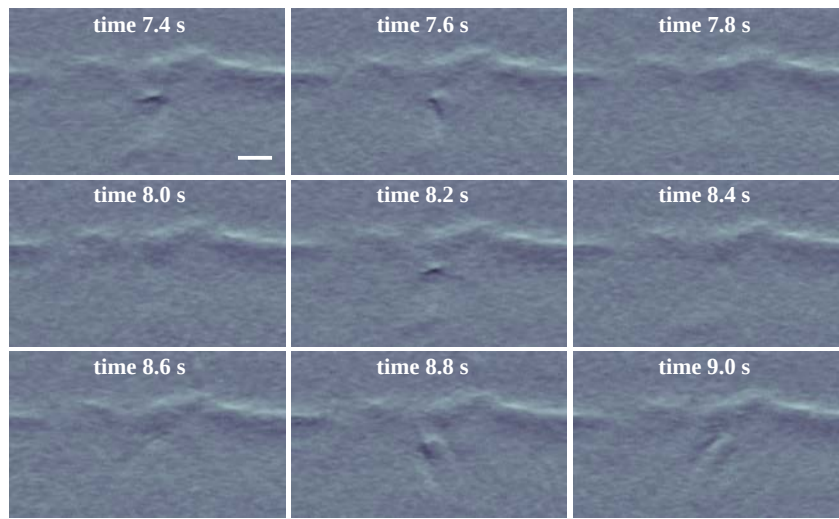


Figure 5.20: Real-time imaging of ink flowing in a tube that is embedded in a chicken fillet. Scale bar: 500 μm .

In order to point out the location of the real-time event, we imaged the chicken fillet inserted with the polythene tube in 3D as shown in figure 5.21. We imaged firstly the polythene tube filled with ink and air bubbles, then we imaged the tube filled with water. Both 3D images were acquired with a frame rate of 5 frames/s. We acquired 205 frames with a step size of 19.5 μm resulting in a field view of 4 mm X 4 mm and a total imaging time of 41 seconds for each measurement.

Figure 5.21 (a) shows the 3D optoacoustic image of a tube filled with ink and a few air bubbles, and figure 5.21 (b) shows the tube filled with water. We can see that the two 3D optoacoustic images match well with the tube positions (indicated by white arrows). We could see the tube filled with ink in deeper tissue due to the strong optical absorption of ink. Figure 5.21 (c) shows a picture of the chicken fillet embedded with a tube full of ink.

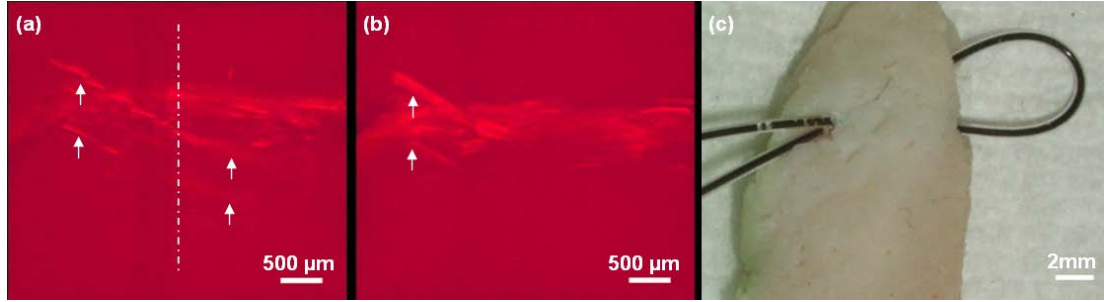


Figure 5.21: 3D imaging of a chicken fillet. (a) Side view of the 3D optoacoustic image when the tube was filled with ink and air bubbles; (b) Side view of the 3D optoacoustic image when the tube was filled with water; (c) A photo of the chicken fillet inserted with a polythene tube. White arrows point to tube positions. The dash line shows where the real-time monitoring was taking place.

5.2.5 Imaging applications

Besides a chicken fillet inserted with a ink tube we also imaged a pupa, a fruit fly, and the author's left palm to verify the performance of the system.

Imaging of a pupa and a fruit fly

The scan range of pupa imaging is 2.95 mm, and we imaged 131 frames with a frame rate of 4 frames/s and a step size of 24.2 μm , resulting in a total scan range of 2.95 mm X 3.17 mm and imaging time of 32 seconds. Figure 5.22(a) and (c) show the 3D visualization of pupa from two visual angles and (b) shows a picture of the corresponding pupa. Scales and salivary glands of the pupa can be identified in the 3D optoacoustic images.

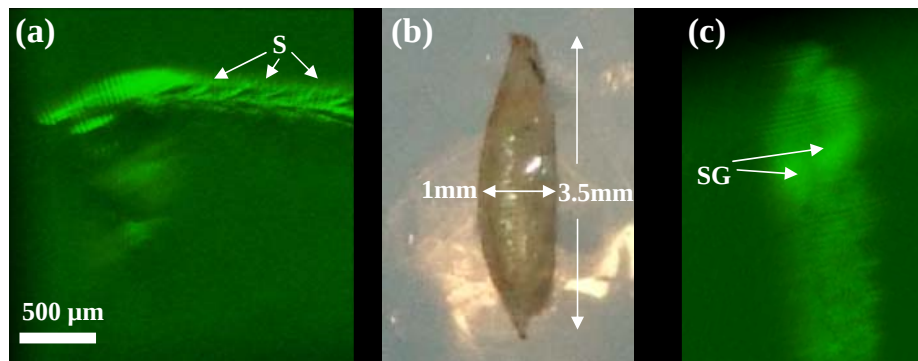


Figure 5.22: 3D optoacoustic imaging of a pupa. (a,c) Optoacoustic images of a pupa in different visual angles; (b) A picture of the imaged pupa. Abbreviations: scale (S), salivary glands (SG).

We also imaged a fruit fly for 114 frames with a frame rate of 4 frames/s and a step size

of $24.3 \mu\text{m}$. The whole scan range is $3.2 \text{ mm} \times 2.77 \text{ mm}$ and the image took 29 seconds. Figure 5.23 (a) and (b) shows the fruit fly in 3D with different visual angles and figure 5.23 (c) is a photo of the imaged fly.

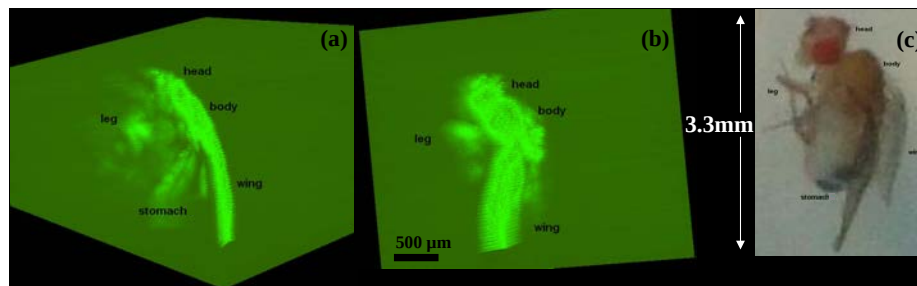


Figure 5.23: 3D visualization of a fruit fly. (a,b) Optoacoustic images of a fruit fly in different visual angles; (c) A photo of the corresponding fruit fly.

