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**Abstract.** Optical clearing, in combination with recently developed optical imaging techniques, enables visualization and acquisition of high-resolution, three-dimensional images of biological structures deep within the tissue. Many different approaches can be used to reduce light absorption and scattering within the tissue, but there is a paucity of research on the quantification of clearing efficacy. With the use of a custom-made spectroscopy system, we developed a way to quantify the quality of clearing in biological tissue and applied it to the mouse brain. Three clearing techniques were compared: BABB (1:2 mixture of benzyl alcohol and benzyl benzoate, also known as Murray's clear), pBABB (peroxide BABB, a modification of BABB which includes the use of hydrogen peroxide), and passive CLARITY. We found that BABB and pBABB produced the highest degree of optical clearing. Furthermore, the approach allows regional measurement of light attenuation to be performed, and our results show that light is most attenuated in regions with high lipid content. We provide a way to choose between the multiple clearing protocols available, and it could prove useful for evaluating images that are acquired with cleared tissues.

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## 1 Introduction

The ability to analyze tissue in three dimensions (3-D) at the mesoscopic scale has proven essential in the study of healthy and diseased organisms and organs. Techniques such as *ex vivo* magnetic resonance imaging and x-ray computed tomography (CT) have been successful in a number of applications, but are limited by low resolution or inability to use common staining techniques. These limitations are overcome by optical imaging techniques such as optical projection tomography<sup>1</sup> and light

sheet fluorescence microscopy.<sup>2</sup> However, to allow for sufficient depth penetration, these imaging techniques require specimens to be rendered transparent via a process known as optical clearing, which can be achieved using a number of techniques. In general, these techniques aim to minimize scattering and absorption of both illumination and detection light within the sample and can achieve light penetration increase up to several millimeters.<sup>3</sup> Light in the body is absorbed by structures such as red blood cells and is scattered at the interface of materials with different refractive indices. Some approaches obtain refractive index matching by replacing extracellular water ( $n \approx 1.35$ ) with solutions with a higher refractive index, closer to that of the cell membrane ( $n \approx 1.46$ ). As lipids are the main scatterers in biological tissue,<sup>4</sup> other approaches obtain a uniform refractive index by their removal.<sup>5</sup>

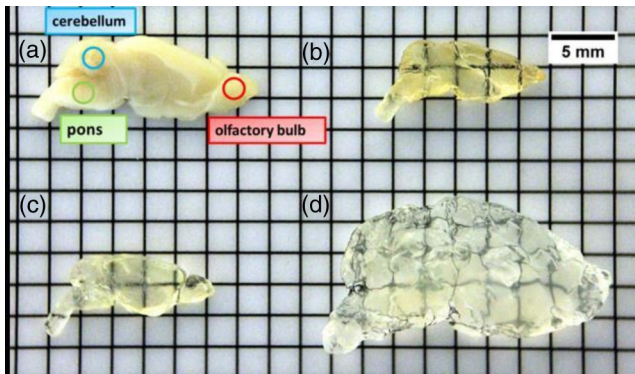
In this paper, the efficiency of three clearing techniques is compared: BABB (1:2 mixture of benzyl alcohol and benzyl benzoate, also known as Murray's clear),<sup>3</sup> pBABB (a modification of BABB with hydrogen peroxide), and passive CLARITY.<sup>6,7</sup> A new method for measuring the clearing degree using a custom-made spectroscopy system was developed and used to evaluate the obtained transparency in the mouse brain. Similar measurements can be acquired with optical CT,<sup>8</sup> but the depth of this modality might be not suitable for some samples. In this paper, three regions of the mouse brain were studied: olfactory bulb, pons, and cerebellum. These areas differ in their microscopic structures and amounts of myelin, which surrounds the axons and has a high lipid content. The absorption spectra for each of the mouse brain regions and for each clearing method were compared and used for quantification of the quality of clearing. Moreover, the change in the sizes of the samples induced by each clearing technique was also evaluated.

