On-line optical clearing method for whole-brain imaging in mice

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Abstract: The combination of optical clearing with light microscopy has a number of applications in the whole-brain imaging of mice. However, the initial processing time of optical clearing is time consuming, and the protocol is complicated. We propose a novel method based on on-line optical clearing. Agarose-embedded mouse brain was immersed in the optical clearing reagent, and clearing of the brain was achieved ~100 μm beneath the sample surface. After imaging, the cleared layer was removed, thereby allowing layer-by-layer clearing and imaging. No pre-immersion was required, and we demonstrated that on-line optical clearing can reduce the whole-brain imaging time by half.

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1. Introduction

Although single neurons are the fundamental elements of the nervous system, studying individual neurons does not allow us to understand how the brain works [1]. Indeed, numerous studies have shown that neural networks are the basis of behavior and cognition, with some complex behaviors requiring the connection of neural networks across the whole brain [2]. As such, an interconnection diagram of the whole brain is of particular interest in neuroscience [3]. To date, various imaging approaches have been introduced to obtain such a diagram. For example, optical microscopy has received a considerable attention as its rich intrinsic and extrinsic optical contrast reveals specific neurite projections and neurons of different subtypes [1,4–6]. Additionally, as the diameter of an axon projected from the soma is ≤1 μm [7], a spatial resolution of hundreds of nanometers is required to visualize it [8]; optical microscopy can fulfill this requirement [9]. However, the high scattering of biological tissue remains an obstacle in the imaging of thick samples with optical microscopy [10–12]. Light rapidly attenuates and diffuses in biological tissue, thereby degrading the contrast and spatial resolution of the optical images [13].

A number of optical clearing approaches have recently been developed to render brain tissue optically clear, which then allows for light to pass through thick samples with little diffusion. For example, Scale [14], SeeDB [15], 3DISCO [16], CUBIC [17], and their variants [18–23] are based on the immersion of specimens in a solution in which the gradual penetration of the liquid matches the tissue’s refractive index. Although benzyl alcohol-benzyl benzoate has been employed as an organic solvent to clear biological specimens, it reduces the stability of the signal from fluorescent proteins [24,25]. In contrast, amines and sugar alcohols have been utilized as aqueous solvents to maintain fluorescent signals, but the extent of optical clearing remains limited in these solvent [14,15,25]. However, CLARITY [25], SWITCH [26], and their variants [27] use electrophoresis to remove substances with high refractive indices from tissue. This harsh treatment requires a tissue-gel hybridization step, and the tissue electrophoresis process itself is difficult [25,26]. In both of these cases, the sample is cleared in the early stage to match the refractive index, and optical microscopy...
is subsequently employed to obtain the data set. However, the processing time in the early stage becomes longer as the sample thickness increases, and the protocol becomes increasingly complicated. Moreover, the imaging quality gradually deteriorates with increasing depth [28].

One potential solution is combining optical microscopy with ultrathin sectioning, as cutting the imaged sample surface effectively prevents reduced image quality [29]. However, continuous sectioning is time-consuming and challenging, and it is necessary to optimize the sample to exhibit sufficient mechanical strength. In micro-optical sectioning tomography (MOST) and array tomography, the samples must be embedded in resin prior to imaging [3,30,31]. Additionally, the majority of treated samples are cut using sharp diamond knives, which are expensive, and led to cutting samples into stripes using cheaper small diamond knives, which is also time-consuming. Therefore, combining of optical clearing with thin sectioning and optical microscopy is expected to address the above issues, although an additional optical clearing stage is required.

Thus, we herein report the development of a novel method of whole-brain imaging in mice based on on-line optical clearing. More specifically, the agarose-embedded mouse brain is immersed in an optical clearing reagent with predicted brain tissue clearing as deep as ~100 μm beneath the sample surface. The cleared layer is imaged and removed by a vibratome, which allows for layer-by-layer clearing and imaging of the whole brain. We propose that complete mouse brain data will be efficiently obtained through parallel imaging, sectioning, and clearing, with little early stage preparation in the early stage.