Both the pupa and fruit fly are translucent, and hence do not have much optical absorption in the green wavelength range. Blood vessels have strong optical absorption and are ideal natural contrast agents for optoacoustic imaging. Therefore we imaged the author's left palm in 3D. The hand was pushed to a tiny fixed water holder in order to avoid movement of the hand. The water holder consists of two pieces of iron rings, one ring is 6 mm thick with four magnet disks embedded, the other ring is 1 mm thick. In this way we can flexibly clamp a fresh kitchen plastic foil in between to hold the water without leakage. The ultrasound gel is placed on top of the area to be imaged in order to avoid air gaps between the palm and the kitchen foil. We took 89 frames with a frame rate of 0.6096 frame/s and a step size of $24.7191 \mu\text{m}$. The scan range of the rotor is 3 mm and the linear stage 2.2 mm. The total imaging time is 146 seconds.

Figure 5.24(a) shows a picture of the author's palm and figure 5.24(b) is the optoacoustic imaging, where the blood vessel branch labeled as 'b' and blood vessel 'v1' are shown in the photo and blood vessel 'v2' cannot be seen from the photo.

Figure 5.25(a) and (c) show a 3D optoacoustic palm image from different visual angles before applying the VDFT and figure 5.25(b) and (d) show corresponding 3D palm image after applying the VDFT. We can see that after applying the VDFT the SNR of blood vessels are improved.

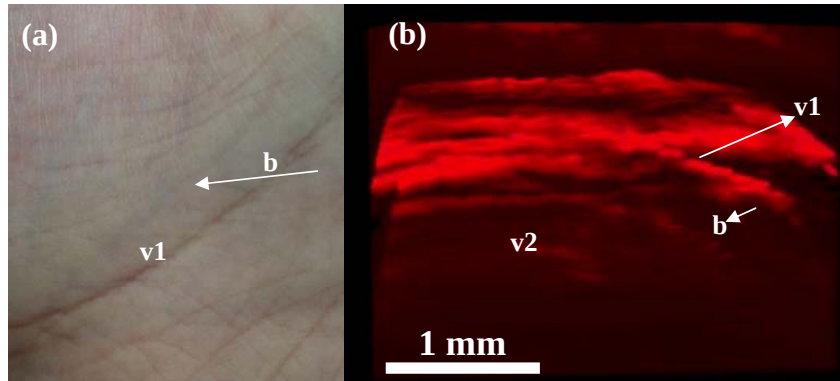


Figure 5.24: A picture of the author's left palm and the 3D optoacoustic image of the palm.

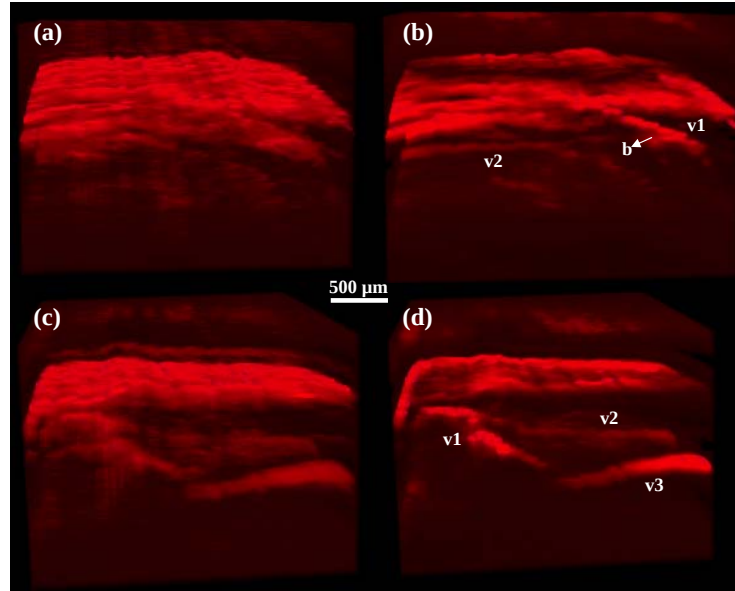


Figure 5.25: Improvement of 3D palm imaging by applying the VDFIT. (a,c) A 3D optoacoustic palm image from different visual angles before applying the VDFIT; (b,d) Corresponding 3D palm image after applying the VDFIT.

5.2.6 Summary

The real-time optoacoustic microscopy system based on a limited angle torque rotor demonstrates its ability to perform real-time imaging with a frame rate of 5 frames/s at a scan range of 4 mm. The system has about $15\ \mu\text{m}$ axial resolution and about $65\ \mu\text{m}$ lateral resolution due to the influence of the fiber tip extruding outside the transducer. The system is suitable to image pupa, fruit flies and human hands three dimensionally.

5.3 A microscopy system based on a piezo linear stage

We switched to a high speed linear stage M-683.2U4 controlled by a C-867.160 piezomotor controller (Physik Instrumente GmbH & Co., Karlsruhe, Germany). This brings several benefits. Firstly, the piezo linear stage is rigid and closed-loop controlled, thus we can flexibly change the beam size at the tissue surface with no influence over the current image display. Secondly, unlike the rotor that has denser scan lines on the border than in the middle within a certain period of time, the piezo stage has a quasi-equally spaced scan lines. Since the maximum scan step determines the scan resolution (should be less than the system lateral resolution), compared with the rotor, the piezo stage needs less scan lines per frame, and this leads to a shorter data acquisition time and less work load. Third, since the stage is closed-loop controlled, the start and stop positions are fixed and optoacoustic imaging results at the same position are repeatable. Fourth, it can be used for free beam illumination. Lastly the optoacoustic images are not compressed like in the rotor-scan case and fulfill the requirement of linear scanning for the VDFT.

The new setup is shown in figure 5.26. All the components are the same as the last system based on a rotor (as shown figure 5.2), except the piezo stage labeled in the figure. In addition, we reduce the length of the lens tube for a more rigid, compact, lighter system.

