Original Research

Quantitative study on the hygroscopic expansion of spurr resin to obtain a high-resolution atlas of the mouse brain

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Abstract

Ultra-thin section-based microscopic imaging is considered one of the most realistic techniques for determining fine architectures of a brain-wide neural network. In this kind of method, the sample is usually embedded in resin and then immersed in water for sectioning and imaging. The effect of resin hygroscopic expansion on data accuracy and integrity is important as it may lead to inconsistent image qualities or degeneration of sectioning properties. But few studies have been conducted on this issue. Here, we have used surface profile measurements combined with sectioning and imaging by micro-optical sectioning tomography (MOST) to quantitatively study the sectioned surface expansion of spurr resin blocks as a result of water immersion for a short time period. The expansion effect on MOST imaging is also presented. The results revealed significant differences in the surface expansion of pure resin blocks with different immersion time durations ($P < 0.001$). During an eight-minute immersion, the surface expansion of the experimental specimens exhibited an approximately linear increase with immersion duration, while MOST images suffered a correlated decrease in brightness. Expansion was restricted to the submicron level with immersion duration of four minutes or less, and the mean and standard deviation of the expansion measurements both reached a maximum at eight minutes. When the immersion duration exceeded eight minutes, the expansion value decreased, which was most likely related to the degeneration of mechanical properties of the resin material on the block surface. This study indicates that it is necessary to select a specific sectioning mode according to the hygroscopic expansion properties of resin materials for maintaining the accuracy and integrity of whole brain atlas data.

Keywords: whole brain atlas, hygroscopic expansion, resin, ultra-thin section, short time immersion, surface profile

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Introduction

The architecture of the brain neural network is the basis for understanding brain functions and diseases, and is also an important field of modern neuroscience research. Studies have indicated that brain functions depend not only on short-range transmissions among neighboring neurons, but also on long-range transmissions of axons extending over different brain regions.[1] Therefore, in recent years, methods to best observe the fine structures of a large volume or even a brain-wide neural network have become a focus of neuroscience research.[2–5]

The complex neural network is mainly composed of neuronal processes of micron-sized diameters, which makes characterizing such fine structures brain-wide an extremely challenging task. Through years of effort, ultra-thin section-based microscopic imaging is now acknowledged as one of the most realistic techniques for achieving this goal.[1,4] In recent years, several new techniques, using ultra-thin sectioning and imaging of samples embedded in rigid resin, have been developed to characterize large volumes of the neural network at the microscopic level. These techniques include array tomography,[6] automated tape-collecting lathe ultramicrotome,[7] micro-optical sectioning tomography (MOST)[8] and knife-edge scanning microscopy.[9] Small errors in registration or a small quantity of data loss may lead to fibre-tracing interrupts; so in these techniques, maintaining precise registration and data integrity is critical for neural network reconstruction at the neurite or even finer level. Unfortunately, when obtaining many serial sections and high-resolution images from large specimens, these techniques necessitate a number of mosaic pictures that are spliced in both the lateral and axial directions. Thus, data registration and data loss remain the greatest challenges associated with these techniques. Resin materials have

an appropriate hardness for ultra-thin sectioning, and cutting specimens in water can effectively reduce the fold and deformation of sections; therefore, a commonly used method is to embed the tissue sample in resin and then section the resin specimen during a several-minute long immersion in water. For example, if a centimetre-sized specimen block of the whole mouse brain is sectioned and imaged by the MOST system with 450-μm section width and 1-μm section thickness, then each section must be immersed in water for 1–2 min, and the total immersion time duration for the whole specimen block may exceed 200 h.[8] The water absorption of the resin may cause swelling and deformation, leading to inconsistent image qualities or degeneration of sectioning properties and hence data misregistration or losses, so the relationship between the water immersion duration and the hygroscopic expansion of resin should be further explored.

Quantitative analyses of the hygroscopic expansion of resin composites have primarily been performed in the field of dental materials and have indicated that all resin composites absorb water and experience hygroscopic expansion in wet environments owing to their chemical properties.[10] Furthermore, the conventional methods employed in this field include measuring the dimensional changes of millimeter-to-centimeter-sized specimens with a laser micrometer or surface profiler, evaluating water absorption by measuring the mass changes of specimens, and using optical scanning to observe deformation patterns of restored tooth surfaces.[11–14] These studies focused on the long-term water immersion (weeks to months) and global changes in the dimensions and mechanical properties of specimens. Because the resin materials of ultra-thin sections are immersed in water, swelling phenomena caused by water absorption may also occur. However, in studies in which ultra-thin sectioning is used, there rarely are quantitative studies focused on the expansion of the local portion (surface or thin sections) of a resin specimen block immersed in water for only a short time.