2. Materials and methods

A schematic diagram of our proposed on-line optical clearing method is shown in Fig. 1. As indicated, the sample is immersed in the clearing reagent. During tissue sectioning with a vibratome, the solution clears the surface of the remaining sample. Thus, upon the completion of sample sectioning, the new sample surface has already been cleared. Data acquisition in the depth direction is performed using our previously reported wide-field large-volume tomography (WVT) system [29]. The WVT system employs structured illumination microscopy (SIM) to accelerate imaging acquisition at a voxel resolution of 0.32 × 0.32 × 2 μm³ in a coronal plane. Its optical sectioning capability is comparable to that of confocal microscopy at the sample surface and circumvents low-throughput point scanning [29]. Clearing biological tissue reduces light scattering and allows high stripe contrast when imaging deeply. The images are saved with their spatial position information [3]. After imaging, the vibratome removes the imaged part of the sample [32], and this process is repeated until whole-brain imaging is complete.
Fig. 1. (a) Schematic of the proposed on-line optical clearing method used for light microscopic imaging of the whole brain. In the on-line optical clearing method, the sample is immersed in the optical clearing solution, and clearing of the sample surface during sectioning. (b) Schematic diagram of serpentine scanning to obtain a complete coronal view and imaging of a thicker layer in the depth direction.

Selecting an appropriate reagent for on-line clearing is of particular importance to obtain whole-brain data sets. Thus we carefully screened existing clearing reagents based on the following three principles: (1) the sample rapidly becomes transparent in the clearing solution, which facilitates sample clearing during tissue sectioning; (2) the clearing reagent does not quench fluorescent proteins; and (3) deformation induced by the clearing reagent is minimized to ensure data continuity.

2.1 Reagents for fast sample clearing

We initially compared the rapid sample clearing capabilities of four water-soluble reagents. In general, increased concentrations of sorbitol, sucrose, and fructose resulted in higher refractive indices. Therefore, we selected saturated solutions in addition to pure glycerol. To demonstrate the rapid clearing capabilities of these reagents, 500-μm thick, Thy1-GFP mouse brain slices were placed on the grid (1 mm), and the brain slices images were acquired before and after immersion. We found that the clearing speed of fructose was superior to that of glycerol, sorbitol, and sucrose, as shown in Fig. 2(a). The animal experiments were approved by the Institutional Animal Ethics Committee of Huazhong University of Science and Technology, and all experiments were conducted in accordance with relevant guidelines and regulations.
We selected an 80% (wt vol⁻¹) fructose + 4 mol l⁻¹ (M; 24% wt vol⁻¹) urea and 0.01 M phosphate-buffered saline (PBS, Sigma-Aldrich, USA) as our clearing reagent. Urea was expected to ameliorate the clearing ability and increase the refractive index [21]. We compared the refractive indices using an Abbe refractometer (WAY-2S, Shanghai YiCe Apparatus & Equipment Co., China), and measured the viscosity using a viscosity detector (NDJ-5S, LICHEN, China). As shown in Figs. 2(b) and 2(c), urea increases the refractive index but does not influence the viscosity of the solution. To verify its clearing capability, we measured the relative transmission rise of a 100 μm brain slice before and after optical clearing using an optical power meter (Nova II, Ophir Optronics, Israel) at 491 nm (Fig. 2(d)). We found that after clearing for 1 min, the relative transmission was improved by 35%.
For imaging, the key parameter in on-line clearing is the thickness that can be reached. Thus, we measured the imaging contrast of the brain sample using our previously developed WVT system at different depths before and after clearing [29]. For this purpose, after anesthesia, the mice (Thy1-GFP) were perfused with 0.01 M PBS and 4% PFA. The brain was then removed, fixed with 4% PFA for 24 h, and washed with PBS for 12 h. Following oxidation of a 5% agarose solution with sodium periodate (Sigma-Aldrich, USA), the brain was embedded in the agarose, placed in a 55 °C water bath, and removed after the natural coagulation of agarose was observed. The brain sample was initially scanned to a depth of 100 μm beneath the surface for continuous imaging in the PBS solution. After replacing the solution with our optical clearing reagent, we repeated with the same exposure and at the same position. The results are shown in Fig. 3, where parts (a)–(f) and (g)–(l) are displayed at intervals of 20 μm before and after clearing, respectively. In the case of on-line clearing, cell signals can be detected 100 μm beneath the surface (Fig. 3(l)); these signals are not detected without clearing as shown in Fig. 3(f). As the root mean square value is a common indicator of image quality [33], we calculated the root mean square values of the images and obtained a corresponding curve for root mean square attenuation with depth by Gaussian fitting. As shown in Fig. 3(m), the image quality for on-line clearing decreased less with increased depth compared to the no clearing samples. Between Figs. 3(g) and 3(l), which were acquired 100 μm apart, the root mean square value decreased by ~15%.