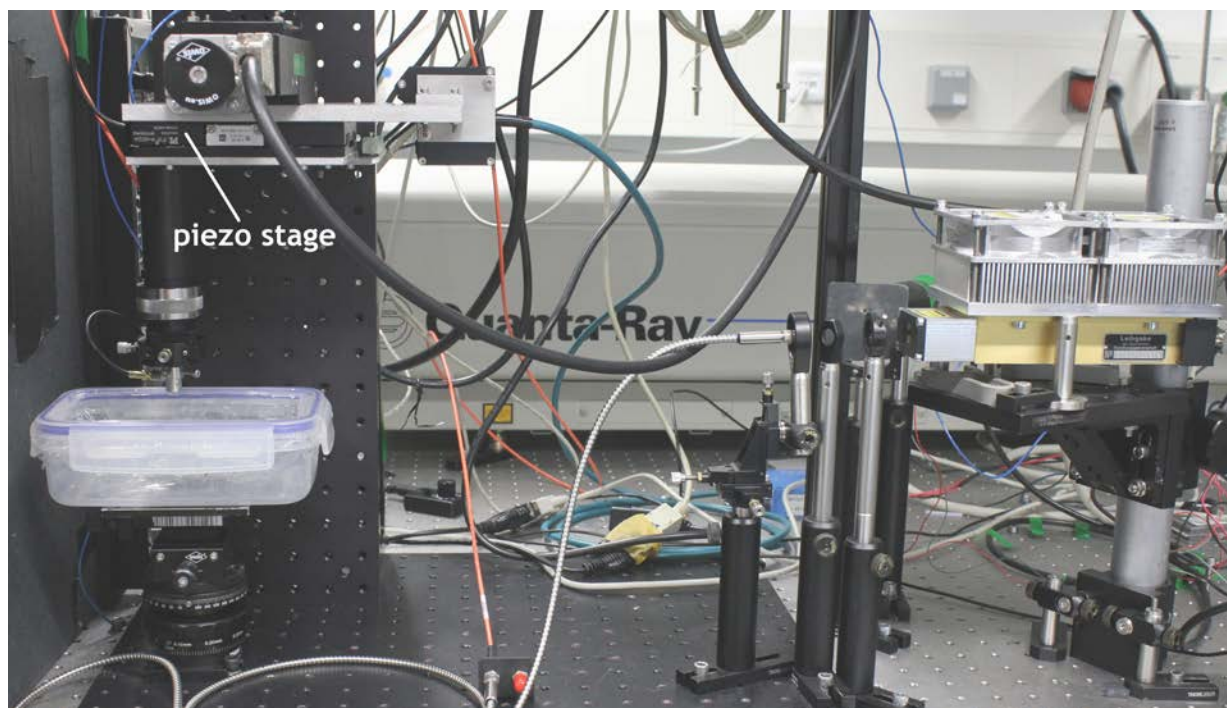


Figure 5.26: A picture of the real-time optoacoustic microscopy system based on a piezo stage.

Also, in order to avoid the influence of the fiber tip to the lateral resolution, we place the fiber inside the transducer. Figure 5.27 shows a picture of the illumination coming out of the transducer.



Figure 5.27: A picture of the multimode fiber illumination coming out of a transducer in water.

5.3.1 System calibration

We calibrated the spatial resolution of the new system at the focal point of a spherically focused transducer (50 MHz center frequency) using a black-dyed sphere with a diameter of $10\text{ }\mu\text{m}$. Figure 5.28 shows an optoacoustic image of such sphere at the focal point of the transducer. The resolved lateral and axial resolutions are $50.6\text{ }\mu\text{m}$ and $11\text{ }\mu\text{m}$, respectively. The lateral resolution is calculated by subtracting the diameter of the microsphere from the FWHM for a Gaussian fit of the optoacoustic signal, which is obtained by a horizontal cut at the sphere center. The axial resolution is calculated by subtracting the radius of the sphere from the positive width of the depth-resolve optoacoustic signal.

We measured the focal length of the system using a $70\text{ }\mu\text{m}$ diameter suture. As shown in figure 5.9 from section 5.2.2 that true optoacoustic signals are negative below the focal point, we inverted the measured optoacoustic signals below the focal point to get reasonable visual images. Figure 5.29 (a) shows optoacoustic imaging of the suture at different depths to demonstrate the focal length of the spherically focused transducer, as well as its sensitivity. Figure 5.29 (b) shows optoacoustic images of the same suture after applying the VDFT. We can see that the lateral resolution is improved dramatically when the suture is off focus. Although around the focal point the optoacoustic signals are stronger than off-focus ones, there are more scan lines further away from the focal point used for the summation in SAFT. Thus after applying the VDFT the signals near the focal point are

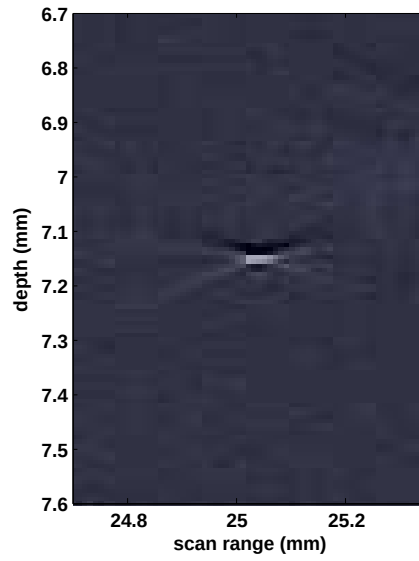


Figure 5.28: Calibration of the system resolution at the focal point of a spherically focused transducer (50 MHz center frequency) using a black-dyed sphere with a diameter of $10\ \mu\text{m}$.

weaker than other positions.

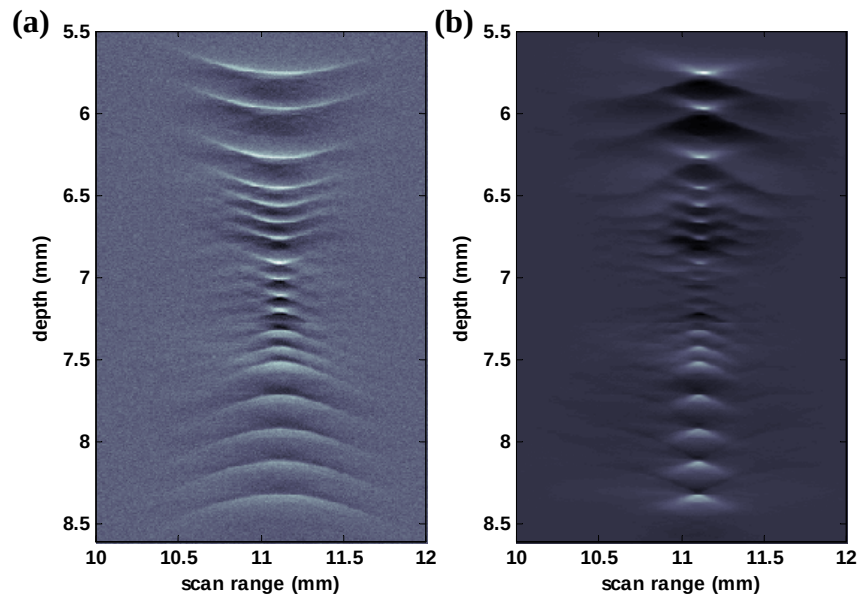


Figure 5.29: Focal length calibration using a $70\ \mu\text{m}$ diameter suture. (a) Optoacoustic imaging of the suture at different depths; (b) After applying the VDFT.

5.3.2 Real-time optoacoustic imaging

We imaged ink flow in a polythene tube (ID 0.28 mm, OD 0.61 mm, Smiths Medical Deutschland GmbH, Grasbrunn, Germany) embedded in a piece of fresh chicken breast with a frame rate of 5 frames/s at a scan range of 3.75 mm. One end of the tube was free of connection and the other end of the tube was connected with a syringe full of ink. During imaging we constantly pulled the syringe back and forth to fill the tube with ink or air. Figure 5.30 shows selected optoacoustic images, where we can see the ink is about to appear and then disappears. At the end of the figure a picture of the chicken fillet inserted with polythene tube is shown. Due to low contrast between the polythene tube and its background, we use two black arrows to indicate the tube positions. Compared with the real-time optoacoustic images in section 5.2.4 there is no obvious image distortion in this experiment when the air is present, because the chicken fillet is fresh and has less acoustic impedance mismatch with the polythene tube and the air.