We assumed that water immersion would cause a surface expansion of the spurr resin block and affect the imaging, but its effects could be ignored when the immersion time duration is controlled appropriately. This calls for a specific design of reasonable sectioning mode. Based on this view, we designed an experimental method based on surface profile to precisely measure the sectioned surface expansion of spurr resin (widely used for electron microscopy)[15] blocks after only a short-time immersion (minutes), in which the MOST system was used for sectioning and a stylus surface profiler was used for measurements. We also sectioned and imaged the immersed surface by the MOST system to evaluate the immersion effects on the tomography image and atlas acquisition of the whole mouse brain.

Materials and methods

Specimen preparation

We used spurr resin (Ted Pella Inc., Redding, CA, USA),[15] a hydrophobic epoxy resin, to prepare the experimental specimen. For 100% pure spurr resin, 43.8 g was composed of 10 g ERL-4221 (vinylcyclohexene dioxide), 7.6 g DER-736 (diglycidyl ether of polypropylene glycol), 26 g NSA (non-enyl succinic anhydride) and 0.2 g DMAE (dimethylaminoethanol). The pure resin was poured into a silicone mould and then polymerized in an oven at 60°C for 36 h.[8,16] By using this procedure, we prepared pure resin blocks with sizes of 6 mm×8 mm×10 mm, and paraffin wax was applied to the block surface as a waterproofing layer.

Using the same 100% spurr resin under the same polymerization conditions, we then prepared a resin block in which a Golgi–Cox stained mouse brain was embedded. The whole brains used for preparing the tested specimen and MOST formal data acquisition were from Chinese KM mice (about 40 days old). Prior to infiltration and embedding with spurr resin, the mouse brain was processed by a series of procedures, including fixation and impregnation with Golgi–Cox solution, darkening and dehydration.[8,16] All procedures were performed in accordance with the guidelines of the Experimental Animal Ethics Committee of Huazhong University of Science and Technology.

MOST and the specimen sectioning principle

In this paper, all experiments were based on the MOST system for section processing and imaging. Developed by our center, MOST is an automated imaging system used for acquiring a high-resolution atlas of the whole mouse brain. The system integrates two important modules: ultra-thin sectioning and light microscopic imaging (Figure 1a). The basic working principle of MOST is the simultaneous sectioning and reflection imaging of a strip on the knife blade (Figure 1b). The specimen block is immersed in water during the entire process of sectioning and imaging. The system section thickness has a range of 0.5–5 μm, and lateral resolution reaches 0.7 μm.[8]

The strip width in MOST sectioning is usually between 400 and 500 μm (limited by the field of optical imaging). To acquire a whole slice image of centimeter-sized specimens, multiple sectioning steps must be performed on the same slice, as shown in Figures 1b and c. Ideally, the knife blade is positioned parallel with the specimen surface during sectioning. In reality, however, the knife blade is much wider than the strips, necessitating that the knife blade be held at a deflection angle θ with the moving plane of the specimen (X–Y plane), as shown in Figure 1d. Because θ is small, this set-up does not affect the alignment between adjacent strips. The MOST technique only uses one side of the knife blade cutting into the specimen for sectioning and imaging, while the other side, which is turned up, neither sections the specimen nor touches the block surface. In view of the deflection angle θ, sectioning of the specimen block by MOST can be represented with the model in Figure 1e.

In MOST, each sectioned surface has an immersion time duration of the order of minutes. Because the surrounding surface of the block is covered with a waterproof layer, only the top surface of the block has direct contact with water. As shown in Figure 1c, each slice of the specimen is broken into multiple strips by sectioning, and the immersion time duration of each strip lasts from the moment its