## 2 Methods

All experimental study protocols were conducted in accordance with institutional, UK home office regulations. MF1 nu/nu mice were individually heparinized and terminally anesthetized. Once anesthesia was confirmed, surgical procedures for intracardial perfusion were performed for systemic clearance of blood. Thirty milliliters of phosphate buffered saline (PBS) (37°C) were administered at a flow rate of 5 ml/min, followed by administration of 40 ml of 4% formaldehyde solution. The brains were extracted and postfixed for 12 h in 4% formaldehyde (4°C), then rinsed in PBS, and sliced to obtain 2 mm sagittal slices. The two central slices close to the midline, one per hemisphere, were used for the light attenuation quantitation study. For each clearing technique, between 6 and 12 mice were evaluated. Tissues were then optically cleared with three procedures. For BABB clearing, samples were dehydrated in methanol (MeOH) for 48 h at room temperature and transferred for clearing in BABB (1:2 mixture of benzyl alcohol and benzyl benzoate) for 48 h. For clearing with pBABB, samples were first treated with a solution known as Dent's bleach, which comprises a 4:1:1 combination of MeOH, dimethyl sulfoxide (DMSO), and hydrogen peroxide. This solution was applied prior to dehydration with MeOH and clearing with BABB. Dent's bleach is widely used for whole-mount immunohistochemistry, where hydrogen peroxide is important to block endogenous peroxidase activity and DMSO to enhance tissue penetration,<sup>9</sup> but to the authors' knowledge, it has not previously been used for optical clearing. The samples cleared with CLARITY were prepared according to the passive

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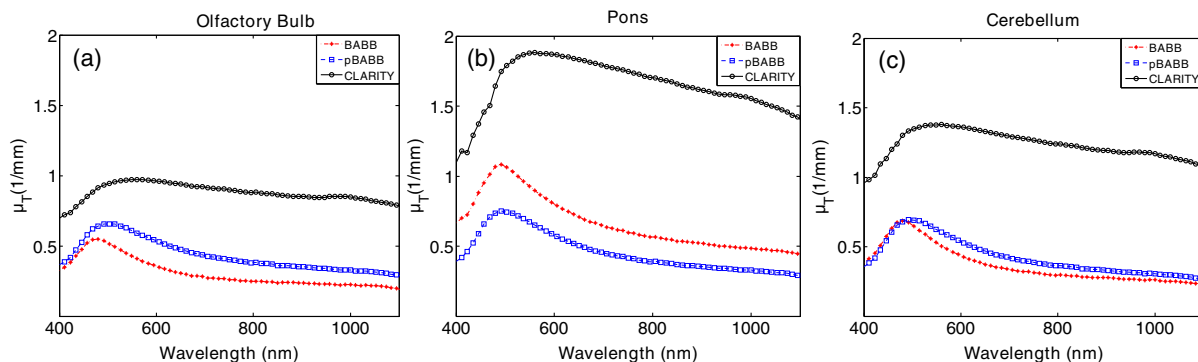
**Fig. 1** Images of 2 mm mouse brain slices before and after optical clearing. The brains, originally opaque (a), were optically cleared with (b) BABB, (c) pBABB, and (d) CLARITY to achieve tissue transparency. The areas where spectroscopic light attenuation measurements were performed are shown in (a). The images have the same spatial scale.

protocol,<sup>7</sup> which aims to remove lipids by passive thermal clearing (37°C) and preserve tissue structure by hydrogel embedding.

Light transmission spectra were acquired with a custom-made spectroscopic system in three areas of the brain: pons, cerebellum, and olfactory bulb (Fig. 1).

The light emitted by a halogen lamp was coupled to a 200- $\mu\text{m}$  fiber and collimated to a 3-mm-diameter beam that illuminated the brain slice. The cleared sample was sandwiched between two glass cover slips and placed in a custom-designed, 3-D printed sample holder with 2-mm spacing. Care was needed when handling the samples cleared with CLARITY, since these tissues lose their rigidity after the clearing process. To allow for regions within the sample to be selectively illuminated by the beam, the holder was placed onto two linear translation stages. Transmitted light was fiber coupled and directed to a spectrometer (MayaPro, Ocean Optics, Duiven, The Netherlands). Since light scattering and absorption are wavelength dependent,<sup>10</sup> data were acquired with the spectrometer in the wavelength range of 400 to 1100 nm. Background and reference spectra were taken with no sample in the holder and with BABB, respectively. Post-processing of the data was performed with MATLAB. The attenuation coefficient  $\mu_T$  of each brain tissue slice with thickness  $x$  (mm) was estimated using Eq. (1):

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



**Fig. 2** Light attenuation spectra acquired in three brain regions show the comparison of the tissue transparency degree obtained with three clearing techniques. The spectra were calculated with Eq. (1) and averaged over all measured brains.

where  $I$ ,  $I_0$ , and  $I_b$  are the spectra acquired with light transmission through the sample, with light through the reference solution (BABB), and in the absence of light (the dark signal), respectively, and  $\lambda$  is the wavelength.

