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TITLE: Universal hand-held three-dimensional optoacoustic imaging probe for deep tissue human angiography and functional preclinical studies in real time

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KEYWORDS: Optoacoustic tomography, photoacoustic imaging, hand-held probe, volumetric imaging, real-time tomography, five dimensional imaging, clinical imaging, functional imaging, molecular imaging, preclinical research.

SHORT ABSTRACT:

We provide herein a detailed description of the experimental protocol for imaging with a newly developed hand-held optoacoustic system for three-dimensional functional and molecular imaging in real time. The demonstrated powerful performance and versatility may define new application areas of optoacoustic imaging technology in preclinical research and clinical practice.

LONG ABSTRACT:

The exclusive combination of high optical contrast and excellent spatial resolution makes optoacoustics ideal for simultaneously attaining anatomical, functional and molecular contrasts in deep optically opaque tissues. While enormous potential has been recently demonstrated for application of the optoacoustic method in small animal research, vast efforts have been also undertaken in translating this imaging technology into clinical practice. We present here a newly developed optoacoustic tomography approach capable of delivering high resolution and spectrally enriched volumetric images of tissue morphology and function in real time. A detailed description of the experimental protocols for operating the imaging system in both hand-held and stationary modes is provided and showcased for different potential scenarios involving functional and molecular studies in murine models and humans. The possibility for real time visualization in three dimensions along with the versatile handheld design of the imaging probe make the newly developed approach unique among the pantheon of imaging modalities used in today's preclinical research and clinical practice.

INTRODUCTION:

Optoacoustic imaging attracts growing interest from the biological and medical research communities, as manifested by the ever increasing number of publications encompassing variety of new applications that exploit the unique advantages offered by the technology [1-4]. In particular, the capacity to image spectrally distinctive photo-absorbing agents with high spatio-temporal resolution opens unprecedented capabilities for functional and molecular imaging [5-9] _ENREF_5_ENREF_5.

Indeed, translation of the optoacoustic technology into clinical practice comes with promising prospects in diagnostics and treatment monitoring of many diseases. Yet, the limited propagation of photons in optically scattering and absorbing tissues and the generally weak responses associated with the optoacoustic phenomenon, limit the applicable depth of the method. As a result, hand-held optoacoustic probes have been attempted to image parts accessible from outside of the body [10,11] while endoscopic systems are used to provide images from within the body by inserting them via natural orifices [12]. Some low absorbing parts of human body, such as female breast, are also accessible by tomographic optoacoustic scanners [13,14]. Of particular interest is the hand-held approach as it enables large versatility, similarly to ultrasonography. Here, adaptation of the common ultrasound linear array probes for optoacoustic imaging remains challenging, mainly due to fundamental differences in tomographic imaging requirements between ultrasound and optoacoustics, resulting in the so called out-of-plane artifacts, lack of sensitivity and quantification and overall poor imaging performance [28]. In fact, due to high optical contrast of tissues, high quality optoacoustic imaging implies acquisition of three-dimensional data from the largest possible solid angle around the imaged object. Fortunately, optoacoustics is ideal for three dimensional imaging as, in principle, it allows for collection of volumetric data from a single interrogating laser pulse.

Recently, we introduced the first handheld optoacoustic probe for three-dimensional (volumetric) imaging in real time[15]. The system is based on a two-dimensional array of piezoelectric elements arranged upon a spherical surface (blue dots in Figure 1a). The size and orientation of the individual elements guarantees effective signal collection from a centimeter-scale volume surrounding the center of the sphere (black cube in Figure 1a). Optical excitation of the imaging region is provided with a fiber bundle inserted through a central cylindrical cavity of the array. An actual picture of the array of transducers along with the optical fiber bundle is shown in Figure 1b. Thereby, the efficient excitation and detection configuration allows deep-tissue imaging with single-shot excitation (one laser pulse), so that real-time imaging at a frame rate determined by the pulse repetition frequency of the laser is further enabled with a graphics-processing-unit (GPU) implementation of the reconstruction procedure [16]. A cylindrical casing with a transparent polyethylene membrane (Figure 1c) is attached to the transducer array to enclose an acoustically transmitting liquid medium (water). The membrane is further coupled to the tissue by means of acoustic gel. A picture of the optoacoustic probe as being used in hand-held operation mode is shown in Figure 1d.