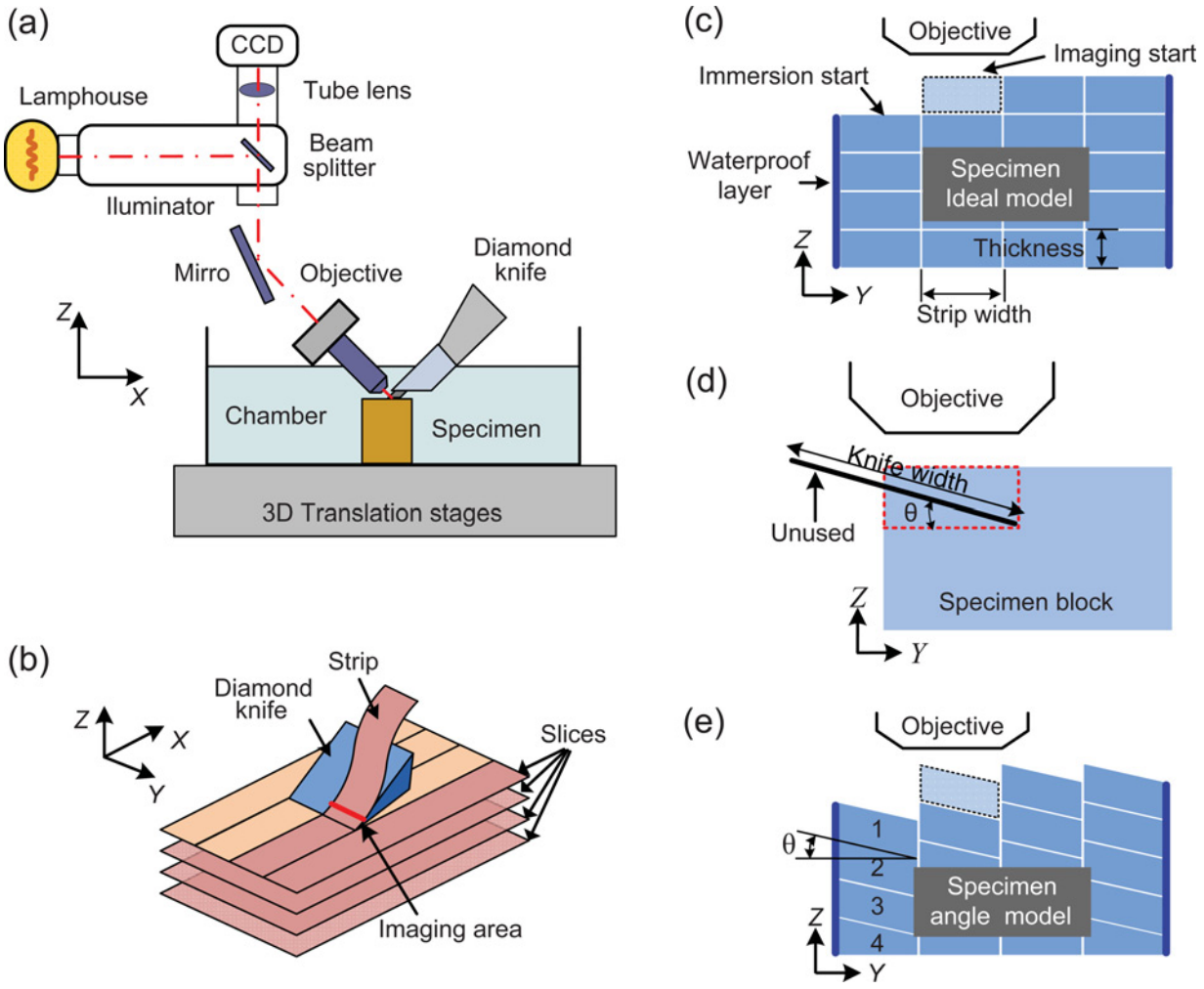

Figure 1 (a) Micro-optical sectioning tomography system construction. Sectioning is performed by the diamond knife and three-dimensional translation stages with high precision; light microscopic imaging is performed by the modified microscope and line-scan charge-coupled device (CCD). (b) Basic principles of sectioning and imaging. The high-precision three-dimensional translation stages control the specimen moving and positioning, and the fixed diamond knife sections the specimen and generates thin strips. Simultaneously with sectioning, the strip on the knife blade is reflection imaged by the microscope and recorded by the line-scan CCD. Usually, a whole slice image of a large specimen (such as mouse brain) is composed of multistrip images. (c) The ideal model of the sectioned specimen block. An immersion starting strip and imaging starting strip are labelled. (d) Use of the knife blade. The dotted box is the ideal shape of the strip. (e) The model considered with the deflection angle θ of the knife blade. For clear display, an exaggerated deflection angle θ is used in (d), (e). (A color version of this figure is available in the online journal)

surface is first exposed to water to the moment it is sectioned. If the strip-by-strip sectioning does not move to the next slice until a whole slice has been sectioned (termed layer-by-layer sectioning), then the immersion time duration of each strip in a given slice is constant, i.e., the immersion time duration of each strip is the same as the time for sectioning the whole slice.

