Optical clearing of the mouse brain and light attenuation quantitation

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ABSTRACT

Optical clearing allows the reduction of light scattering in biological tissue, enabling 3D morphological information to be obtained deep within tissue using techniques such as optical projection tomography and light sheet microscopy. However, the extent of the clearing is dependent on the technique that is used. There is therefore a need for methods to quantify the quality of the clearing process and thereby to compare clearing techniques. In this study, we developed such a method using a custom spectroscopy system and applied it to compare three techniques that were applied to mouse brain: BABB (Murray's clear), pBABB (a modification of BABB which includes the use of hydrogen peroxide), and passive CLARITY.

Keywords: Optical clearing, refractive index, mouse brain, spectroscopy, optical imaging, CLARITY, BABB

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1. INTRODUCTION

Morphological analysis of deep structures with high resolution is of significant interest for gaining greater understanding of biological processes in healthy and diseased tissues. Many optical imaging techniques have recently been developed to overcome the limitations of conventional techniques such as Magnetic Resonance Imaging (MRI), X-ray computed tomography (CT), and ultrasound imaging. For example, optical projection tomography (OPT)[1] and light sheet fluorescence microscopy (LSFM)[2] can allow for three-dimensional (3D) imaging of cellular, molecular, and genetic processes across the whole organisms or locally in excised tissues.

Optical clearing can be invaluable to increase light depth penetration with some optical imaging techniques such as OPT and LSFM. Techniques for optical clearing can significantly reduce scattering of both illumination and detection light within the sample. Some methods reduce scattering by replacing extracellular water ($n \approx 1.33$) with solutions that have refractive indices closer to that of the cell membrane ($n \approx 1.46$). As lipids that are found in cell membranes are dominant scatterers in biological tissues[3], other optical clearing methods have obtained approximately uniform refractive index profiles by the removing them[4]. Assessing the transparency of a tissue is particularly important when imaging cleared samples with systems like Optical Projection Tomography or Light Sheet Fluorescence Microscopy, as reduced light scattering can lead to higher spatial resolution and greater contrast[5].

In this paper, three clearing techniques are compared in terms of tissue transparency and change in morphology that they provide. These methods are BABB (Murray's clear)[6], pBABB (a modification of BABB with hydrogen peroxide) and CLARITY[7,8]. Light attenuation in mouse brain slices was measured spectroscopically, using a custom-made system. Three regions of the mouse brain were studied, which were chosen because they are known to have different tissue morphologies and lipid content. The attenuation spectra for each of the mouse brain regions and for each clearing technique were compared. The change in tissue size was evaluated by image analysis of cleared tissue slices.

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2. MATERIALS AND METHODS

2.1 Animal treatment

All experimental study protocols were conducted in accordance with institutional, United Kingdom home office regulations. MF1 *nu/nu* mice were were individually heparinized (Wockhardt, Heparin Sodium) by intraperitoneal injection (50 ml/specimen, 1000 U/ml) and terminally anaesthetized via intraperitoneal injection of 100 mg/kg sodium pentobarbital (Animalcare, Pentoject) diluted in 0.1 ml phosphate buffered saline (PBS). Once anesthesia was confirmed, surgical procedures for intracardial perfusion were performed for systemic clearance of blood. An incision was made below the xyphoid process, and the thoracic cavity was exposed by cutting through the diaphragm. The heart was exposed by folding up the sternum; a blunt needle inserted from the left ventricle was used to cannulate the aorta. The right atrium was severed to provide blood outflow. PBS (30 ml; 37°C) was administered with a perfusion pump (Watson Marlow, 5058) at a flow rate of 5 ml/min, followed by administration of 40 ml of 4% formaldehyde solution. Individually harvested brains were stored for 12 hours in 4% formaldehyde (10 ml; 4 °C) for post fixation.

2.2 Sample preparation and clearing

The mouse brain samples were rinsed three times in PBS (10 minutes each) prior to clearing. For spectroscopic light attenuation measurements, harvested brains were sliced with a brain slicer matrix (Zivic Instruments, BSMAS002-2) to obtain sagittal slices that were 2 mm in thickness; the two central slices close to the midline were used (Figure 1). For each clearing method, between 6 and 12 mice were used.