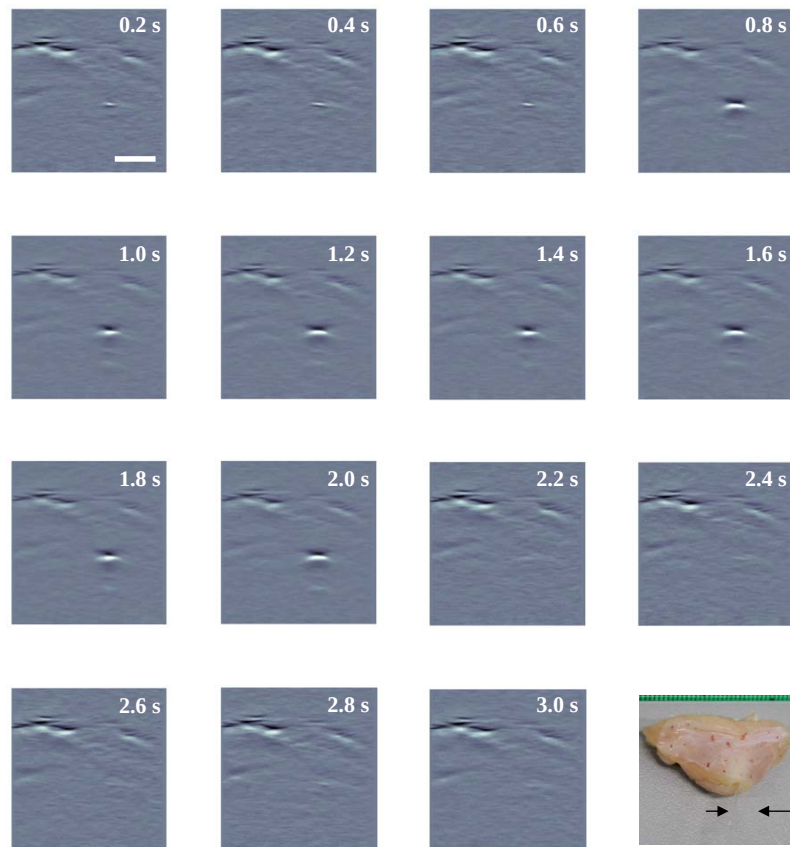


Figure 5.30: Real-time imaging of ink flow in a polythene tube embedded in a piece of fresh chicken. Black arrows in the last subfigure indicate the locations of the polythene tube. Frame rate: 5 frames/s. Scale bar: 1 mm

The real-time optoacoustic microscopy system based on a piezo stage has the same frame-rate limitation as the system based on a rotor as described in section 5.2.4.

5.3.3 Verification of 3D reconstructed optoacoustic images

We imaged printed letters and the inner skin of a white mouse to demonstrate the correct 3D positioning of the system. Figure 5.31 shows a photo of the printed letters *hen*. The scan range of each 2D optoacoustic image is 5 mm with 20 μm step size for adjacent scan lines. We acquire 91 frames with 50 μm distance between neighboring frames. The total field of view is 5 mm X 4.5 mm. Figure 5.31(b) shows the maximum amplitude projection (MAP) of the 3D reconstructed optoacoustic image before applying the VDFT. The MAP represents the position of printed letters *hen* well, but the out-of-focus artifacts lead to thicker printed lines. The VDFT decreases these artifacts and the new MAP corresponds better with the photo of the printed letters.

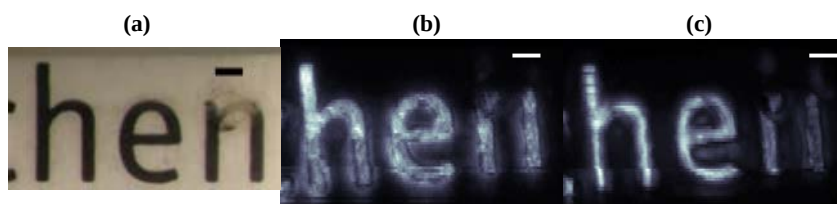


Figure 5.31: Optoacoustic imaging of printed letters. Field of view: 5 mm X 4.5 mm. (a) A photo of the printed letters being imaged; (b) The optoacoustic MAP of the letters before applying the VDFT; (c) The optoacoustic MAP of the letters after applying the VDFT. Scale bar: 0.5 mm.

We also imaged the inner skin of a white mouse body piece to further verify the correct image positioning. Figure 5.32(a) and (b) show a picture and the MAP of the 3D reconstructed optoacoustic image of the inner skin. The scan speed is 1.1 frames/s and the field of view is 8 mm X 6 mm with 524 frames of optoacoustic images. The step size between adjacent frames is 11.45 μm . Since the skin was not fixed and blood diffused to the surrounding tissue, both the photo and the MAP show the degradation of the inner skin. However, figure 5.32(c) demonstrates that the positions of blood vessels in the MAP match well with their positions in the picture.

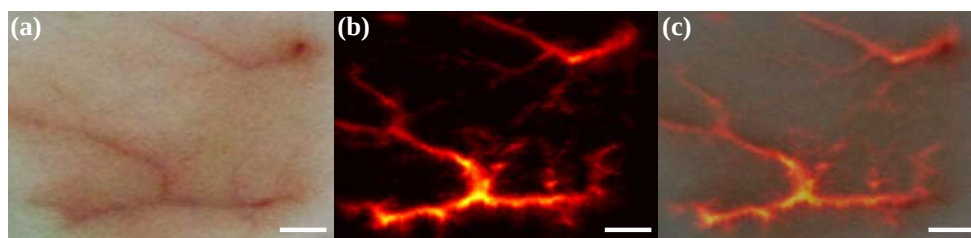


Figure 5.32: Imaging of the inner skin of a white mouse body piece. Total field of view: 8 mm X 6 mm. (a) A picture of the inner skin of a white mouse body piece; (b) The optoacoustic MAP of the skin; (c) An overlap of (a) and (b). Scale bar: 1 mm.

5.3.4 Optoacoustic microscopic imaging of mice *post mortem*

We imaged the shaved front breast of a white mouse *post mortem* with a frame rate of 1 frame/s. The total field of view is 8 mm X 18 mm with 1209 frames of optoacoustic images. Figure 5.33(a) shows the MAP of the mouse skin. We also applied the VDFT to improve the out-of-focus imaging quality (figure 5.33(b)). Black arrows in the figure point at blood vessels that do not show in the original image, and blue arrows point at places where the image quality improves after applying the VDFT. Since some part locates at the focal point where the VDFT cannot improve the lateral resolution; furthermore, signals at the focal point are very sensitive to the VDFT if noises are present, we can see that some part of the optoacoustic image gets smeared. Therefore we combined images before and after applying the VDFT to reduce this effect as shown in figure 7(c). The combination was made by summing up the image values before applying the VDFT with a weighting factor of 1.2 and the image values after applying the VDFT.