Experimental methods

Surface profile measurements on pure resin blocks

We used a surface profiling method to measure the step height produced by micron-sectioning the surface of a pure spurr resin block. Detailed procedures for this method are shown in Figure 2, including resin block surface processing (Figure 2a) and step height measurement (Figure 2b). The pure spurr resin block was sectioned by MOST in the layer-by-layer mode, with a section thickness of 1 μm and a strip width of 450 μm. Initially, the block surface was sectioned to be flat. Next, a whole slice was sectioned, and then the sectioning was immediately paused once the block was positioned at the next slice location. Hence, the immersion time duration was controlled by the pause time. Next, sectioning was continued, and as soon as a half-slice had been sectioned, the process was stopped and the block was immediately removed from the water for surface profile measurement (Figure 2a). At this point in processing, a step formed at the boundary of the newly and formerly sectioned surfaces. Immersion of the newly sectioned surface on the step boundary was ignored, and the immersion time duration of the formerly sectioned surface on the step boundary (referred to as the immersion duration) was measured as the time required to section a whole slice plus the pause time. We used a stylus surface profiler, a commercial tool of step height measurement with a step resolution of 1 nm (Dektak 150; Veeco Instruments Inc., Tucson, Arizona), to measure the step height with different immersion durations (Figure 2b),

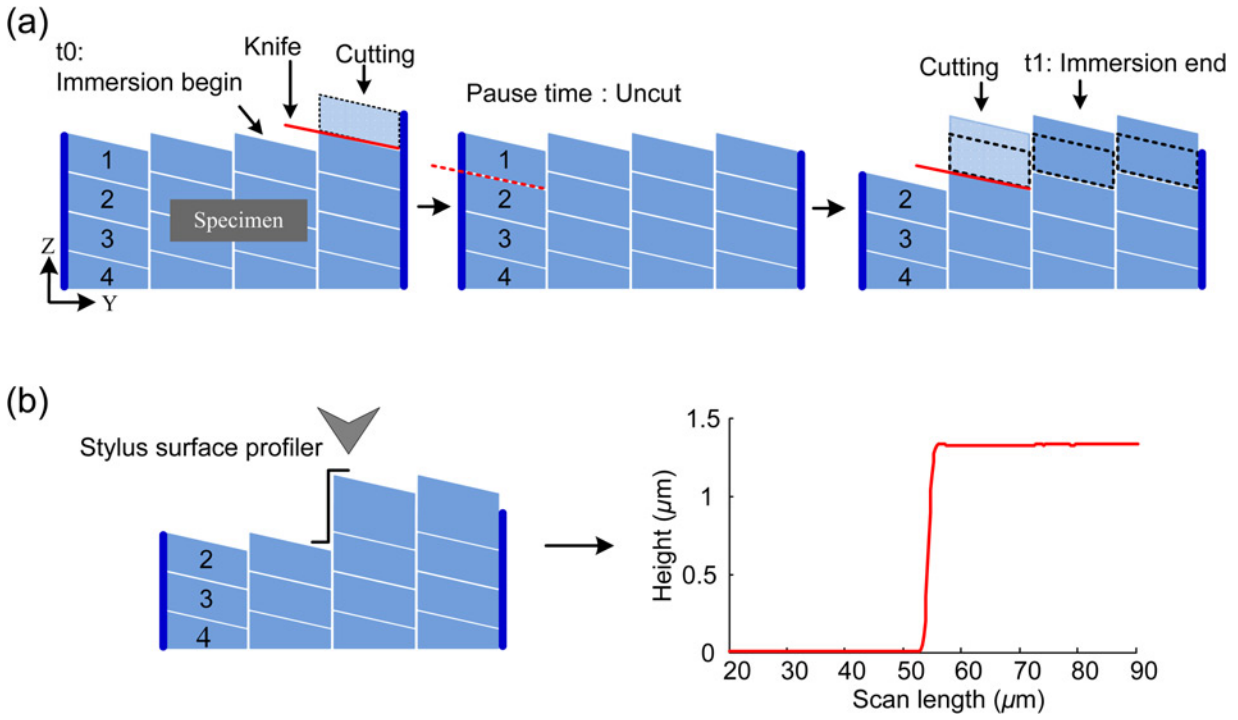

Figure 2 Procedures of the expansion measurement experiment. (a) Processing the block surface and producing steps. The immersion duration is equal to the time for processing a whole slice plus the pause time (i.e. the immersion duration is from t0 to t1). (b) Step height measurement using a stylus surface profiler (left) and an example profile across the step surface (right). Stylus surface profiler is a conventional tool for measuring morphology heights of solid surfaces such as film thicknesses, and usually has a nanometer to angstrom resolution. The profiler used here is capable of measuring steps below 1 nm and provides a step-height repeatability of 0.6 nm. It can measure samples up to 200 mm in diameter and up to 90 mm in height. (A color version of this figure is available in the online journal)

which further allowed us to evaluate the hygroscopic expansion of the resin block surface. The experiment tested seven groups of step heights resulting from immersion durations between 0–12 min, with five pure resin blocks tested in each group. The zero-minute group was established by sectioning the block in a waterless chamber, and in the other groups, immersion was carried out in distilled water. The temperature of the water and the measurement environment were approximately 21–23°C.

