22

Optical clearing aided photoacoustic imaging in vivo

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CONTENTS
Introduction..........................................................................................................................................................411
Optical clearing–aided optical resolution photoacoustic imaging.................................................................411
Optical clearing–aided acoustic resolution photoacoustic imaging.................................................................415
Conclusions..........................................................................................................................................................417
Acknowledgments................................................................................................................................................417
References.........................................................................................................................................................418

Introduction

Combining the advantages of optical excitation and of acoustic detection, photoacoustic imaging (PAI) has recently shown its unparalleled strength in providing structural [1], molecular [2], functional [3–5], and metabolic information [6]. Based on its focusing mechanism, PAI can be classified into two different implementations: optical-resolution PAI (OR-PAI) and acoustic-resolution PAI (AR-PAI) [1]. In OR-PAI, the lateral resolution is determined by the diffraction-limited optical focusing. As photons travel in tissue, the focusing capability degrades due to optical scattering. In addition, the maximum penetration depth of OR-PAI in biological tissue is mainly limited by the optical transport mean free path, affected by both absorption and scattering. Since the tissue scattering is typically an order of magnitude larger than the absorption, it is the scattering that predominantly limits the penetration depth of OR-PAI. In AR-PAI, the resolution is determined by acoustic detection, so the optical scattering does not have a significant impact on the resolution. However, it does limit the maximum penetration depth of AR-PAI for the same reason as in OR-PAI [7].

To reduce the scattering, tissue optical clearing (TOC) techniques have been widely used in many high-resolution optical imaging modalities, such as laser speckle contrast imaging and optical coherence tomography [8]. The basic mechanism of TOC is to diffuse a high-refractive-index optical clearing agent (OCA) into the tissue, which reduces the refractive index mismatch between intracellular components and extracellular fluids and thus decreases the scattering. In addition, tissue dehydration caused by OCAs has also been used to explain TOC [8]: on one hand, it increases refractive index matching due to decreased volume fraction of free water in the interstitial fluid; on the other hand, it increases packing of scatters, which may engender spatial correlations between scatterings, both of which reduce scattering further.

In this chapter, we discuss the state-of-the-art methods of using optical clearing in photoacoustic imaging in vivo. We first introduce how optical clearing has been applied to enhance the imaging depth and lateral resolution of OR-PAI. Then we discuss how optical clearing improves penetration of AR-PAI. Finally, we summarize the application of optical clearing in photoacoustic imaging and suggest future research directions.

Optical clearing–aided optical resolution photoacoustic imaging

In 2013, Zhou et al. first applied optical clearing in a typical OR-PAI implementation [9], as shown in Figure 22.1. Briefly, a tunable dye laser (CBR-D, Sirah GmbH) pumped by a Nd:YLF laser (INNOSAB, Edgewave, GmbH) was used as the light source. After being focused by a condenser lens, filtered by a pinhole, and reflected by a mirror, the laser beam was finally focused by an objective lens into the sample. Ultrasonic detection was achieved by a wide-band ultrasonic transducer (V214-BC, Panametrics-NDT Inc.), which was placed confocally with the optical objective lens. Each laser pulse yielded a one-dimensional depth-resolved PA image (A-line) by recording the time course of the photoacoustic signal. A three-dimensional (3D) image was obtained by raster scanning the sample and piecing together A-lines.

They first demonstrated the efficiency of optical clearing in a phantom experiment. A U.S. penny [Figure 22.2(a)] covered by a piece of freshly harvested mouse skin was imaged at 570 nm. The phantom was immersed in the glycerol–water solution. As shown in the baseline image [Figure 22.2(b)], there were almost no PA signals from the coin because of the strong scattering of the mouse tissue. During optical clearing, the PA signals from the coin became stronger and stronger, and more...
features became resolvable, as shown from Figure 22.2(c)–(d). Figure 22.2(e) shows the average total PA signal amplitude from the coin versus time; after 250 min, the amplitude increased by more than three-fold over the baseline value. Note that the PA signals were still increasing at the point when they stopped the experiment. This phantom experiment clearly illustrates that the scattering of the tissue was reduced by optical clearing, and thus the penetration depth was enhanced.