2.2 Influence of the clearing reagent on fluorescent proteins

We then compared fluorescence intensities before and after clearing. For this purpose, the mouse brain (Thy1-GFP) was treated as described above prior to slicing (100 μm depth) using a vibrating slicer (Leica VT1000 S, Germany). The slices were collected, attached to slides, then imaged by confocal microscopy (Zeiss, Germany) before and after clearing with an 80% fructose + 4 M urea and 0.01 M PBS solution for 1 min under the above conditions. The imaging depth was ~15 μm below the surface. As shown in Fig. 4, no weakening of the fluorescence signal was observed following sample treatment using our optical clearing.
reagents. As the degree of transparency increased in the samples, scattering was reduced, damage of the excitation light was reduced, and the emitted light of the neuronal GFP protein scattered less. Moreover, the clearing reagent caused the sample to shrink in all directions, so the detected signal intensity was stronger.

2.3 Control of sample deformation to ensure data continuity

The majority of clearing reagents introduce deformation to a sample; thus, we chose to embed the sample in agarose to both prevent deformation [32] and provide support while cutting prior to sample impression. During imaging, redundant information was employed for data registration, and the images were segmented using the threshold method [34]. All cells had a diameter of ~5–20 µm, and the pixel size was 0.32 × 0.32 µm for the 20 × objective (Olympus UPLSAPO 20XO), giving each cell an area of ~190–3000 pixels. The center of mass in each segmented cell was used to estimate the position, and the position was stored in k-d tree format to improve inquiry [34]. A nearest-neighbor search was employed to establish cell correspondence [34], from which a 3D displacement field was formed by fitting a cubic surface of the point cloud using the RegularizeData3d package by MATLAB [35]. Figure 5(d) shows a merged map of the foreground color of Fig. 5(b) (redundant area before sectioning) set to red and the foreground color of Fig. 5(c) (corrected after sectioning) set to green. Yellow indicates effective merging. Using these point clouds, the tiles were registered. Subsequently, the medial-lateral, dorsal-ventral, and anterior-posterior axis displacements from the upper layer to the next layer were calculated before and after cutting in Figs. 5(h), 5(i), and 5(j). The data were averaged from four sets of mouse brain (Thy1-GFP) samples. We found that the on-line clearing solution caused the sample to shrink in all directions, particularly in the depth direction. Due to the limited field view in the high numerical aperture objective lens, the coronal plane of intact mouse brain could not be entirely imaged in one time. In the actual process, mosaic stitching will be used. Usually, the deformation caused by clearing will lead to the missing or misplaced data. This indicates that data redundancy is necessary to ensure data continuity. In our system, the redundant information is approximately 25 µm in the coronal plane of adjacent mosaics and 30 µm in depth, respectively.
3. On-line imaging of the whole brain

To demonstrate the ability of on-line optical clearing technique used for light microscopy to obtain whole-brain architecture, we integrated the on-line optical clearing technique into WVT to obtain a data set for an adult (8-week-old) mouse brain (Thy1-GFP). The mouse brain was processed as described above. The sample was then treated with the clearing reagent. During tissue sectioning with a vibratome [33], the 80% fructose + 4 M urea and 0.01 M PBS solution cleared the surface of the remaining sample. In our WVT system, a UPLSAPO 20XO objective (Olympus, Japan) was used with an imaging depth of 80 μm and a cutting depth of 50 μm. For data registration, a 30-μm depth of redundant information was employed. The additional redundant data account for approximately 23% of the total data. This system therefore allowed the whole brain to be cleared and imaged layer by layer. Image preprocessing was implemented in C++ after obtaining the whole-brain data. Based on the sequence of images in space and the overlaps, we stitched mosaics of each coronal section to obtain an entire section [29]. The data obtained for the mouse brain were then down sampled to 10 μm × 10 μm × 10 μm and loaded into Amira software (v 5.3.2, FEI, France) to rebuild the sample based on the sliced sequence. We use the T7600 workstation (two Intel E5-2687w CPUs, 256GB memory and an Nvidia K6000 graphics card, Dell Inc., USA). It required approximately 12 hours to reconstruct the mouse brain data. Thus, Fig. 6 shows a complete data set for the mouse brain. Indeed, we found that on-line optical clearing was easily applied to WVT to obtain the whole mouse brain data set. Additionally, immersion of the mouse brain in the optical clearing reagent cleared the surface of the remaining sample during tissue sectioning. This indicates that the complete mouse brain data set was efficiently obtained through parallel imaging, sectioning, and clearing.
We also found that the on-line clearing technique reduces tissue scattering to achieve axial scanning beneath the sample surface. Indeed, we compared the various imaging times required both with and without clearing under the same conditions (Table 1). From these results, it is apparent that the on-line clearing technique enables one-time imaging of a thicker layer in the depth direction, which reduces the number of layers cut and shortens the overall process. Additionally, objective lens scanning with PZT (30 ms) for the axial image was significantly faster than stage scanning (150 ms) for mosaic imaging. Considering all factors outlined in the table, the on-line clearing technique clearly improved the efficiency of the WVT system.

