Effects of the murine skull in optoacoustic brain microscopy

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ABSTRACT

Advances in research on neurological diseases require the ability to acquire minimally invasive longitudinal data with high reproducibility. Despite the great promise behind the recent introduction of optoacoustic technology into the arsenal of small-animal neuroimaging methods, high resolution noninvasive optoacoustic microscopy studies are still limited to a thin layer of pial microvasculature due to presence of the skull. The presented research examines a variety of acoustically and optically attenuating effects introduced by the murine skull and evaluates performance limitations in transcranial optoacoustic imaging under both, acoustically- and optically-determined resolution scenarios. It is shown that strong low-pass filtering characteristics of the adult murine skulls may significantly deteriorate the achievable spatial resolution in non-invasive animal studies. While only brain vasculature with diameters >60µm were effectively resolved through the intact adult skull, significant improvements to the minimum achievable resolution are seen through cranial windows and thinned skull experiments.
I. INTRODUCTION

Advances in research regarding the treatment and progression of neurological diseases require the ability to acquire non- or minimally-invasive, longitudinal data with high reproducibility [1]. Therefore, imaging techniques that allow for time-resolved measurements of cerebral changes in intact brains under normal intracranial pressure conditions are of high importance. While whole-body methods, such as fMRI [2] and diffuse optical imaging [3], may offer fully noninvasive endogenous measurements of the brain anatomy and its macroscopic function, the contrast and temporal resolution provided are not sufficient for resolving fast hemodynamic and functional changes in the microvasculature. In contradistinction, imaging of cortical structures and fast neuronal activation with diffraction limited optical resolution using two-photon excitation microscopy [4], [5] has seen enormous progress, but photon scattering greatly limits the effective imaging depth of even the most advanced optical microscopy systems. To this end, optoacoustic microscopy has shown capacity for transcranial imaging of the mouse brain [6], [7]. While optical resolution optoacoustic microscopy (OR-OAM), employing tightly focused light, may achieve sub-micron spatial resolution [8], similarly to other optical microscopy techniques, its potential as a volumetric imaging modality is hindered by the severely restricted depth penetration. As a result, studies are commonly performed in young mice [9], through thinned skulls [10] or otherwise concentrate on demonstrating high-resolution functional imaging performance in a thin layer of pial vasculature immediately adjacent to the skull [11]. An alternative imaging strategy relies instead on unfocused illumination and detection of diffraction-limited acoustic fields, which can be done tomographically [12] or by means of raster scanning of spherically focused ultrasonic
detectors, as in acoustic resolution optoacoustic microscopy (AR-OAM) [13]. In this way, diffraction-limited acoustic resolution can be achieved at significantly greater depths, independently of photon scattering [14], [15]. Yet, the presence of an acoustically mismatched skull was previously shown to also significantly deteriorate imaging performance in this modality [16], [17].

II. MATERIALS AND METHODS

In order to analyze and quantify effects of the murine skull in various illumination and detection scenarios, an optoacoustic microscopy system with hybrid optical and acoustic resolution was used [14], [15]. The system comprises an ultrawideband spherically focused PVdF transducer with a focal distance of 7.8 mm, central frequency of 30 MHz and detection bandwidth >100% (Precision Acoustics, United Kingdom), providing diffraction-limited lateral acoustic resolution of 48 µm. For data acquisition in pure acoustic resolution mode, broad and unfocused illumination is applied to the imaged object via a liquid light guide (Lumatec, Germany) positioned in its vicinity at an angle of 90° to the acoustic axis (Fig. 1a). To achieve optical resolution performance, a different light delivery system is used instead, which consists of a single-mode photonic crystal fiber inserted through the central bore through the transducer’s aperture (Fig. 1b). The fiber output is focused onto the object’s surface using a gradient-index lens (GRINTECH GmbH, Germany), yielding an optical focus with a full width at half maximum (FWHM) size of 20µm and a Rayleigh length of 2.2 mm. For achieving three-dimensional imaging, the optoacoustic head is scanned laterally in two dimensions and time-resolved ultrasound signals are recorded at each scanning position.
To determine the skull-induced deterioration in the acoustic resolution performance, an agarose phantom was prepared containing a single 10 µm microsphere placed in the focus of the ultrasonic transducer. Two data sets were then acquired - with and without a piece of excised adult mouse skull with a measured thickness of 230 µm, which was placed between the phantom and the transducer and coupled using distilled water.