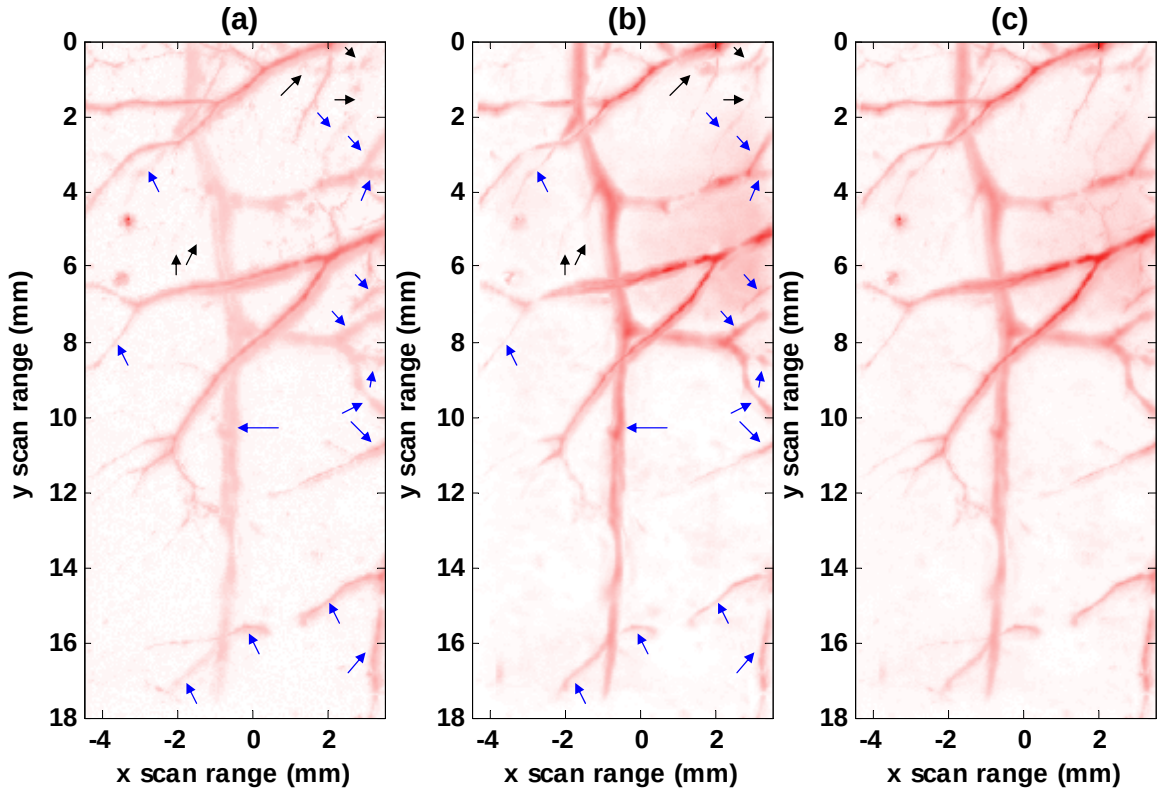


Figure 5.33: Optoacoustic imaging of the front breast (left side) of a shaved white mouse *post mortem*. Total field of view: 8 mm X 18 mm. (a) The MAP without applying the VDFT; (b) The MAP applying the VDFT; (c) Combination of (a) and (b). Black arrows in the figure point at blood vessels that do not show in the original image, and blue arrows point at places where the image quality improves after applying the VDFT.

In order to demonstrate the 3D imaging capability of the system, we imaged a formalin-

fixed mouse ear embedded in pure agar. The total field of view is 8 mm X 10 mm with 756 frames at 0.75 frame/s. Figure 5.34 shows a picture and the MAP of the 3D reconstructed optoacoustic image of the mouse ear. Figure 5.35 shows the 3D rendering of the mouse ear at different perspectives.

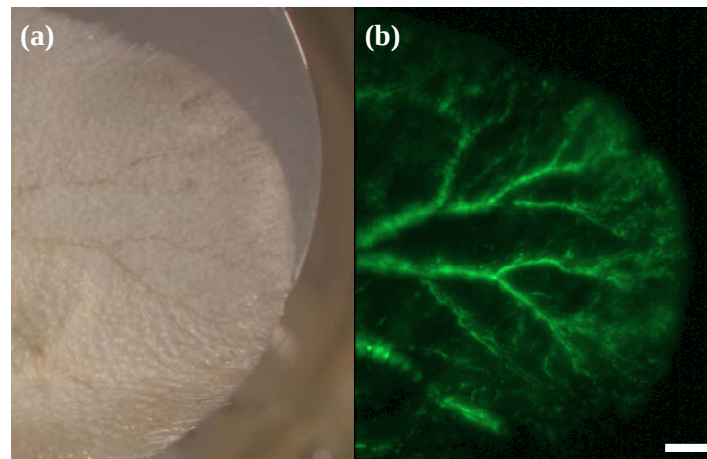


Figure 5.34: Imaging of a fixed mouse ear embedded in agar. Total field of view: 8 mm X 10 mm. (a) A picture of the fixed mouse ear; (b) The MAP of the 3D reconstructed optoacoustic image. Scale bar: 1 mm.

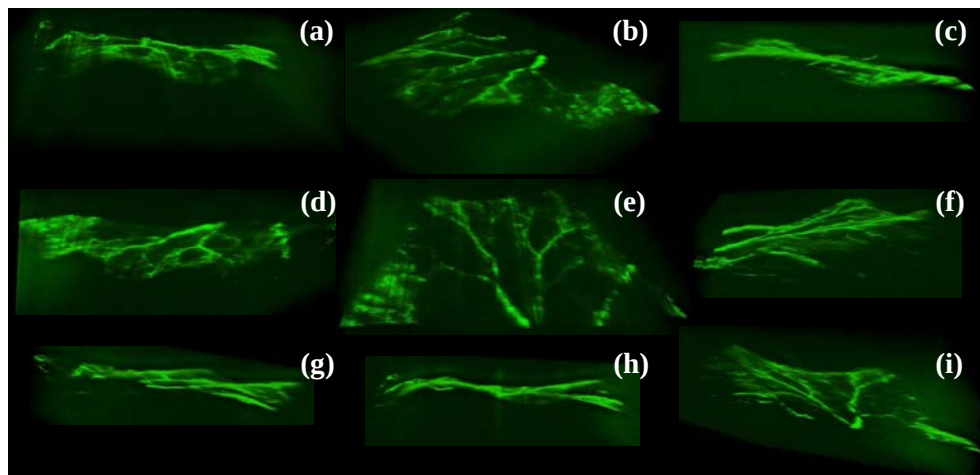


Figure 5.35: 3D rendering of the fixed mouse ear at different perspectives.