The demonstrated three dimensional hand-held optoacoustic imaging combined with real time functional imaging capacity come with important advantages for clinical diagnostics and a number of potential applications are envisioned for various indications, such as peripheral vascular disease, lymphatic system disorders, breast cancer, skin lesions, inflammation or arthritis [17]. Furthermore, the fast imaging capacity enables visualization of dynamic biological events with the probe arranged in a stationary position. Combined with fast wavelength-tuning optical parametric oscillator (OPO) laser technology, this approach allows for real-time imaging of biodistribution. Thereby, new possibilities may equally emerge in small animal imaging applications, e.g. in studying tissue hemodynamics, *in vivo* cell tracking, visualization of pharmacokinetics, organ perfusion, targeted molecular imaging of tumors and cardiovascular system, or neuroimaging.

In this work we provide a detailed description of the experimental imaging protocol to operate with the spherical array optoacoustic hand-held probe and showcase performance in several typical clinical and small animal imaging scenarios.

PROTOCOL:

The detailed procedure for operating with the volumetric hand-held optoacoustic probe is described below. This procedure is performed according to approved institutional regulations regarding animal and human experiments.

1. System preparation.

1.1) Switch on the laser for a warm-up period of ~15 min prior to operation for stabilizing the output light beam.

1.2) Place the water enclosing part with the isolating membrane at the right position to set the region of interest at the desired depth. Fill the water enclosing part with deionized water by means of a pump. Then ensure proper sealing or recirculation of water to maintain a constant pressure.

2. Imaging preparation.

2.1) Human imaging preparation.

2.1.1) If necessary, remove hair from the to-be-imaged part with a shaving lotion in order to avoid an undesired background in the images.

2.1.2) Establish the imaging wavelength(s) to be used in the experiments, the pulse repetition rate of the laser and the number of signal averages depending on the application. Select the parameters for the acoustic data acquisition system - high input impedance for broadband

signal detection, dynamic range optimized for the amplitude of the signals, sampling frequency and number of samples adapted to the bandwidth and duration of the acquired signals.

2.1.3) Ensure that both the operator and the patient use protective goggles adapted to the optical excitation wavelength(s). Set the laser power so that the light fluence at the tissue surface is below 20 mJ/cm^2 for near-infrared wavelentgths in order to satisfy safety exposure limits for human experiments.

2.1.4) Apply ultrasound gel onto the skin around the region to be imaged in order to provide efficient acoustic coupling. Place the optoacoustic probe in the region of interest.

2.2) Animal imaging preparation.

2.2.1) Ensure that the care and experimental procedures with animals are in agreement with institutional and government rules and regulations.

2.2.1) If necessary, remove the fur of the animal in the region to be imaged with a shaving lotion. Protect the eyes of the animal with ointment (Bepanthen® cream, Bayer AG, Germany), which prevents dryness and damage from exposure to intense pulsed laser radiation.

2.2.2) Anesthetize the animal by using intraperitoneal injection (IP) of ketamine/xylazine (100 mg/kg KG Ketamine + 5 mg/kg KG Xylazine) prior to the experiment or use isoflurane anesthesia (2-3 % (by volume) with 0.9 l/min gas flow) during the experiment. Confirm anesthetization by checking the reflex of the hind limb of the animal.

2.2.3) Establish the wavelength(s) to be used in the experiments, the pulse repetition rate of the laser and the number of averages depending on the application. Select the parameters for the data acquisition system, high input impedance for broadband signal detection, dynamic range adjusted to the amplitude of the signals, sampling frequency and number of samples adapted to the bandwidth and duration of the acquired signals.

2.2.4) Set the laser power to a value satisfying exposure limits according to approved regulations regarding animal experiments.

2.2.5) Apply ultrasound gel onto the skin around the region to be imaged in order to provide efficient acoustic coupling and place the optoacoustic probe in the region of interest.

3. Pre-view operation mode

3.1) Open the laser shutter so that optical excitation is enabled.

3.2) Start the pre-view software with a GPU implementation of processing algorithms to allow visualizing three-dimensional images at a frame rate corresponding to the pulse repetition rate of the laser.

3.3) Move the probe and/or the object to be imaged in order to optimize visualization performance and localize the structures of interest to be imaged.

4. Data acquisition.

4.1) Data acquisition for scanning (hand-held) mode.

4.1.1) If needed, inject a contrast agent prior to acquisition to enrich the contrast in the region of interest. The contrast agent must be approved for human and/or animal use by the respective government committee.

4.1.2) Start the data acquisition maintaining the execution of the pre-view software. Gently move the probe around the imaging region to track the structures of interest. Note that when images at multiple laser wavelengths are acquired simultaneously, the speed of probe motion in the hand-held mode has to decreased significantly (preferably below 2 mm/sec for laser pulse repetition rate of 50 Hz) in order to avoid motion-related artifacts in the spectrally unmixed images.