### 3 Results and Discussion

Transmission spectra acquired in the brain samples showed that the efficacy of optical clearing varied depending on the clearing technique and tissue region (Fig. 2). Moreover, samples cleared with pBABB and BABB resulted in lower attenuation coefficients than those cleared with CLARITY (Table 1). For instance, the mean attenuation coefficients in the pons were  $1.09 \pm 0.28$  and  $0.75 \pm 0.10 \text{ mm}^{-1}$  for BABB and pBABB, respectively; they were  $1.89 \pm 0.19 \text{ mm}^{-1}$  for CLARITY. Furthermore, the shapes of the spectra acquired from CLARITY samples differed prominently from that of brains cleared with BABB and pBABB, with the attenuation peak occurring at a higher wavelength than for BABB-cleared samples (480 nm for BABB, compared with 560 nm for CLARITY in the pons). The dominant contribution to the errors (Table 1) was intersample variability.

The data presented in this study show that light attenuation in cleared brain tissue was greatest in samples cleared with passive CLARITY, and lowest in those cleared with pBABB or BABB. The significantly lower absorption by BABB samples at higher wavelengths ( $>600 \text{ nm}$ ) could perhaps be exploited for the optimization of imaging of fluorophores with emission at such wavelengths. The resulting absorption coefficients relative to BABB- and pBABB-cleared samples present a considerable disparity when compared with the ones relative to CLARITY-cleared samples.

Structural tissue modifications such as dehydration or lipid extraction lead to tissue swelling or shrinkage. Evaluation of the change in the size of brain samples was assessed from tissue cross-sectional areas before and after clearing. Measurements showed a decrease in size with BABB and pBABB clearing (58% and 56%, respectively), most likely caused by the dehydration step, and a large increase in size was found with passive CLARITY (229%). Regarding the swelling induced by the CLARITY protocol, care is needed in the hydrogel preparation step and in the incubation temperature of the sample for hydrogel polymerization (37°C). The same degree of shrinkage and expansion was observed in cleared whole brains (data not shown). Both of the size changes caused by the examined techniques could be confounding factors in morphological studies

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

Region	Technique	$\lambda_{\text{MAX}}$ (nm)	$\mu_{\text{T}}$ ( $\lambda_{\text{MAX}}$ ) (mm <sup>-1</sup> )
Olfactory bulb	BABB	476	0.55 $\pm$ 0.23
	pBABB	497	0.66 $\pm$ 0.12
	CLARITY	558	0.97 $\pm$ 0.11
Pons	BABB	487	1.09 $\pm$ 0.28
	pBABB	497	0.75 $\pm$ 0.10
	CLARITY	553	1.89 $\pm$ 0.19
Cerebellum	BABB	482	0.68 $\pm$ 0.29
	pBABB	497	0.70 $\pm$ 0.09
	CLARITY	549	1.38 $\pm$ 0.22

and should be taken into account when choosing a clearing approach for a particular application.

Of the three brain regions investigated, the pons showed the lowest clearing efficacy, most likely due to its greater gray matter density. Gray matter contains large quantities of myelin, a lipid-based substance that contributes significantly to optical scattering.<sup>10,11</sup> In this study, 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. Moreover, DMSO acts as a solvent increasing the clearing solution penetration without damaging the tissue.<sup>12</sup> This methodology is also particularly advantageous as it is straightforward and inexpensive, and the clearing times are considerably shorter when compared with techniques such as CLARITY. However, BABB, as with most of the techniques that make use of organic solvents for refractive index matching, has the main disadvantage of decreasing the half-life of the fluorescent signal,<sup>13</sup> most probably due to peroxide contamination.<sup>14</sup> Despite the highest degree of tissue clearing which can be obtained with pBABB, this approach is only applicable to autofluorescence studies to look at tissue morphology, due to the bleaching step with hydrogen peroxide. Conversely, the CLARITY protocol produces minimal protein loss and preserves native fluorescence.<sup>5</sup> The fluorescence quenching that occurs with each clearing agent is an important factor that needs to be taken into account when choosing a clearing approach.<sup>15</sup>

#### 4 Conclusion

This study demonstrated a novel method to quantify the efficacy of optical clearing protocols, which is an essential step in the process of choosing a clearing approach for a specific application and in the stage of imaging data evaluation. 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 600 nm. It is also been

shown that the level of optical clearing varies within the brain, most likely due to the structure and composition of each particular area. The study focused on three optical clearing techniques and on optical clearing of brain, but could easily be applied to any other method or organ. This work could help guide the choice of the most suitable optical clearing method for a specific application. Furthermore, assessing the transparency of a tissue is particularly important when imaging cleared samples with systems like optical projection tomography or light sheet microscopy, as reduced light scattering can lead to higher spatial resolution and greater contrast.<sup>16</sup>

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