| Table 1. Times required both with and without clearing |
|-------------------------------------------------------|
| Without clearing | With on-line clearing |
|------------------|-----------------------|
| Imaging depth    | 10 μm                 | 80 μm                 |
| Sectioning thickness | 10 μm          | 50 μm                 |
| Stage scanning time | 150 ms           | 150 ms               |
| PZT scanning time   | 30 ms                | 30 ms                |
| Exposure time      | 10 ms                | 10 ms                |
| Sectioning speed   | 20 mm/min            | 20 mm/min            |
| Imaging time/working time | 30%          | 67%                  |
| Mouse brain acquisition time | 3 d         | 1.5 d               |

4. Discussion and conclusion

The combination of optical clearing and light microscopy has numerous applications in whole-brain imaging of mice. In the case of high-speed volumetric serial two-photon (STP) tomography [35], a clearing medium comprised of dimethyl sulfoxide, D-sorbitol, and aqueous buffer was employed to obtained the mouse brain data set. Additionally, for oblique light-sheet tomography [36], the CUBIC clearing protocol was modified for light-sheet microscopy with vibratome sectioning. However, in both cases, sample clearing was required...
for over one week prior to imaging. In contrast, no sample clearing was required in advance for our on-line optical clearing technique, thereby reducing the labor cost attributed to sample processing.

Our system employed structured illumination microscopy to obtain the whole-brain data from mice in 1.5 days at a voxel resolution of $0.32 \times 0.32 \times 2 \mu m^3$. We compared the scanning time of other whole-brain imaging techniques that use a clearing method. In the oblique light-sheet tomography, the whole mouse brain data set was acquired at $0.75 \times 0.75 \times 2.5 \mu m^3$ resolution in approximately 14 hrs. The imaging speed of our system is slower than that of a light-sheet microscope [36], but the resolution of light-sheet microscope is limited because it lacks objectives with both long working distance and high numerical aperture [8,36]. In volumetric two-photon tomography, the point scanning method took 7 days to acquire the whole-brain data set at a voxel resolution of $0.3 \times 0.3 \times 1 \mu m^3$ [35]. Our clearing strategy is directed to the sample surface. There is indeed a photobleaching effect on the next layer, but little light can reach the deep tissue. The piezoelectric scanner rapidly completes the axial scanning, and then the program automatically moves to the next position. Each mosaic requires approximately 1 s. As shown in Fig. 3, the cellular signal intensity did not decrease significantly in continuous axial scanning. According to the root mean square values of the images, it decreased by ~15%. The effect of photobleaching on tissue is not serious.

Additionally, the Scale [14], SeeDB [15], and CUBIC [17] protocols employ gradient solutions to gradually clear samples, while CLARITY [25] and SWITCH [26] require electrophoresis to remove substances of high refractive indices from the tissue. Although soaking in water-soluble small molecules is less efficient than the above-mentioned methods, a completely clear brain sample is not required due to the use of a vibratome. More importantly, our on-line clearing technique does not require significant manual operations or additional clearing times, thereby creating a suitable balance between the degree of transparency and convenience.

In our proposed system, the oil objective lens (UPLSAPO 20XO) performs better in the refractive index medium of 1.518; the refractive index of fructose urea solution is ~1.48. A mismatch in the refractive indices may introduce spherical aberration into the system, thereby reducing the final system resolution [22]. Thus, using a solution with a matching refractive index is expected to further improve both the on-line clearing performance and the optical resolution [23]. We also note that the working distance of the current objective lens is 0.17 mm. With further improvements in the manufacturing of objective lenses, the imaging of thicker tissue samples will soon become possible, thereby further improving the efficiency of the system through reduced sectioning and stage positioning. Although we did not demonstrate this application in large animals, it may be much more useful in those scenarios. Optically clearing a whole macaque brain is challenging, since its volume is hundreds of times larger than a mouse brain. It is more feasible to only clear the superficial layer of brain tissue. We will focus on this project in future work.

In summary, we successfully developed a novel imaging method based on on-line clearing for whole-brain imaging in mice. We found that sample clearing was not required in the early stage, as the on-line clearing solution rapidly cleared the sample surface in the time required to remove the previously imaged layer. As such, a complete data set was efficiently obtained through parallel imaging, sectioning, and clearing. Combining this technique with light microscopy successfully yielded a whole-brain data set. Finally, we successfully integrated the on-line clearing technique into WVT and found that the system efficiency doubled.

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Disclosures

The authors declare that there are no conflicts of interest related to this article.

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