4.2) Data acquisition for stationary mode.

4.2.1) Mount the imaged object (e.g. animal) and the hand-held probe onto the holder and start the acquisition maintaining the execution of the pre-view software.

4.2.2) Maintain the optoacoustic probe and the imaging part in the same position during the experiment to visualize dynamic biological events in the region of interest.

4.2.3) If needed, inject a contrast agent to track its dynamic distribution in the region of interest.

5. Finalizing the experiment.

5.1) Stop the laser.

5.2) Remove the optoacoustic probe from the imaged region and the anesthesia supply for animal experiments.

5.3) Position the animal under an infrared heater to keep it warm and prevent the contact with other animals until it fully recovered from the anesthesia. Do not leave the animal unattended during recovery from the anesthesia.

6. Off-line data processing.

6.1) Process the acquired data with an accurate reconstruction algorithm to obtain a more quantitative representation of the distribution of absorbed energy. Process the images for different wavelengths to obtain functional and molecular parameters such as the blood oxygen saturation or distribution of an optoacoustic contrast agent.

REPRESENTATIVE RESULTS:

Representative results, demonstrating the capabilities of the described volumetric hand-held optoacoustic probe, are showcased in this section. In all cases, the light fluence on the skin surface was kept below the safety exposure limit of 20 mJ/cm^2.

The performance of the probe in real-time tracking of peripheral human vasculature is showcased in Figure 2. In this experiment, the hand of a healthy human volunteer was scanned at a single wavelength of 800 nm with the laser operating at 10 pulses per second[16]. The representative maximum intensity projection (MIP) of the reconstructed images in all three directions are displayed in Figure 2. Real-time visualization during the measurement is enabled with a GPU implementation of the filtered back-projection algorithm[16].

The real-time multispectral imaging capacity is showcased in Figure 3. Specifically, measurements were performed by scanning the probe along the wrist of a healthy volunteer having blood vessels with different sizes and oxygen saturation levels as well as a melanin-rich skin pigmentation[9]. A 50 Hz pulse repetition rate laser with a wavelength-tuning capability in a per-pulse basis was employed in this case. The laser was tuned to multiple wavelengths between 730 and 850 nm, corresponding to a monotonic decrease in the absorption of melanin, a monotonic increase in the absorption of oxygenated haemoglobin and a characteristic peak in the absorption of deoxygenated haemoglobin. The MIP images along the depth direction for 3 different wavelengths, corresponding to the same position of the probe, are displayed in Figure 3a. Figure 3b shows the unmixed distribution of oxygenated haemoglobin (HbO_2), deoxygenated haemoglobin (HbR) and melanin in red, blue and yellow, respectively, whereas it was further assumed that the absorption was solely due to these three chromophoric components.

Figure 4 illustrates the capability of imaging dynamic processes *in-vivo*. Herein, the circulation in the middle finger was obstructed by means of a rubber band and released during data acquisition[17]. A sequence of single wavelength images was acquired at 10 frames per second as determined by the pulse repetition rate of the laser. Four MIP images along the lateral and depth directions spaced by 1 second are showcased, where the second image corresponds to the instant after the circulation was restored. The wavelength was set to 900 nm, so that

amplitude of the optoacoustic signals is increased both with blood volume and blood oxygenation.

Finally, Figure 5 demonstrates the ability of the introduced system to track perfusion dynamics in a three-dimensional region of a mouse by using ICG as a contrast agent. An eight week-old female nude CD-1 mouse was used for the in-vivo experiments. The experimental procedure was in agreement with institutional and Bavarian government rules and regulations. The brain vasculature was imaged by positioning the mouse in a supine position and 2% Isofluorane in pure oxygen was used for anesthesia. Bepanthen® cream (Bayer AG, Germany) was used to protect the eyes of the mouse. 10 nmol of ICG diluted in 50 ml of saline was injected 5 s after starting the optoacoustic data acquisition. For each set of wavelengths, the ICG distribution was unmixed by assuming that the optical absorption is only due to this agent as well as the oxygenated and deoxygenated forms of haemoglobin. The MIP images along the depth direction corresponding to the unmixed ICG distribution for 5 different instants are shown in Figure 5a (time after injection is also indicated). The absorption spectrum of ICG in plasma is displayed in Figure 5b. This particular experiment demonstrates that the suggested approach is capable of simultaneously rendering truly five-dimenional (i.e. spectrally enriched time-resolved three dimensional) tomographic data, which is subsequently used to reconstruct and spectrally unmix the distribution of various intrinsic chromophores and exogenous agents in real time.