Sectioning and imaging in MOST

For the pure resin block and the resin block containing the Golgi–Cox-stained mouse brain, the surfaces with different immersion durations were sectioned and imaged by MOST under the same imaging conditions. For imaging, an immersion objective (40×, 0.8 NA) and a time-delay integration line-scan charge-coupled device were used.[8]

Results

The change in step height of the pure resin block surface with increasing immersion duration is shown in Figure 3. Owing to the deflection angle of the knife blade, the step height of non-immersed specimens produced by 1-μm sectioning was approximately 1.3 μm.

For the pure spurr resin, there were significant differences in surface expansion with different water immersion durations ($P < 0.001$). During the four-minute water immersion, the step height of the experimental specimens (pure resin blocks) increased linearly with the immersion duration, while the expansion value (the increment of immersed step height compared with the unimmersed step height) was a submicron value. For immersion duration between four and eight minutes, the step height increased approximately linearly with increasing duration. At the eight-minute immersion duration, the mean and standard deviation of step heights both reached maximum values. For immersion duration greater than eight minutes, the step height stopped increasing and suddenly decreased instead.

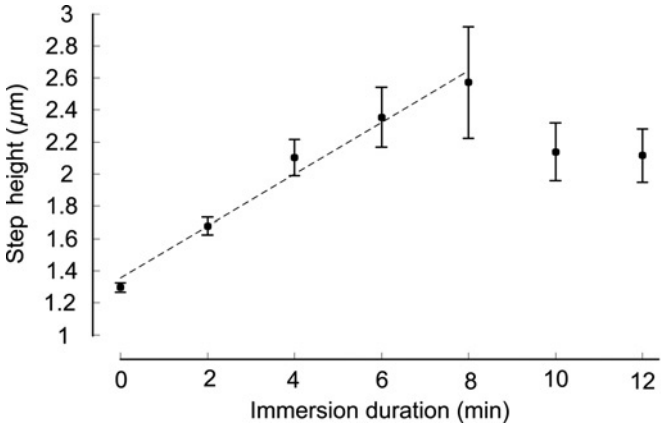

Figure 3 The surface step heights of pure spurr resin blocks immersed in distilled water with different immersion durations

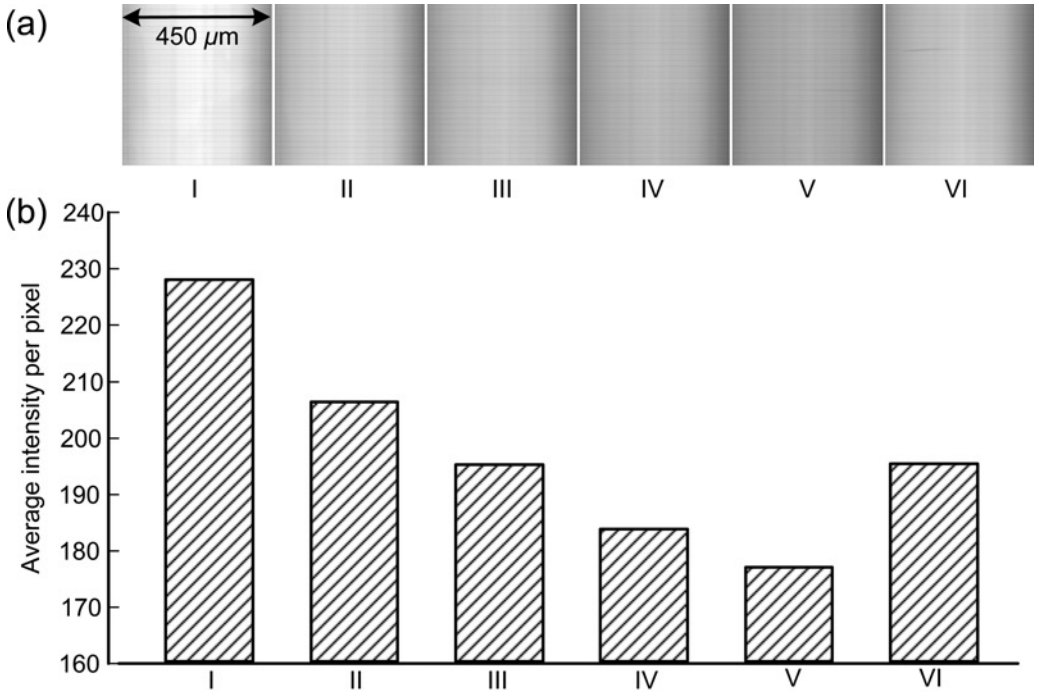

Figure 4 Brightness change in micro-optical sectioning tomography (MOST) images from the pure resin block surface with increasing immersion duration. (a) Six MOST images of a pure resin block surface immersed in water for 45 s (I), two minutes (II), four minutes (III), six minutes (IV), eight minutes (V) and 10 min (VI), respectively. These pictures, respectively, show a part of a 450-μm-wide strip and are small images sampled from raw images. (b) The average intensity per pixel of raw images of the pictures in (a)