Next, they used a nude mouse in an *in vivo* experiment. Because the diffusion of glycerol across the epidermal layer is very slow, they directly injected the glycerol–water solution into the mouse scalp to create a local optical clearing window. Figures 22.3(a) and (b) show the maximum amplitude projection (MAP) of the PA images of blood vessels in the mouse scalp before and after optical clearing, respectively. For a better visualization of the deeper vessels in the scalp, most of the superficial capillaries seen in Figure 22.3(b) were digitally removed, with the result shown in Figure 22.3(c). From Figures 22.3(a)–(c), a clear PA signal amplitude enhancement after optical clearing can be observed. Three representative lines were taken from Figures 22.3(a)–(c) for further comparison, as shown in Figures 22.3(d)–(f). The averaged PA amplitude had increased by about eight times after optical clearing.

The optical clearing effect was quantified along the depth direction, as shown in Figures 22.3(g)–(h). To quantify the total signal enhancement after optical clearing, the averaged PA signal amplitude along the depth was calculated and is shown in Figure 22.3(i). Based on Beer’s law, the fitted attenuation coefficients before and after optical clearing were 57.4 cm$^{-1}$ and 103.1 cm$^{-1}$, respectively. At first glance, the

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**FIGURE 22.1** System schematic used in the experiment. CorL, correction lens; OL, objective lens; RAP, right-angle prism; RhP, rhomboid prism; SOL, silicone oil layer; UT, ultrasonic transducer. Reprinted with permission from reference [9] © The Optical Society.

**FIGURE 22.2** Optical clearing aided PAI of a coin covered by mouse skin. (a) Photograph of the one-cent coin used in the experiment. (b)–(d) PA images of the coin at different times during optical clearing. (e) Time course of the PA amplitude averaged over the whole image during optical clearing. The higher PA amplitude indicates that the optical clearing increased penetration depth. Reprinted with permission from reference [9] © The Optical Society.
attenuation appears to have increased after optical clearing. However, since dehydration due to optical clearing caused the total thickness of the tissue to decrease by about 2.57 times [Figures 22.3(g)–(h)], the scatter and absorber number density should increase by about 2.57 times. If both the scattering and absorption cross sections had remained constant after optical clearing, the attenuation coefficient should have increased by 2.57 times rather than the 1.80 times observed in the experiment. Thus, the scattering or absorption cross section was reduced after optical clearing. Because optical clearing did not affect the absorption cross section, it was concluded that the 0.77 times less increase in the attenuation coefficient resulted from the decrease in the scattering cross section.

The enhanced PA detection sensitivity after optical clearing enabled better capillary imaging [Figure 22.3(b)]. Figure 22.4(a) and (b) are close-up images of the red-dashed-square areas in Figures 22.3(a) and (b), respectively. The large vessels in the periphery of both images indicate that they are from the same area in the mouse scalp. As shown in Figure 22.4(a), very few capillaries can be detected before optical clearing. However, after optical clearing, more capillaries can be distinctly observed, as shown in Figure 22.4(b). As shown in Figures 22.4(c)–(d), the vessel density increased by about ten times after clearing, and the averaged PA signal amplitude from individual capillaries increased by a factor of 22. The much denser capillaries and stronger PA amplitudes in Figure 22.4(d) indicate that, after optical clearing, the local laser fluence at the capillaries was increased. There were two possible reasons for the increased local laser fluence: (1) enhanced focusing on these capillaries due to the dehydration-induced shrinkage of the tissue, and (2) reduced scattering loss. As shown in Figure 22.3(i), because of dehydration, the averaged thickness of the tissue decreased from about 0.88 mm to 0.37 mm. Assuming the light beam intensity had a Gaussian shape, it was estimated that the average fluence increase caused by shrinkage was about five times. Therefore, it can be concluded that the remaining 4.4-fold increase in the PA signal amplitude was due to the decreased scattering coefficient. Because the background noise remained constant during the experiment, the noise-equivalent sensitivity depended only on the PA signal amplitude. Therefore, sensitivity should also increase 4.4 times due to the decreased scattering coefficient.

