High-contrast differentiation resolution 3D imaging of rodent brain by X-ray computed microtomography

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Abstract: The biomedically focused brain research is largely performed on laboratory mice considering a high homology between the human and mouse genomes. A brain has an intricate and highly complex geometrical structure that is hard to display and analyse using only 2D methods. Applying some fast and efficient methods of brain visualization in 3D will be crucial for the neurobiology in the future. A post-mortem analysis of experimental animals’ brains usually involves techniques such as magnetic resonance and computed tomography. These techniques are employed to visualize abnormalities in the brains’ morphology or reparation processes. The X-ray computed microtomography (micro CT) plays an important role in the 3D imaging of internal structures of a large variety of soft and hard tissues. This non-destructive technique is applied in biological studies because the lab-based CT devices enable to obtain a several-micrometer resolution. However, this technique is always used along with some visualization methods, which are based on the tissue staining and thus differentiate soft tissues in biological samples. Here, a modified chemical contrasting protocol of tissues for a micro CT usage is introduced as the best tool for ex vivo 3D imaging of a post-mortem mouse brain. This way, the micro CT provides a high spatial resolution.

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of the brain microscopic anatomy together with a high tissue differentiation contrast enabling to identify more anatomical details in the brain. As the micro CT allows a consequent reconstruction of the brain structures into a coherent 3D model, some small morphological changes can be given into context of their mutual spatial relationships.

Keywords: Computerized Tomography (CT) and Computed Radiography (CR); MRI (whole body, cardiovascular, breast, others), MR-angiography (MRA)
1 Introduction

Computed tomography is widely applied in the human medicine especially to monitor bone injuries, to diagnose head, lung and chest conditions or to detect cancer. In the last decades, this non-destructive imaging technique has been evolved and therefore is able to provide high-resolution \textit{ex vivo} analyses of biological samples. The micro CT brings unique possibilities concerning the imaging of complex biological systems, such as the following cases: a description of a mouse knee cartilage development [1]; a formation of 3D models of mouse chondrocranium, an analysis of the ossification onset and morphological changes in various transgenic mice [2–4]; a noninvasive observation of a human embryo [5].

The principle of the X-ray micro CT imaging is based on taking series of 2D projection radiographs from different angles and their subsequent processing. In each projection, the information about radiographic density is recorded. A mathematical process called tomographic reconstruction forms a 3D matrix which represents a map of volume density. The spatial resolution of the system is determined by the geometry of a cone beam. The achievable resolution is down to 1 \textmu m for lab-based machines. This method has proven to be an effective tool for imaging of native bone tissues [6, 7], since the hydroxyapatite is dense enough to be easily detected. In the case of biological samples and soft tissues \textit{ex vivo}, the micro CT imaging requires an application of some X-ray absorbing contrast agents, e. g. phosphotungstic acid (PTA), iodine or osmium [8]. If the contrast is sufficient, it is possible to segment different structures within the 3D tomographic data by using an appropriate software.

The major advantage of the CT method is the ability to image a bone, soft tissues and blood vessels at the same time. In some cases, it is advantageous to combine the CT method with some other techniques for \textit{in vivo} imaging to visualize better various soft tissues. Especially in the case of cancer diagnosis, the computed tomography is combined with the positron emission tomography (PET/CT) [9].
In biomedical research, the rodent brain attracts a lot of attention because of some human-related pathologies that can be modelled in transgenic animals and also because of the high homology between the human and mouse genome, which causes numerous fundamental neurobiological questions \cite{10}. To take advantage of the information gained from mouse brain research, it is necessary to systematically collect the phenotype information at all biological levels. Conventionally used 2D analysis methods — histology, immunochemistry or transmission electron microscopy — show a high resolution within the plane in which the tissue is sectioned. However, these methods are destructive and two dimensional in their nature.