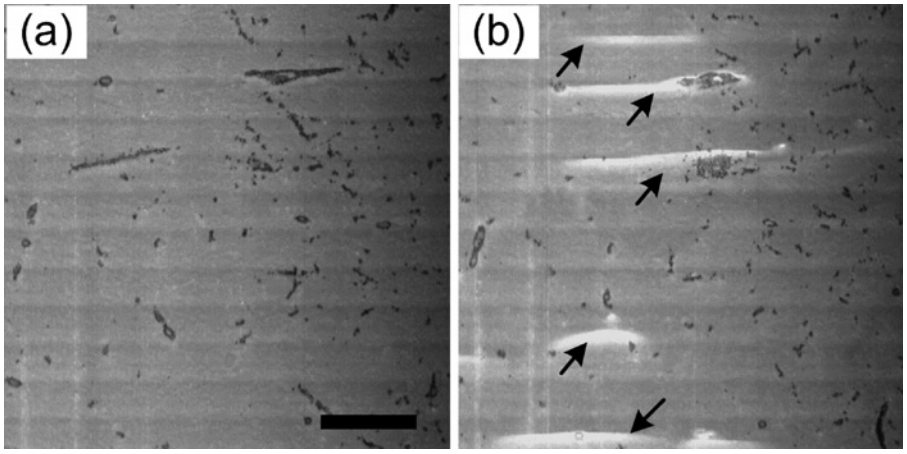

Figure 5 Micro-optical sectioning tomography imaging comparison between six-minute immersion strip (a) and 10-min immersion strip (b) from the resin specimen of Golgi-Cox stained mouse brain. No processing has been applied to the two images, except unified contrast enhancement for visible effects. Scale bar = 45 μm. The arrows indicate the chatter that appeared in the image of the strip under 10-min immersion

The hygroscopic expansion of the pure resin block surface was also measured by observing the MOST image brightness. Figure 4 shows the imaging results obtained under the same experimental conditions with different immersion durations. At immersion duration up to eight minutes, the image brightness decreased with immersion duration because of the increase in strip thickness. When immersion duration exceeded eight minutes, however, the brightness instead began to increase, which correlated with the step measurement results.

Although in Figures 3 and 4, the expansion trend reversed at the eight-minute immersion duration, a longer immersion duration did not reduce the effects of hygroscopic expansion on imaging. Rather, the expansion reversal could be followed by a degeneration of the sectioning properties of the block surface. Figure 5 shows images of the resin block containing Golgi-Cox-stained mouse brain tissues, which are located in the same position on two slices. The block surface with a six-minute immersion duration was sectioned smoothly and imaged in high quality (Figure 5a). With a 10-min immersion duration, however, the section quality significantly degenerated, and chatter appeared as a result of strip wrinkling or jitter, as shown by obvious light spots in the image