In order to examine the effects the skull might have on the microscopy system's actual imaging performance, we imaged several sutures (50 µm diameter) embedded at different depths into a scattering, tissue-mimicking agar phantom (1% absolute intralipid concentration). Raster scanning of the spherically-focused transducer was performed to acquire volumetric image data using broad sample illumination (acoustic resolution mode). The phantom was scanned twice: once directly and once with a piece of excised skull placed above the central crossing of the sutures, which covered almost the entire phantom diameter. During image reconstruction, the out-of-focus signals were further recovered using a synthetic aperture-focusing algorithm [18].

Finally, in accurately analyzing the applicability of optoacoustic microscopy for non-invasive imaging of fine brain vasculature in optical resolution mode, a perfusion fixation procedure [19] was adapted for labeling the brain's vasculature ex vivo. The procedure thus enables studying the effects of the murine skull independently from potential artifacts related to in vivo imaging, such as motion. Adult CD1 mice were used for perfusion. The animals were sacrificed with an overdose of ketamine and xylazine, followed by IP injection of 200 heparin units, and then perfused through the left ventricle with heparinized PBS (5 units heparin/ml) using a perfusion pump (bhr Labor-Technik GmbH, Germany). The perfusion agent was then changed to a 1:4
The mixture of Pelikan 4001 ink (Pelikan Holding AG, Switzerland) to agarose, prepared using 1.5% of low gelling agarose (SeaPrep, Lonza, Switzerland) in PBS. Following the mixture's complete perfusion, the agarose was allowed to cool and set for 45 minutes at 4°C, thereby fully replacing the circulating blood with highly absorbing ink while the agarose retains the integrity of the solution and the structure of the vasculature (Fig. 4g). Furthermore, the speed of sound difference of water and agarose is negligibly small, thus adverse effects of the perfusion method were minimized. The skull was exposed and prepared for imaging by either leaving it intact, removing it completely or thinning it using a high-speed handheld drill (IDEAL Micro Drill, Cellpoint Scientific, USA). Acquisition time was kept to under a minute for the entire 3D volumes (ROI 5x3 mm), with PBS being used as the coupling medium to diminish any destructive effects of distilled water on the biological tissue.

III. RESULTS

The results of the microsphere experiment shown in Figure 2, reveal that the skull disperses the optoacoustically generated ultrasound wave and effectively acts as a low-pass filter. As higher frequencies suffer higher attenuation, the peak of the attenuated signal becomes broader and lower in amplitude when compared to the non-attenuated signal (Fig. 2a). The axial FWHM of the Hilbert-transformed signals, governed by the acoustic resolution, amounts to 35 ns (53 μm) and 62 ns (93 μm) for the non-attenuated and attenuated case, respectively. The frequency-dependent attenuation (or insertion loss) of the skull was subsequently calculated by dividing the two detected signals in the Fourier domain, demonstrating significant attenuation of high frequency signal components by the skull (Figs. 2b and 1a). For comparison, the
murine skull’s attenuation was roughly estimated by scaling acoustic attenuation values found in the literature for a human skull [20] down to the actual sample thickness used in our experiments (solid black line in Fig. 2b). The measured dispersion generally follows the predicted trend, except for some low frequency peaks and troughs around 5 MHz, putatively representing thickness-dependent Lamb modes of the skull acting as a solid plate [21]. As the frequency increases, the agreement between experimental and predicted curves becomes mainly qualitative, with the order of magnitude remaining well in agreement for frequencies above 25 MHz.

In addition to these frequency-dependent attenuation effects, it can be observed that the skull introduces both time shifts and ringing artifacts in the arrival of the optoacoustic responses. The speed of sound is significantly higher in bone as compared to water [16], thus, optoacoustic signals travelling through the skull will arrive earlier at the detector surface as compared to signals travelling the same distance through water alone. Indeed, the time shift as seen in Fig. 2a is calculated to be \( \Delta t = 72 \text{ nsec} \). The speed of sound \( c_{sk} \) in the skull is given by

\[
c_{sk} = \frac{c \cdot w}{w - c \cdot \Delta t},
\]

where \( w \) is the skull’s thickness and \( c \) is the speed of sound in water. Using the measured thickness of the adult murine skull (230 \( \mu \)m), we calculate the speed of sound in the skull to be 2828 m/s, closely corresponding to the 2800 - 2900 m/s range found in literature [20], [16]. Moreover, when a microsphere absorber is placed in the immediate vicinity of the outer skull surface, ringing is created due to reflections of the generated optoacoustic signal at the two skull surfaces, which could be mistakenly interpreted as shadow absorbers inside the brain (Figs. 2c and 1a).