In the case of post-mortem 3D imaging of mouse brain, the micro CT is not the only method that could be applied. The magnetic resonance imaging (MRI) could be used for a similar purpose as well. In the past, the micro CT was used as an accessory technique to MRI for the imaging of the mouse brain. The micro CT was employed for the imaging of the skull \cite{6,7} or for the visualization of vascular system of mouse brain filled with radio-opaque silicone rubber Microfil \cite{11–17}. With the recent progress in development of chemical contrasting protocols for brain tissue \cite{6,18–22} or application of micro CT imaging as a tool for location of cerebral ischemia \cite{22,23} or brain tumors \cite{21,24}, micro CT imaging is becoming a vital research tool for mouse brain imaging in general.

In this article, we will focus on the mouse brain 3D imaging with a high-resolution laboratory micro CT system combined with an optimization of sample staining. We use different staining protocols based on iodine solution and phosphotungstic acid. The benefits of such approaches will be specified. We will summarize the comparison of the different staining procedures and we will also compare the gained data with the corresponding magnetic resonance data. Furthermore, the power of presented method will be demonstrated by a number of 3D models of selected brain structures with specifically complex 3D geometries that we have reconstructed and analyzed.

2 Materials and methods

2.1 Sample preparation for micro CT measurement

An adult mouse was sacrificed with isoflurane overdose. The brain was dissected from skull and collected into ice-cold PBS. Subsequently, the sample was fixed in freshly prepared 4% paraformaldehyde (PFA). The sample was dehydrated in ethanol grade (12 h) and stained in 1% iodine solution in 90% methanol. After 24 hours in the solution, the sample was rinsed in ethanol rehydration series to end up in sterile distilled water. For the sake of micro CT measurement, the brain was embedded in 1% agarose gel and placed in a plastic tube. All animal (mouse) concerned work had been approved and permitted by the Ethical Committee on Animal Experiments (Norra Djurförsöksetiska Nämnd, ethical permit N226/15 and N5/14) and was conducted according to The Swedish Animal Agency’s Provisions and Guidelines for Animal Experimentation recommendations.

2.2 Micro CT measurement and data processing

Micro CT measurement was performed with a GE Phoenix v\textsuperscript{\textregistered}tomex L 240 (GE Sensing & Inspection Technologies GmbH, Germany), equipped with a nanofocus X-ray tube with maximum power of 180 kV/15 W. The data were acquired using a high contrast flat panel detector DXR250 with 2048 px × 2048 px, 200 μm × 200 μm pixel size. The micro CT scan was carried out in an air-conditioned cabinet (21°C) at 60 kV acceleration voltage and 200 μA tube current. Exposure
time was 900 ms and 3 images were averaged for reducing the noise. Two different measurement procedures were performed. The first one was used for the evaluation of staining protocol. The brains contrasted by various techniques were scanned with a voxel resolution of 18 \( \mu \)m. After the evaluation, the second measurement procedure was applied for one brain with a voxel resolution of 6.5 \( \mu \)m. 2200 projections were taken over 360° in this case. A tomographic reconstruction was realized by software GE phoenix datos|x 2.0 (GE Sensing & Inspection Technologies GmbH, Germany). Reconstructed slices were imported into a VG Studio MAX 3.1 (Volume Graphics GmbH, Germany). In this software, the 3D data were aligned so that orthogonal slices matched the horizontal \((xy)\), coronal \((yz)\) and sagittal plane \((xz)\) resulting in series of 1700 coronal, 1090 sagittal and 680 horizontal tomographic sections of the brain. These data were compared with an anatomical atlas, and 3D models of anatomical structures were obtained by a segmentation based on the global thresholding.

2.3 MRI measurement

The post-mortem mouse brain within the skull was measured by a high-resolution MRI scanner Bruker Avance 9.4 T (Bruker Biospin MRI, Ettlingen, Germany). The resolution of the obtained data was 0.027\( \times \)0.027 mm (matrix 512\( \times \)512 pixels). \( T_1 \)-weighted anatomical images of 25 parallel 2D slices were taken using the FLASH sequence with slice thickness 0.5 mm, interslice distance 0.5 mm, repetition time (TR) 461.3 ms, effective echo time (TE) 6.1 ms, 50 averages, flip angle 35.0°, echo spacing 6.1 ms. The total measurement time was 9 hours and 25 images were obtained.