Finally, the lateral resolution enhancement after optical clearing was analyzed. By zooming in on the deeper vessels in Figures 22.3(a)–(b), it can be seen that the vessels before optical clearing were much more blurred than those after optical clearing, as shown in Figures 22.5(a) and (b). Again, the averaged PA amplitude was increased by about five times [Figures 22.5(c)–(d)]. More importantly, the lateral resolution improvement was analyzed by comparing the vessel diameters. As shown in Figure 22.5(e), a representative vessel had nominal diameters of 75 μm and 30 μm before and after clearing.
FIGURE 22.4 Optical clearing reveals more capillaries. (a–b) Close-ups of PAM images in the red dashed boxes in Figures 22.3(a–b). (c–d) PA amplitudes along the red dashed lines in (a–b). Reprinted with permission from reference [9] © The Optical Society.

FIGURE 22.5 Optical clearing improves the lateral resolution for deep vessels. (a) Close-up of PAM image in the green dashed box in Figure 22.3(a). (b) Close-up of PAM image in the green dashed box in Figure 22.3(c). (c–d) PA amplitudes along the red dashed lines in (a–b). (e) Combined curves in (c) and (d) after normalization. Reprinted with permission from reference [9] © The Optical Society.
respectively. The measured vessel diameter decreased at least by 2.5 times after optical clearing. Because the measured vessel diameter was the convolution of the actual lateral resolution with the true vessel diameter, the actual resolution improvement should be better than 2.5 times.

Optical clearing–aided acoustic resolution photoacoustic imaging

Liu et al. did a very comprehensive test of the use of optical clearing in AR-PAI [10]. A typical dark-field illumination AR-PAI was used, as shown in Figure 22.6(a). Briefly, a conical lens and an optical condenser were used to provide the dark-field illumination. A focused ultrasonic transducer (center frequency, 50 MHz; NA, 0.47; V30011, Olympus, USA) was placed in the center of the optical condenser to detect the induced ultrasonic waves. Between the sample and the transducer, the ultrasonic wave was coupled by a water tank with a bottom window sealed with a polyethylene membrane. As shown in Figure 22.6(b), the rat skin specimens were immersed in two layers of OCA. A black taper was used to measure the amplitude, while some carbon fibers with a diameter of 6μm were used to measure the spatial resolution.

Five commonly used OCAs (glycerol, PEG-400, DMSO, glucose (40%), and oleic acid) were first characterized on AR-PAI ex vivo. As shown in Figure 22.7, DMSO, PEG-400, and glycerol enhanced the photoacoustic signal, while glucose and oleic acid caused subtle decrease in the amplitude similar to PBS. Meanwhile, no obvious changes were found in the lateral resolution after OCAs were applied.

Based on the ex vivo experiments, PEG-400, glycerol, and DMSO were selected for in vivo experiments. Because PEG 400 does not easily penetrate the dermis, thiazone was chosen as a penetration enhancer for the in vivo experiment. To further improve dermal penetration, massaging was applied with OCAs. The experimental results from PEG-400, glycerol, and DMSO are shown in Figure 22.8, Figure 22.9, and Figure 22.10, respectively.

As shown in Figure 22.8(a) and (b), the mixture of PEG-400 and thiazone produced an obvious optical clearing effect. As shown in Figure 22.8(c), (d), and (g), although the photoacoustic amplitude of subcutaneous vessels is greatly improved, the measured vascular diameters are the same, indicating there are no lateral resolution changes. Based on the B-scan images shown in Figure 22.8(e) and (f), the blood vessels seem to be closer to the skin surface, probably due to the dehydration effect. A statistical analysis from ten randomly chosen vessels confirms that there is significant photoacoustic amplitude improvement but no big changes in lateral resolution, as shown in Figure 22.8(h).