5.3.5 Optoacoustic microscopic imaging of a human hand

3D imaging of a human palm

The author's left palm shown in figure 5.36(b) is imaged under the optoacoustic microscope. Figure 5.36(a) shows a typical 2D optoacoustic image of the author's left palm where an imaging depth of 3.2 mm is achieved.

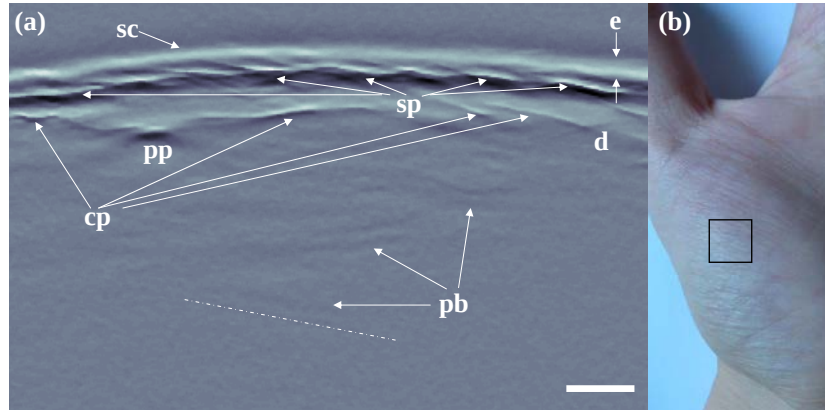


Figure 5.36: (a) 2D optoacoustic imaging of a human palm. Above the dot-dash line there is a structure located 3.2 mm beneath the skin; (b) A picture of the imaged palm with a black frame indicating the area that was used for 3D optoacoustic imaging henceforth. Abbreviations: sc, stratum corneum; e, epidermis; d, dermis; sp, subpapillary plexus; pp, princeps pollicis; cp, cutaneous plexus; pb, pollicis brevis. Scale bar: 1mm.

The 3D optoacoustic image was acquired with a total field of view of 8 X 8 mm, shown in the black frame, with 353 frames at a frame rate of 1.1 frames/s. The step size between each frame is $22.7 \mu\text{m}$. The whole image takes about 6.5 minutes. From figure 5.36(a) we can see that many subpapillary plexus beneath the palm skin make it difficult to resolve deeper blood vessels, thus we applied a skin removal algorithm to remove the strong surface signals to increase the SNR from deep tissue. The algorithm finds out the first maximum of the optoacoustic signal, which comes from the stratum corneum in our experiment, then signals within certain thickness above and below this maximum will be suppressed by zero, due to the reason that the epidermis thickness of the palm stays quite constant. The value of the thickness can be changed for optimization purpose (see figure 5.38).

Figure 5.37 shows examples of applying the skin removal algorithm, where the signals from subpapillary plexus are removed. Figure 5.38 compares the MAP of the palm before and after applying the skin removal algorithm. Also different thickness values, within which the signals are suppressed, are employed. The original image only shows the palm print, by gradually increasing the thickness the blood vessels beneath the skin pop up. Since the palm is not fixed well in this experiment, we can see the palm movements from the MAP image.

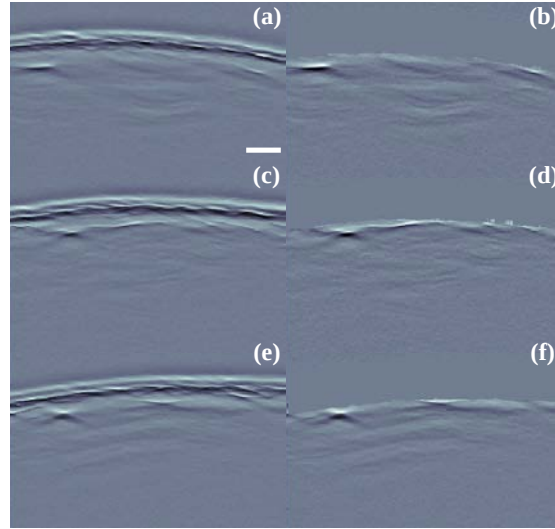


Figure 5.37: Examples of applying the skin removal algorithm to optoacoustic images that have strong surface signals. (a,c,e) 2D optoacoustic images of the author's left palm; (b,d,f) 2D optoacoustic images of the palm after applying the skin removal algorithm. Scale bar: 1mm.

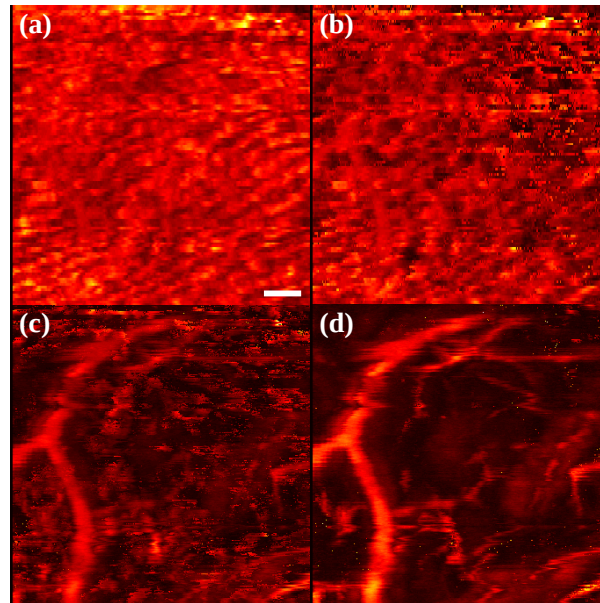


Figure 5.38: Comparison of the MAP of palm imaging when the skin removal algorithm is applied. (a) No skin removal algorithm is applied; (b) Remove signals beneath the skin for a thickness of $330\ \mu\text{m}$; (c) Remove signals beneath the skin for a thickness of $420\ \mu\text{m}$; (d) Remove signals beneath the skin for a thickness of $540\ \mu\text{m}$. Scale bar: 1mm.

Figure 5.39 shows the 3D rendering of the optoacoustic palm image before and after applying the skin removal algorithm. We can see that after removing strong surface signals,

small blood vessels are enhanced.

Since we imaged different areas from the author's palm, we cannot directly compare the obtained 3D optoacoustic image from the rotor (figure 5.24) with the one from the piezo stage. The blood vessels in figure 5.24(a) are relatively large and can be seen by the naked eye, additionally these vessels are far from the focal zone of the transducer, therefore by applying the VDFT technique the intensity of out-of-focus vessels are enhanced. In figure 5.36(b) we cannot see any vessels beneath the skin although there are profound blood vessels in the imaging area. Due to the absorption of subpapillary plexuses, the small blood vessels beneath the skin is suppressed, therefore skin removal algorithm needs to be applied to increase the SNR of small vessels. Since the vessels beneath the skin locate around the focal zone of the transducer, VDFT is not necessary.