3 Results and discussion

For a comprehensive visualization of the mouse brain structures done by a conventional micro CT, a chemical contrasting step is usually required. The differentiation of soft tissues by an imaging method delivers the biologically-relevant information only if the contrasting is sufficient. Our priority in this research was achieving the ultimate differential contrast enabling the identification of all major cell types or the tissue modes.

Inorganic iodine and phosphotungstic acid (PTA) are the most broadly used agents in the field of post-mortem tissue contrasting for the following X-ray investigation [25]. Previously, it was suggested to use alcohol solutions containing these compounds to enable a rapid diffusion into the sample [8]. If iodine is compared to PTA, PTA appears as a larger molecule with much slower tissue penetration rates [8]. Ten different staining protocols were tested to visualize mouse brain structures by micro CT imaging (table 1). Samples were stained in PTA, iodine or combination of both of solutions for various periods of time.

In order to compare the contrast among these samples, we analyzed all of them in the same condition. The voxel resolution of obtained CT data was 18 \( \mu \)m, which turned out to be enough to compare the quality of different contrasting protocols (figure 1). In summary, the staining in iodine exhibited more clear contours of fibres than in the case of PTA-stained samples. Fixation of samples in 4\% PFA demonstrated higher contrast to tissues fixed in 10\% PFA. The best resolution was obtained in 1\% iodine in 90\% methanol solution. As this staining showed the most contrastive and sharp edges of anatomical structures at coronal brain sections, we selected the sample stained with this protocol for further analysis.
Figure 1. Comparison of the coronal sections of samples following different staining protocols. (A) 1% PTA in 90% MeOH for 21 days, (B) 1% PTA in 90% MeOH for 14 days, (C) 1% PTA in 100% MeOH for 16 days, (D) 1% PTA for 10 days + 1% iodine in 90% MeOH for additional 24h, (E) 1% iodine in 100% EtOH for 24h, (F) 1% iodine in 100% EtOH for 48h, (G) 1% iodine in 100% EtOH for 7 days, (H) 1% iodine in 90% MeOH for 24h, (I) 1% iodine in 100% EtOH overnight, (J) 1% iodine in 100% EtOH for 24h.
Table 1. List of different processing and staining protocols for brain visualization in micro CT.

|   | Pre-fixation | Post-fixation | Dehydration | Staining                      | Time          |
|---|--------------|---------------|-------------|-------------------------------|---------------|
| A | 4% PFA       | 4% PFA (24h)  | EtOH / 12h  | 1% PTA in 90% MeOH            | 21 days       |
| B | /            | 4% PFA (24h)  | EtOH / 12h  | 1% PTA in 90% MeOH            | 14 days       |
| C | 10% PFA      | 10% PFA (24h) | EtOH / 12h  | 1% PTA in 100% MeOH           | 16 days       |
| D | 4% PFA       | 4% PFA (24h)  | EtOH / 12h  | 1% PTA + 1% iodine in 90% MeOH| 10 days PTA/24h iodine |
| E | 4% PFA       | 4% PFA (24h)  | EtOH / 12h  | 1% iodine in 100% EtOH        | 24h           |
| F | 4% PFA       | 4% PFA (24h)  | EtOH / 12h  | 1% iodine in 100% EtOH        | 48h           |
| G | 4% PFA       | 4% PFA (24h)  | EtOH / 12h  | 1% iodine in 100% EtOH        | 7 days        |
| H | 4% PFA       | 4% PFA (24h)  | EtOH / 12h  | 1% iodine in 90% MeOH         | 24h           |
| I | 10% PFA      | 10% PFA (12h) | EtOH / 2h   | 1% iodine in 100% EtOH        | overnight     |
| J | 10% PFA      | 10% PFA (12h) | EtOH / 2h   | 1% iodine in 100% EtOH        | 24h           |

Four coronal sections (figure 2) from different parts of the mouse brain demonstrate the extent of resolution quality of contrasting protocol as captured by a micro CT scan. Identification of the

![Figure 2](image_url)
described structures in the coronal sections was based on the annotation from Allen Mouse Brain Atlas [26]. For instance, corpus callosum, cerebral peduncle and anterior commissure appeared among the most contrasted and easily identifiable structures. Furthermore, the smaller and finer structures such as mamillothalmic tract, column of the fornix or caudoputamen were successfully recognized as well. Because of the grey matter’s high contrast, the ventricles (e.g. lateral ventricle and third ventricle) were also evident.