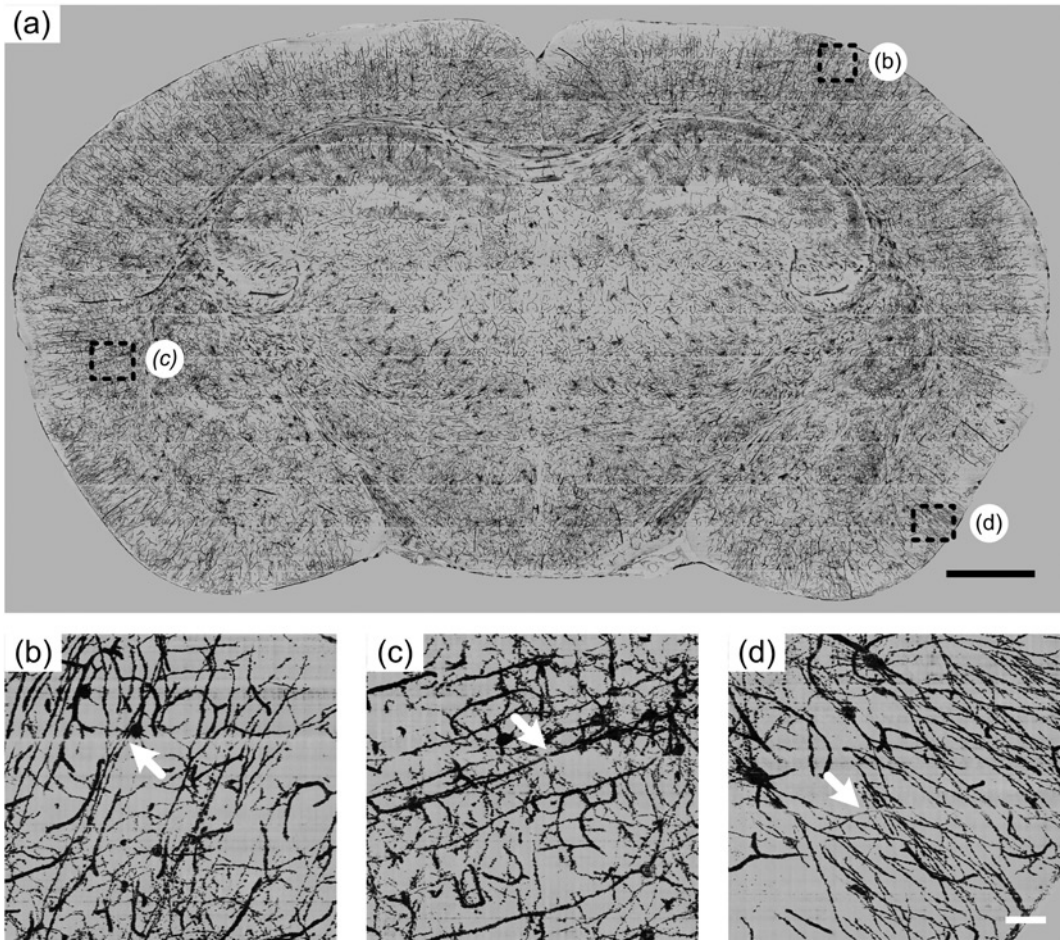

Figure 6 (a) A coronal plane acquired by micro-optical sectioning tomography from a specimen of Golgi–Cox stained mouse brain, corresponding to the plane of interaural 1.98 mm in the mouse atlas, which is a sampled image of minimal projection of serial 30-layer coronal slices, scale bar = 1 mm. (b), (c), (d) Show three different details of the region inside the dashed rectangles. Arrows indicate the boundaries between neighboring strips, scale bar = 50 μm

(Figure 5b), which further contributed to significant information losses.

From these data, we found that the hygroscopic expansion of spurr resin affected the image qualities and the degree of expansion as well as its effects was related to the water immersion duration. Additionally, we can clearly conclude the following: when sectioning and imaging a spurr resin specimen, the immersion duration of each sectioned strip should be both constant and less than four minutes for data accuracy and integrity, which require a proper scheme for sectioning control. To avoid non-uniform expansion deformation caused by different immersion durations, the MOST system adopts the simple layer-by-layer sectioning mode to ensure that all strips in the same slice are exposed to water for the same immersion duration. Furthermore, by adjusting parameters such as sectioning speed and strip width, the immersion duration of each slice can be strictly controlled to avoid data misregistration and data loss. This sectioning control enables MOST to achieve the uniform data acquisition of large specimens with high qualities. Figure 6a shows a minimal projection image of the whole coronal plane from a dataset of Golgi-Cox-stained whole mouse brain acquired by MOST.[8] The coronal plane was in the widest part of the mouse brain (corresponding to the plane of interaural 1.98 mm in the mouse atlas)[17] and complete imaging of the whole slice took approximately 1.5 min. There is almost no dislocation or break down of neuronal processes found throughout the whole plane. Figures 6b–d show three different details in the plane, and they all retain clear tissue morphology without chatter. Furthermore, neuronal processes display good continuity across the boundary between neighboring strips, which is consistent with the accurate tracing of neuronal processes.

Discussion

In the technique of ultra-thin section-based microscopic imaging, the hygroscopic expansion of resin may affect data accuracy and integrity, but these effects have rarely been studied. In this paper, we designed an experimental method based on the microtome and surface profiler to precisely measure the surface expansion of resin blocks caused by a short time immersion. Using this method, we evaluated the sectioned surface expansion of pure spurr resin blocks with different water immersion durations. Additionally, the pure spurr resin block and resin block containing

mouse brain tissues were sectioned and imaged by the MOST system to directly compare sectioning and imaging qualities among specimens with different immersion durations.