As shown in Figure 22.9(a) and (b), the glycerol produced very little optical clearing effect visually. However, as shown in Figure 22.9(c), (d), and (g), the photoacoustic amplitude of subcutaneous vessels is greatly improved. Different from the mixture of PEG-400 and thiazone, there was no obvious dehydration effect, as shown in Figure 22.9(e) and (f).

FIGURE 22.6 (a) Schematic of the imaging head. (b) Diagram of the sample setup. A, absorber; CL, conical lens; MP, metal plate; OC, optical condenser; S, sample; SH, sample holder; U, ultrasonic transducer; WT, water tank. Reprinted with permission from reference [10] © The Optical Society.

FIGURE 22.7 Changes in the photoacoustic amplitude with different OCAs. Not all OCAs enhanced the amplitude. Reprinted with permission from reference [10] © The Optical Society.
FIGURE 22.8 Comparative images and statistical analysis for in vivo imaging with a mixture of PEG-400 and thiazone. (a) and (b) are photographs of skin before and after immersion. (c) and (d) are photoacoustic maximum amplitude projection (MAP) images projected along the depth direction. (e) and (f) are B-scan images denoted by the dashed lines in (c) and (d). The horizontal axis is the scan direction, and the vertical axis is the depth. (g) shows transverse plots of the vessel indicated by the green arrows in (c) and (d). (h) shows the statistical results for ten randomly selected vessels. The scale bar is the same for (a)–(f). Reprinted with permission from reference [10] © The Optical Society.

FIGURE 22.9 Comparative images and statistical analysis for in vivo imaging with glycerol. The descriptions of the subgraphs are the same as in Figure 22.8. Reprinted with permission from reference [10] © The Optical Society.
A statistical analysis from ten randomly chosen vessels shows that there is about 10% photoacoustic amplitude improvement but no big changes in lateral resolution, as shown in Figure 22.9(h).

Surprisingly, the in vivo experiment from DMSO presents contradictory results compared to ex vivo. As shown in Figure 22.10(a) and (b), there is no obvious optical clearing effect with DMSO. The photoacoustic amplitude of subcutaneous vessels greatly decreased after applying DMSO, as shown in Figure 22.10(c), (d), and (g). As shown in Figure 22.10(e) and (f), the subcutaneous blood vessels are deeper from the skin surface, which is probably from the swelling caused by stimulation. Based on the statistical result in Figure 22.10(h), the photoacoustic signal decreased to only 36% of the original ones. The physiological response of applying DMSO (such as swelling) probably caused the decrease of photoacoustic signals. Thus, DMSO may not be appropriate for AR-PAI as an OCA.

Conclusions

In summary, optical clearing has shown great capability in reducing optical scattering, thus improving photoacoustic signals in both OR-PAI and AR-PAI. In addition, because the lateral resolution is determined by optical focus in OR-PAI, utilizing optical clearing can also lead to an increase of lateral resolution in OR-PAI. Finally, it has been found that not all optical agents are beneficial in photoacoustic imaging, such as DMSO, glucose, and oleic acid. Glycerol has been proven to be able to improve photoacoustic amplitude in both OR-PAI and AR-PAI.

Because photoacoustic imaging performance is determined by both optical excitation and acoustic detection, a clearing agent that can reduce both optical and acoustic scattering is desired. So far, a majority of studies have focused on the optical side, and few works have been carried out from the acoustic perspective. It has been shown that a common optical clearing agent will introduce more acoustic attenuation due to dehydration, thus deteriorating the imaging performance. In 2016, Yang et al. proposed a new optical clearing agent that can be used to increase not only the transmittance of light, but also that of ultrasound in the skull in AR-PAI [11]. However, no obvious resolution improvement was observed. In the future, an optical clearing agent that can also reduce acoustic scattering in the skull would be very helpful for brain imaging with photoacoustic imaging techniques.

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