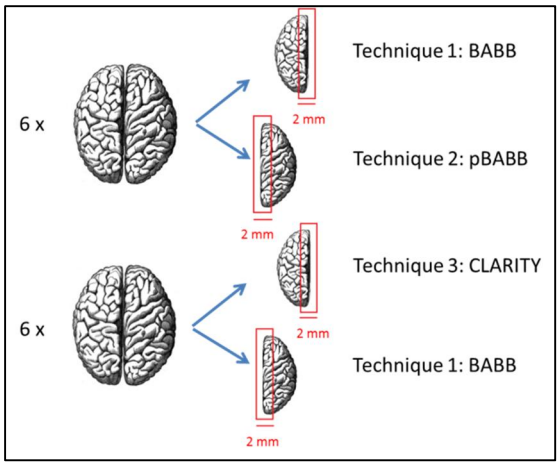

Figure 1. Schematic diagram of the experimental protocol. The two central slices taken from each hemisphere (red rectangles) were cleared for spectroscopic measurements of light attenuation.

Tissue clearing was performed with established procedures. In particular, BABB clearing preparation consisted of dehydration in methanol (MeOH) for 48 hours at room temperature and clearing in BABB (1:2 mixture of benzyl alcohol and benzyl benzoate) for 48 hours. For pBABB clearing, samples were submerged in a 4:1:1 mixture of methanol: dimethyl sulfoxide (DMSO): hydrogen peroxide (Dent's bleach) for 1 hour at room temperature, washed twice with MeOH and dehydrated in MeOH for 48 hrs. Samples were then transferred for clearing to BABB for 48 hours. The CLARITY protocol[4] was modified for passive clearing, as described by Tomer et al.[8]. This passive CLARITY protocol included thermal clearing of lipids at 37 °C and preservation of tissue structure with hydrogel embedding.

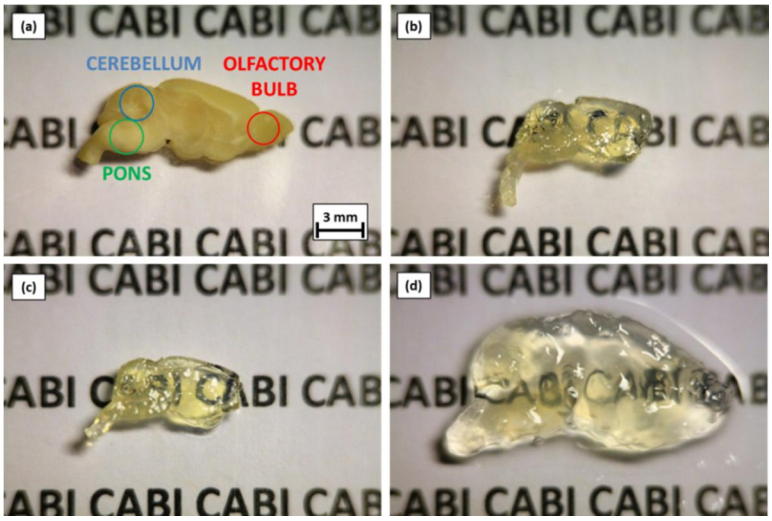

Figure 2. Images of cleared and uncleared mouse brain slices acquired with white light. Prior to optical clearing, the tissue was opaque **(a).** Optical clearing with **(b)** BABB, **(c)** pBABB, and **(d)** CLARITY significantly increased tissue transparency. The areas where spectroscopic light attenuation measurements were performed were the same in all slices; they are shown in **(a)**. The images have the same spatial scale.

2.3 Spectroscopic light attenuation

Spectroscopic light attenuation measurements were performed with a custom system (Figure 3) in three areas within the brain: the olfactory bulb, the cerebellum and the pons. The system consisted of a halogen lamp (LH-2000, Ocean Optics), coupled to a 200 µm optical fiber and collimated to a diameter of 3 mm. The cleared mouse brain slices were sandwiched between cover slips so that their thickness was 2 mm, and they were positioned vertically in a 3D printed holder. In the case of the CLARITY technique, the slices needed to be compressed due to the tissue expansion that resulted from clearing. To allow for different regions within the sample to be selectively illuminated by the beam, two x-y translation stages were attached to the holder.