Figure 3 shows volumetric optoacoustic images for the embedded 50 \( \mu \)m suture phantom experiments, color-coded for depth. To compare the two scenarios, lateral
FWHM measurements were estimated at the positions marked by white arrows (Fig. 3a). The FWHM only increases by 13% on average when the skull fragment obstructs the transducer's view. Such minor broadening of 50 μm diameter structures when imaging through the skull is generally expected since absorbers of this size emit optoacoustic responses at much lower frequencies as compared to the 10 μm sphere. In comparing Fig. 3b to 3a, it becomes apparent that the reconstructed sutures also exhibit minor irregularities (yellow arrows), which might be attributed to the local refraction effects with the curvature and porosity of the skull possibly influencing the appearance of the reconstructed features. A thorough investigation of such irregularities remains however outside the scope of our current work.

Even though the brain vasculature is clearly visible in maximum intensity projection (MIP) images acquired through the intact skull (Figs. 4a and b), the fine capillary structure is not visible. When a partial cortical window is created by thinning the skull, both acoustic attenuation and photon scattering are locally reduced and finer vasculature becomes visible in the region of the thinned window (Figs. 4c, d). Naturally, the finest capillary structures are visible when no bone is obstructing the view of the transducer (Figs. 4e, f). As a measure of the effective lateral resolution, the FWHM for exemplary smallest visible vessels was calculated for all the three cases and were found to be 63.1 μm, 28.7 μm and 11.8 μm for the intact skull, thinned skull and excised brain, respectively.

IV. DISCUSSION AND CONCLUSIONS

In conclusion, it was shown how the adult murine skull affects the effective lateral and axial resolution in non-invasive optoacoustic microscopy studies, which may
limit the capacity for transcranial imaging of small capillary structures in deep brain. The skull's acoustic low-pass filtering effects limit the resolution in all forms of optoacoustic microscopy; however, in the special case of optical-resolution microscopy, the blurring of the cortical vasculature in the lateral direction is not solely attributed to the frequency-dependent acoustic attenuation but rather to the light scattering properties of the cortical bone, which similarly reduces the effective lateral resolution in non-invasive \textit{in vivo} experiments. The additional image degradation can be attributed to reflections at the skull-tissue-interface experienced by strong optoacoustic sources inside and on top of the skull; where the resultant time-domain ringing conceivably masks brain vasculature located below the skull. Measures such as skull thinning, cortical windows and using infant animals with thinner skulls may assist to reduce the skull-related effects, yet some of these methods may compromise the non-invasive nature and versatility of the imaging studies.

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Fig. 1: Schematics of the experimental approaches. (a) Acoustic resolution optoacoustic microscopy configuration using broad illumination. Optoacoustic sources located just above the skull generate ringing artifacts and shadow signals due to reflections at the skull surfaces (vertical line in the blue box denotes temporal position of the skull). Acoustic dispersion of the skull creates frequency dependent attenuation with higher frequency signals (generated by smaller objects) experiencing stronger attenuation compared to lower frequency content (generated by larger objects), see green box. (b) Optical resolution optoacoustic microscopy configuration using focused light illumination. Focused light is scattered by the skull, broadening the excitation beam and affecting the lateral resolution performance.

Fig. 2: Results of the microsphere experiments demonstrate acoustic dispersion and ringing effects of adult murine skull. (a) Optoacoustic signal traces generated in the presence and absence of the skull. The attenuated peak is lower in amplitude and broader when compared to the non-attenuated peak (marked by arrows). The signal shift $\Delta t$ is labeled. (b) Frequency dependent attenuation (insertion loss) of the skull was calculated by dividing the spectra of optoacoustic signals in the Fourier domain and compared to the predicted thickness corrected human skull dispersion behavior [20]. (c) Time domain signal of a microsphere placed in the immediate vicinity of the skull surface clearly shows signal ringing due to reflections at the skull-tissue interface.
Fig. 3: Effects of acoustic attenuation on imaging performance in the acoustic resolution mode. (a) Three-dimensional optoacoustic image (color-coded for depth) of four absorbing sutures randomly arranged inside tissue-mimicking scattering agar phantom. (b) The corresponding image acquired in the presence of skull. Scale bars correspond to 1.5 mm. Yellow arrows mark irregularities in the skull measurements. White arrows mark the position of FWHM measurements: 365 μm, 261 μm, 210 μm and 402 μm, 308 μm, 236 μm in (a) and (b) respectively.

Fig. 4: Murine skull affects the effective lateral resolution performance in optical resolution optoacoustic microscopy scans of biological targets. Maximum amplitude projections of optical resolution images of the perfused brain made through an intact skull (a-b), thinned skull (c-d) and without presence of the skull (e-f). Boxes in a,c,e denote the location of images shown in b, d, f,. Arrows mark the location of the FWHM measurement. Scale bars are 500 μm and 250 μm for a, c, e and b, d, f respectively. Photograph of an excised mouse brain, perfused with a mixture of agarose and ink is shown in (g).