The experimental results show that the key to maintaining accurate registration and data integrity is to avoid non-uniform expansion deformation and degeneration of section properties, and these two factors are both related to water immersion duration. The differences in surface expansion values of the pure spurr resin blocks with different immersion durations are significant ($P < 0.001$). Figure 3 shows that the surface expansion increased approximately linearly with time up to eight minutes of water immersion. Figure 4 shows that sectioned strips from the immersed surface had different image brightnesses along with different immersion durations, which corresponds to the brightness change of the image background during actual specimen imaging. According to the combined analysis of the results in Figures 3 and 4, if sectioned strips are subjected to similar immersion durations, then differences in strip expansion and image background brightness may be relatively small, which further facilitates the acquisition of high-quality images with easy registration and good uniformity. Figure 3 further indicates that the surface expansion was limited to the submicron level during four-minute immersion, implying that this is a critical threshold for acquiring fine structure images with high qualities. When the immersion duration is between four and eight minutes, the surface expansion reaches the 1-μm level, and the image background brightness becomes relatively weak, which may enhance difficulties in image segmentation and identification. Despite this, in submicron resolution imaging and under the condition of uniform expansion, the process tracing can still be achieved by the image processing techniques. However, when the immersion duration reaches or exceeds eight minutes, the block surface tends to shrink rather than expand. This transition may be related to the degeneration of the mechanical properties of the block surface. It is presumed that substantial water absorption weakens the resin matrix and induces hydrolytic degradation,[10,18] thus leading to the chatter shown in Figure 5. In this case, the resin block surface is not suitable for thin sectioning and MOST imaging.

Based on the above results, it is necessary to design or select suitable sectioning modes according to the hygroscopic expansion properties of the resin materials for the implementation of ultra-thin section-based microscopic imaging. Specific to sectioning and imaging the spurr resin blocks by MOST, the immersion duration of each strip should be limited to four minutes or less and held as constant as possible during the sectioning and imaging. In consideration of this requirement, the MOST system uses a sectioning control scheme to obtain a high-resolution atlas of the mouse brain: (1) a simple layer-by-layer sectioning sequence is used to ensure the same immersion duration for all strips in a slice; (2) by adjusting parameters such as sectioning speed and strip width, the time required to process the whole slice is strictly limited to four minutes, i.e., the immersion duration for each strip is less than four minutes during data acquisition; and (3) throughout long-term data acquisition, all adjustment and system-down pauses should be limited to as far under eight minutes as possible, and once the pause time is close to or reaches eight minutes, several slices on the block surface should be marked as abnormal data. With this sectioning control, MOST can acquire satisfactory high-resolution datasets of the whole mouse brain at the neurite level, as shown in Figure 6.

Essentially, adopting the layer-by-layer sectioning sequence ensures the constant and short-strip immersion duration. In particular, MOST establishes layer-by-layer sectioning by using the lower side of the knife blade, as shown in Figure 1d. If the turned-up side of the knife blade was used instead, the lower side (unused part in this case) of the knife blade would damage the unimaged surface of specimen. Thus, layer-by-layer sectioning cannot be used in such an instance, and a more complicated mode such as stair-step sectioning should be used instead.[19] Stair-step sectioning has two main limitations: it is difficult to ensure short and constant immersion duration for all strips in the same slice, and relatively deep steps are produced, such that the effects of lateral surface immersion cannot be ignored, thereby making it more challenging to achieve accurate registration and low data loss.

This study focused on spurr resin, a hydrophobic epoxy resin which is widely used in ultra-thin section-based research such as electron microscopy because of its stable sectioning properties. Our study shows that even the block surface of the hydrophobic spurr resin exhibits significant hygroscopic expansion. For hydrophilic resins, the immersion effects may be even more rapid and distinct. Because the proteins in some samples are easily damaged by hydrophobic epoxy resins,[20] hydrophilic resin materials may be more suitable. Consequently, it is also important to pursue research into the immersion effects on hydrophilic resins, as well as to explore how best to retain resin sectioning properties. Although this paper does not discuss other resin materials, our methods can also be applied in the studies on hydrophilic resins.

The evaluation of ultra-thin sectioning or precise processing quality is usually involved in other studies of histological anatomy related instruments and methods. However, it is difficult to collect ultra-thin sections while maintaining the original sample state; because of the required series of collecting procedures such as flattening the sections in water, these collected sections are nearly impossible to truly reflect the original properties of sectioning (e.g. section thickness and flatness). We introduce the idea of measuring workpiece surface profiles for evaluating the precision in the field of metal mechanical processing to this study, and provide an evaluation method based on the production of micron-sized steps and surface profile measurements. Instead of directly measuring ultra-thin sections, our method offers another solution for evaluating sectioning properties, which can realistically reflect the sectioning properties of the instrument used for processing resin blocks.

In conclusion, our work allows a significant advance in the reliability of ultra-thin section-based microscopic imaging. This kind of imaging method continues to play

an irreplaceable role in researches of the fine architectures in a brain-wide neural network, and will be an important method for studying brain structure, function and pathology in the future.

Author contributions: All authors participated in the work presented here. QL and QW defined the research topic and wrote the paper. QW designed the investigation and analyzed the data. QW and DX performed all the laboratory experiments. AL and HG interpreted the results and co-wrote the paper. All authors have read and approved the manuscript.

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