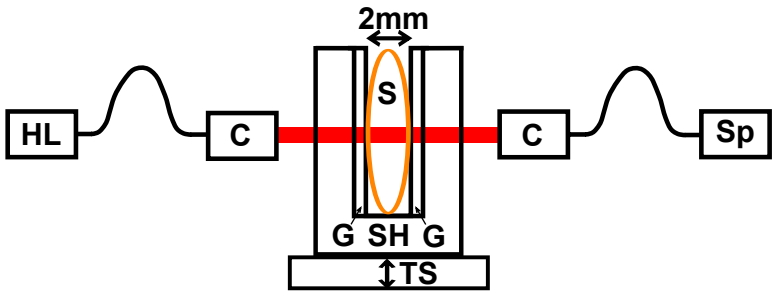

Figure 3. Schematic diagram of the experimental setup used for spectroscopic light attenuation measurements. The light emitted by a halogen lamp (HL) was coupled to a 200 µm fiber and collimated with a collimator (C) to form a 3 mm-diameter beam that illuminated the sample. The cleared sample was sandwiched between two 200 µm glass cover slips (G) and placed in a custom-designed, 3D printed sample holder (SH) with 2 mm spacing. To ensure that the laser was aligned with a particular region of interest, two linear translation stages (TS) were attached to the holder. Transmittance spectra were collimated and acquired with a spectrometer (Sp).

Transmittance spectra in the range of 400 to 1100 nm were acquired with a spectrometer (Maya Pro; Ocean Optics, Dunedin, FL) using a program written in Labview (National Instruments, Austin, Texas). Background spectra with no sample in the holder, and reference spectra using only BABB were acquired separately. For each brain region, 100 spectra were acquired with the same exposure time (6 ms) and averaged.

Post processing of the data, which included background subtraction and subsequent division by reference spectra, was performed with Matlab (Mathworks, Natick, MA). The attenuation of unscattered light was modelled using the Beer-Lambert Law. The attenuation coefficient μ_T (mm^{-1}) of each brain tissue slice with thickness x (mm) was estimated using the following equation:

$$\mu_T(\lambda) = -\frac{1}{x}\ln\left(\frac{I-I_b}{I_0-I_b}\right), \quad (1)$$

where I, I_0, and I_b are the spectra acquired with light transmission through the sample, through the reference solution (BABB), and through air in the absence of light, respectively.

2.4 Tissue volume changes

Changes in tissue volume that resulted from clearing were assessed by measuring changes in the cross-sectional areas of tissue slices before and after clearing, and extrapolating to three dimensions. Areas were measured by analysing tissue images with Image J (National Institutes of Health, Bethesda, Maryland).

3. RESULTS

3.1 Spectroscopic measurement of light attenuation μ_T (λ_{MAX})

Light attenuation spectra acquired in the brain samples varied with the clearing technique, and these variations depended on the tissue region (Figure 4).

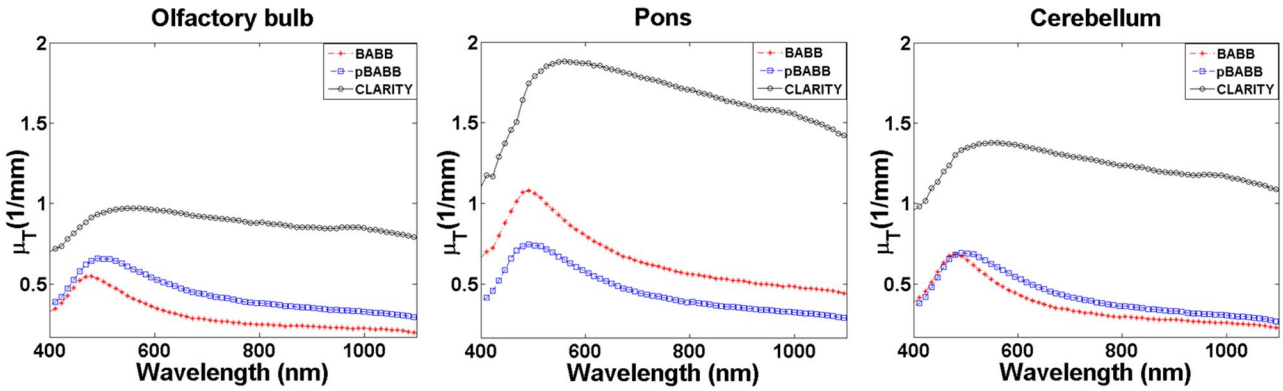

Figure 4. Light attenuation spectra obtained with the three clearing techniques in three brain regions, as calculated with Equation (1) and averaged over all measured brains.