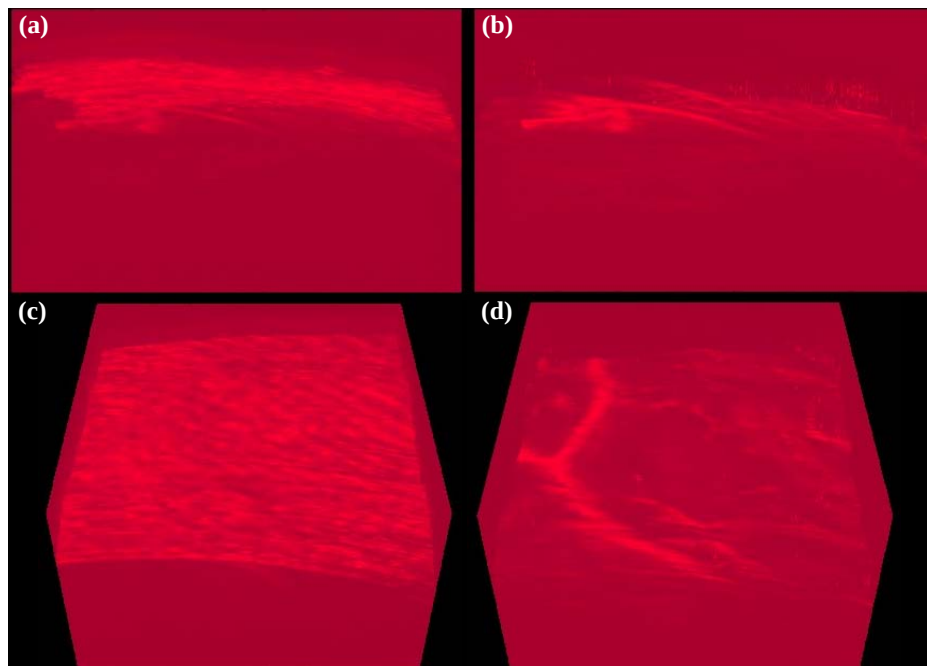


Figure 5.39: 3D rendering of optoacoustic palm imaging. (a,c) Before applying the skin removal algorithm; (b,d) After applying the skin removal algorithm.

Optoacoustic microscopic imaging of a human index finger

Researchers found out that the abnormal capillary shapes and sizes can often address rheumatic diseases and systemic inflammatory diseases [72, 73]. Nailfold capillaroscopy is a common tool to image the nailfold capillaries *in vivo*. The penetration depth of such system is very shallow, with the aid of an optoacoustic microscopy system we can potentially investigate the capillaries and blood vessels lie not only shallow but also deep in the nailfold and get a thorough insight of the vessels around the nailfold. This might bring

more research aspects to study rheumatic diseases.

Figure 5.40 shows 2D optoacoustic images of the author's left index finger, shown in the inset of figure 5.40(a), at different imaging positions. The bone of the finger in figure 5.40(b) is 4.72 mm beneath the stratum corneum and the bumpy shape of the finger nail can be explained by the inset of the figure 5.40(b) pointed out by the curly bracket.

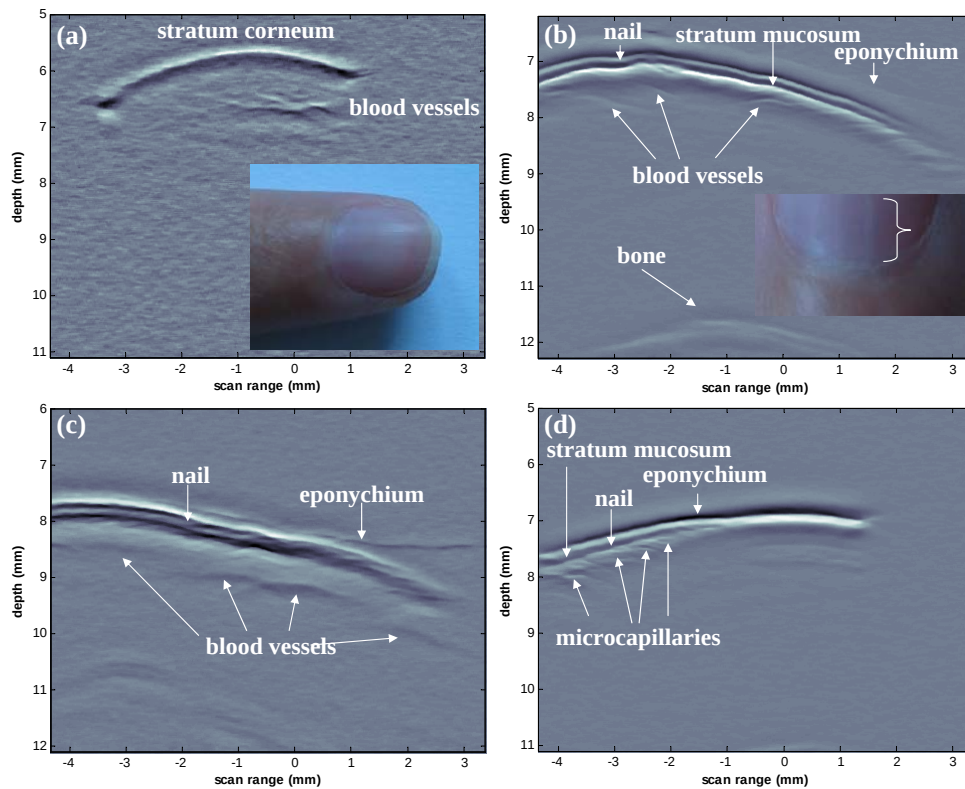


Figure 5.40: 2D optoacoustic images of the author's left index finger at different imaging positions.

Due to the variation of the speed of sound in nail and in soft tissues, the depth of blood vessels can be slightly different. Also due to acoustic wave scattering by the nail, the intensity of the blood vessels can be influenced. The influence of the nail can be further quantified by imaging tissue-mimicking phantoms.

Optoacoustic microscopic imaging of a human opisthenar

We imaged the author's left opisthenar to present different characteristics of human tissues under the optoacoustic microscopy system. Figure 5.41 shows a 2D optoacoustic image of the left opisthenar and corresponding imaged position. We removed optoacoustic signals of

the skin to underline signals from deep tissue, which are suppressed in the original optoacoustic image. We can see from the inset of figure 5.41(a) that there are profound structures beneath the skin. The deepest structure in this image is 5.8 mm beneath the skin. Due to record length setting for this experiment, deeper signals were not recorded; therefore it is not firm whether 5.8 mm is the deepest location the system can reach in this measurement.