Figure 1: Title: Layout of the hand-held three-dimensional optoacoustic probe. Legend: (a) Distribution of the piezoelectric elements (blue dots) with respect to the region of interest (black cube). (b) Actual picture of the transducer array (TA) and fiber bundle (FB). (c) Water enclosing part. (d) Actual picture of the optoacoustic probe as being used in the hand-held operation mode.
Figure 2: Title: Tracking of peripheral human vasculature. Legend: Maximum intensity projections images of optical absorption along the three Cartesian directions for four consecutive images acquired with a laser operating at 10 pulses per second at a wavelength of 800 nm. The gray-scale color scheme represents intensity of optical absorption *H* in the object in arbitrary units.
Figure 3: Title: Hand-held imaging of specific endogenous chromophores. Legend: (a) Maximum intensity projections images of optical absorption along the depth direction for three different wavelengths corresponding to three consecutive pulses of the laser operating at 50 pulses per second (the probe was not moved). (b) Spectrally unmixed images showing distribution of oxygenated and deoxygenated haemoglobin and melanin.
Figure 4: Title: Real-time imaging of blood flow. Legend: Maximum intensity projections images of optical absorption along the depth and lateral directions corresponding to four different instants. The circulation in the middle finger was blocked prior to the experiment and released during the experiment (at 0s).
Figure 5: Title: Real-time imaging of the distribution of optical contrast agent in mice. Legend: (a) Distribution of the ICG contrast agent (maximum intensity projections along the depth

direction) for four different instants after injection of the agent (at 0s). (b) Extinction spectrum of ICG in plasma.

DISCUSSION:

The unique advantages offered by optoacoustic imaging techniques in small animal research have created strong motivation for translating the technology into clinical practice, with a number of diagnostics and treatment monitoring applications envisioned. However, as opposed to mice or smaller animals, which can be surrounded by a sufficient number of illumination sources and detection elements to create an effective tomographic image acquisition geometry, the large dimensions of human body and limited optical penetration hinder implementation of whole-body optoacoustic tomography similarly to MRI or CT. The presented hand-held optoacoustic imaging probe is ideal for human imaging as it shares many of the advantages of ultrasonography, such as portable use, high resolution, non-ionizing excitation and real-time capacity. Nevertheless, as we have demonstrated, the optimal hardware design and reconstruction procedures for optoacoustic imaging significantly differ from those commonly used in ultrasound scanners. In particular, optoacoustics is inherently a three-dimensional imaging modality as complete volumetric tomographic data sets from the imaged object can in principle be generated with a single interrogating laser pulse, as was demonstrated in this work.

As compared with other well-established clinical imaging modalities, such as magnetic resonance imaging (MRI) or x-ray computed tomography (CT), optoacoustic tomography is not a whole-body imaging modality but may provide significantly richer and more specific contrast based on light interrogation with tissues. Indeed, endogenous optical absorption contrast does not only deliver high-resolution tissue morphology but also renders functional and potentially targeted molecular information of high importance for clinical decision making. The molecular imaging capacity is further strongly supported by the significantly larger availability of contrast agent approaches of optical imaging methods as compared those available for the other imaging modalities. Furthermore, the high temporal resolution of the optoacoustic approach demonstrated here, i.e. high frame rate (real-time) three-dimensional imaging, is not generally available with any other modalities currently in clinical or laboratory use. Finally, simultaneous acquisition of multi-wavelength data brings an additional fifth dimension into the real time volumetric visualization that allows performing true three-dimensional spectroscopic observations of tissues chromophore and specific bio-marker biodistributions.

The potential applications of a three-dimensional optoacoustic hand-held probe are not limited to clinical imaging but it may also represent a highly important tool in biological research with animal models. Indeed, animals larger than mice are generally not suitable to be imaged in a tomographic optoacoustic system and the hand-held approach is probably more convenient. Also, volumetric (three-dimensional) imaging of certain regions in real time with optical

contrast and ultrasound resolution represents a unique advantage in the study of drug delivery, hemodynamic changes or pharmacokinetics.

In conclusion, we expect that the introduced methodology for hand-held optoacoustic imaging will prompt clinical translation of the technology and significantly advance pre-clinical and biological research on many frontiers as well.

ACKNOWLEDGMENTS:

The research leading to these results has received funding from the European Research Council under grant agreement ERC-2010-StG-260991.

DISCLOSURES:

The authors have nothing to disclose.

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