For all brain regions, samples that were cleared with pBABB and BABB resulted in lower attenuation coefficients than samples that were cleared with CLARITY. For example, for the cerebellum, the mean attenuation coefficients, as calculated across the entire measured wavelength range, were 0.68 ± 0.29 mm^{-1} and 0.70 ± 0.09 mm^{-1} for BABB and pBABB, respectively, and they were 1.38 ± 0.22 mm^{-1} for CLARITY. Moreover, the maximum attenuation occurred at a higher wavelength for CLARITY than for BABB- and pBABB-cleared samples (Table 1).

Table 1. The wavelength for which the attenuation coefficient was a maximum, λ_{MAX}, varied with brain region and clearing technique, as did the corresponding attenuation coefficients, $\mu_T(\lambda_{MAX})$ (mean ± standard deviation).

Region	Technique	λ_{MAX} (nm)	$\mu_T(\lambda_{MAX})$ (mm^{-1})
Olfactory bulb	BABB	476	0.55 ± 0.23
	pBABB	497	0.66 ± 0.12
	CLARITY	558	0.97 ± 0.11
Pons	BABB	487	1.09 ± 0.28
	pBABB	497	0.75 ± 0.10
	CLARITY	553	1.89 ± 0.19
Cerebellum	BABB	482	0.68 ± 0.29
	pBABB	497	0.70 ± 0.09
	CLARITY	549	1.38 ± 0.22

3.2 Brain volume changes

Each of the clearing techniques under investigation induced tissue volume changes (Table 2). Both BABB and pBABB resulted in reductions in tissue volume (58%). In contrast, the CLARITY resulted in volume increases (229%).

Table 2. Cross sectional area of the brain slices before and after optical clearing, which was performed with the three compared techniques: BABB, pBABB and CLARITY. The data refer to one slice per each technique but are representative of all the cleared samples.

Condition	Technique	Area (mm^2)	Area change (%)
Before clearing		78	--
After clearing	BABB	45	58
	pBABB	44	56
	CLARITY	179	229

4. DISCUSSION

The data presented show that light attenuation in cleared mouse brain tissue was greatest in samples cleared with passive CLARITY, and lowest in those cleared with pBABB or BABB. The shape of the attenuation spectra was different for pBABB/BABB and CLARITY-cleared samples, with the latter producing a wider spectrum with a peak and extended tail at higher wavelengths as compared with those produced with pBABB/BABB. The origin of these spectral differences is unclear.

A new clearing technique called pBABB was also investigated, in which hydrogen peroxide is added to the dehydration medium. This step is beneficial as it enables the bleaching of endogenous tissue pigments, thereby reducing autofluorescence. Moreover, DMSO acts as a solvent increasing the clearing solution penetration without damaging the tissue[9]. This methodology is also particularly advantageous as it is straightforward and inexpensive, and clearing times are considerably shorter when compared to techniques such as CLARITY. The results showed that the degree of clearing obtained with pBABB methodology is greater than those given by BABB and CLARITY, and that differences exist in the shape of absorption spectra, particularly at wavelengths greater than 600nm. Despite the highest degree of tissue clearing which can be obtained with pBABB, this approach may be more suited to autofluorescence studies investigating tissue morphology, due to the bleaching step with hydrogen peroxide.

Despite the limitations of CLARITY that were observed in this study, this clearing technique has a number of important advantages as compared with BABB. For instance, it results in a smaller amount of protein loss and preserves native and introduced fluorescence[4]. Additionally, BABB is disadvantageous from the standpoint of decreasing the half-life of the fluorescent signal.[10]

It was shown that the extent of optical clearing varies spatially within the brain, most likely due to differences in the structure and composition of different areas. These results may have implications for quantitation of fluorescence. Of the three brain regions investigated, the pons showed the lowest clearing efficacy, most likely due to the great amount of white matter. White matter contains large quantities of myelin, a lipid-based substance that contributes significantly to optical scattering[11,12].

The results of this study could be useful for optimizing the choice of wavelengths for optical imaging techniques. For instance, the lower attenuation by pBABB/BABB-cleared samples at higher wavelengths (> 600 nm) could be beneficial for imaging of fluorophores with emission in the far visible range, such as Alexa 647. The results could also be useful as a precautionary guide to morphological studies: the considerable size changes could play a confounding role, depending on the application.

5. CONCLUSION

In this study, the efficacy of optical clearing protocols was quantified with optical spectroscopy. This spectroscopic method could prove to be useful for choosing a clearing protocol and for evaluating images that are acquired with cleared tissues, and it could readily be extended to other to other clearing methods or organs.

6. ACKNOWLEDGMENTS

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