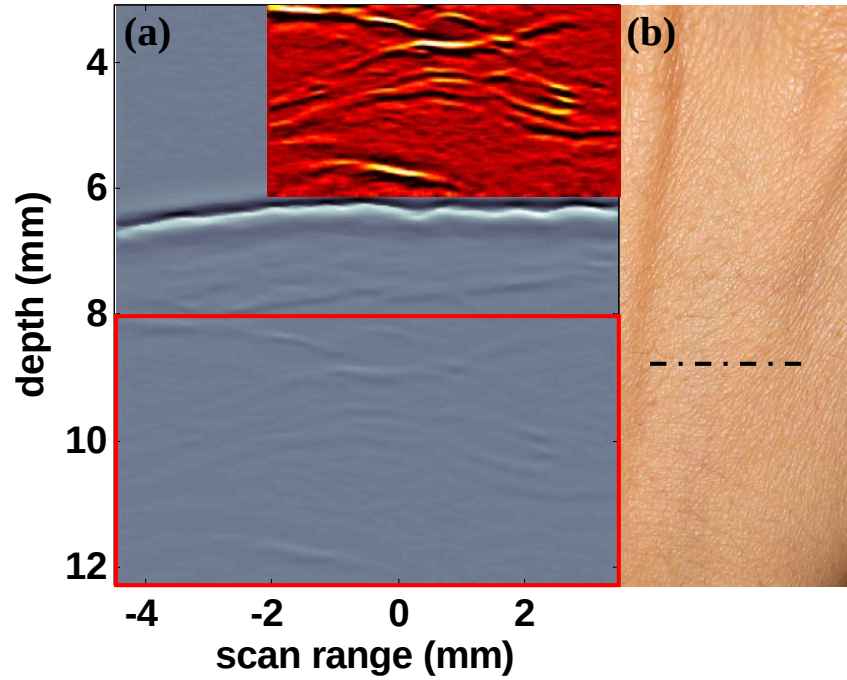


Figure 5.41: (a) A 2D optoacoustic image of the author's left opisthenar. The red-rectangle tagged region is enlarged in the inset with a logarithmic scale; (b) A picture of the left opisthenar. A black dashed line indicates the imaged location.

5.3.6 Summary

The optoacoustic microscopy system based on a closed-loop controlled piezo linear stage enables real-time imaging with a frame rate of 5 frames/s at a scan range of 3.75 mm. The system has a lateral resolution of $50.6 \mu\text{m}$ and an axial resolution of $11 \mu\text{m}$ at the 7.15 mm focal point. The 3D positioning was verified by printed letters and blood vessels in a piece of mouse inner skin. The system was used to image the mouse skin, a mouse ear and a human palm three dimensionally. Also we imaged the author's left index finger around the nailfold and the left opisthenar, which opens the door for clinical applications such as the investigation of human blood capillary microcirculation, macrocirculation, and the study of skin cancer, etc.

5.4 Conclusions and discussions

In this chapter we have demonstrated a real-time optoacoustic microscopy system based on either an angular or a linear scan motion. The system can scan 5 frames/s under a scan range of ~ 4 mm. Faster scan speed is possible by reducing the scan range. The system can have an ultimate 30 frames/s scan speed if we replace the laser with a higher repetition rate. The system is tested for small animal imaging such as pupa, fruit flies and mice. It also enables *in vivo* imaging of human skin. With an ultrasound transducer of higher frequency or a focused free space beam it can be applied for monitoring the fast neural response as well.

Since the laser pulse energy varies $\sim 10\%$, if we have a third channel to feed the energy variation acquired from the photodiode to the optoacoustic signals, the optoacoustic signal changes due to illumination, rather not because of the optical absorption variation, can be eliminated, thus the imaging results can be further quantitatively improved.

Since we are using a single-element transducer, it is flexible to change transducers with different frequencies and bandwidths, i.e. different penetration depths and resolutions, which enables multiscale imaging. With current available ultrasonic detectors, optoacoustic imaging ranging from cell level [82] up to deep tissues [83] is feasible.

The system still has room to improve, such as the imaging speed, multispectral imaging capability, better illumination design and higher system resolution. As described in section 5.2.4, the 1.5 kHz repetition rate of the laser limits the scanning frame rate, in the market there are nanosecond lasers with up to 100 kHz repetition rate available, which can increase the frame rate by an order of magnitude. Also using a laser with a large selection of wavelengths, we can resolve contrast agents and monitor fast functional changes, etc using multispectral imaging as shown in section 4.2. Furthermore, we can improve the lateral resolution by focusing a free space beam to a diffraction limited small spot, where the system lateral resolution around the optical focus equals to the focused optical beam size and far from the optical focus the lateral resolution stays as the ultrasound resolution [68]. Using a focused beam can further reduce the power being used and a more compact and cost-effective laser can be used. Another possibility of improving the system's resolution is to employ ultrasound transducers with higher center frequency, with the compensation of shallower imaging depth [75, 84]. Another alternative is to use multi-ring, multi-band annular array transducers (a transducer contains multiple ring-elements with different center frequencies) to improve the SNR and the resolution of the system [85–87].

CHAPTER 6

Summary

My PhD study focuses on the development of fast, high-resolution optoacoustic imaging systems with high optical contrast and large penetration depth.

The first part of the thesis describes the development of a multispectral optoacoustic tomography system (MSOT), which have successfully achieved the following results:

- A 2D image acquisition within 9 seconds in comparison with 13 minutes reported by other groups [88].
- High-resolution imaging with an in-plane resolution of $32\ \mu\text{m}$ and a vertical resolution of $155\ \mu\text{m}$.
- Optoacoustic imaging of small animals with varying sizes, ranging from $800\ \mu\text{m}$ to $28.5\ \text{mm}$.
- 3D imaging capability, exemplified by imaging a complicated hair structure.
- Molecular imaging of fluorescent dye (Texas Red) in a tissue-mimicking phantom.

We have then extensively studied mice and zebrafish using the MSOT system with the following appealing results:

- 2D optoacoustic imaging of a nude mouse *post mortem* larger than $20\ \text{mm}$.
- 3D whole-body optoacoustic imaging of an adult zebrafish *post mortem* with 3D segmentation of main anatomical structures.
- 3D molecular imaging of transgenic zebrafish with mCherry expressed in the brain area (*zic4* zebrafish) both *post mortem* and *in vivo*.
- Mapping of the resolved mCherry fluorescent protein in *zic4* zebrafish to the 3D segmentation of the fish skin and brain.

The results presented for the MSOT system open the door for the longitudinal study of physiological changes, developmental biology and a variety of disease models.

Although the MSOT system provides fast 2D acquisition and deep penetration based on a continuous data acquisition method and a tomographical reconstruction scheme, it requires acquisition of optoacoustic signals from multiple projections over 360° . Thus real-time imaging cannot be performed with our current low repetition rate (30 Hz) laser system. Accordingly we have developed a high-resolution optoacoustic microscopy system based on a spherically focused transducer with a laser of 1.5 kHz repetition rate. The system utilizes a fast sawtooth scan enabled by either an open-loop controlled rotor or a close-loop controlled piezo stage. We obtained improved microscopic images using the piezo stage. Below are a list of exciting results we have achieved:

- Real-time imaging capability with a frame rate of 5 frames/s at a scan range of ~ 4 mm.
- High-resolution imaging with a lateral resolution of $50.6\ \mu\text{m}$ and an axial resolution of $11\ \mu\text{m}$.
- Optoacoustic microscopic imaging of pupa, fruit flies, and mice.
- 3D human skin imaging with a skin removal algorithm applied to show up deep-seated blood vessels.

The optoacoustic microscopy system can therefore provide noninvasive imaging of small animals with high resolution and is able to image fast functional changes as well. Additionally it has the potential for clinical applications such as the study of human skin cancer.

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