Although both micro CT and MRI belong to the category of methods providing 3D imaging of the internal structures, the character of the output data including the resolution quality is rather different. The geometry of the data element called voxel has a cubic shape (the same dimensions in all three axes) in the CT and a rectangular cuboid shape in the MRI. The MRI data voxel is defined by the pixel size and slice thickness, which is typically significantly larger than pixel dimensions, i.e. the lateral and axial resolution are different. Isotropic 3D imaging is feasible by both methods, however the length of such experiments needed for achieving a good signal-to-noise ratio is limiting. Consequently, the brain structures were analyzed in detail on 1900 coronal CT slices (with voxel size $6.5 \mu m \times 6.5 \mu m \times 6.5 \mu m$) and 25 coronal MRI slices (with voxel size $27 \mu m \times 27 \mu m \times 500 \mu m$). From this point of view, CT data provided enhanced opportunities to study the arbitrary cross-sections within one dataset and also demonstrated an accurate 3D modelling of internal structures.

The voxel values are defined by greyscale values depending on different properties of the material. CT greyscale values are defined by the X-ray absorption properties in relation to the atomic number of the sample material and to the accelerated voltage of the X-ray source [27, 28]. Based on this fact, brighter tissues refer to denser materials. In the case of anatomical T1 weighted MRI, the amount of the obtained signal depends on the time needed for realigning proton spins in main magnetic field (realigned to Boltzmann equilibrium) [29, 30]. For instance, the fat realigns its spins quickly, and therefore it appears bright on a $T_1$ weighted image [29, 30]. Water realigns much slower, and therefore it has a lower signal and is represented by darker values.

As for the measurement time, it depends on the particular method. Considering ex vivo approach plus the highest possible resolution of both systems, the typical brain measurement took approx. 9 hours for the MRI and 2 hours for the CT.

To compare corresponding MRI and micro CT data (figure 3, table 2), we selected four pairs of corresponding coronal slices of the adult mouse brain. Corpus callosum, anterior commissure, medial mammillary nucleus, posterior commissure and cerebral peduncle were clearly recognizable on both micro CT and MRI images. All the mentioned structures demonstrated well-defined edges in micro CT data. Lateral ventricle, lateral olfactory bulb, optic tract, stria medullaris, internal capsule and cerebral peduncle were also detectable in both micro CT and MRI data. However, unlike the micro CT images, MRI pictures did not display a clear identification of above mentioned structures. The structures such as myelinated fibres in caudoputamen, third ventricle, column of the fornix, dentate gyrus, mamillothalmic tract and other locations were clearly distinguished in the micro CT. Moreover, the compartmentation of dorsal hippocampal commissure from corpus callosum and alveus was possible only in micro CT images.

As the next step, we selected several structures for segmentation to demonstrate possible outputs from micro CT data (figure 4, figure 5, figure 6). Tissue segmentation and construction of accurate
Figure 3. Comparison between corresponding coronal sections in MRI (A, C, E, G) and contrasted (1% iodine in 90% methanol) micro CT (B, D, F, H) of mouse brain.
Table 2. Identification of anatomical structures in mouse brain in micro CT and MRI images (*not suitable for segmentation, hard to detect).

| structure                   | micro CT | MRI |
|-----------------------------|----------|-----|
| corpus callosum             | ✓        | ✓   |
| anterior commissure         | ✓        | ✓   |
| medial mammillary nucleus   | ✓        | ✓   |
| posterior commissure        | ✓        | ✓   |
| cerebral peduncle           | ✓        | ✓   |
| lateral ventricle           | ✓        | ✓/* |
| lateral olfactory bulb      | ✓        | ✓/* |
| optic tract                 | ✓        | ✓/* |
| stria medullaris            | ✓        | ✓/* |
| internal capsule            | ✓        | ✓/* |
| cerebral peduncle           | ✓        | ✓/* |
| myelinated fibres in caudoputamen | ✓  | ×   |
| third ventricle             | ✓        | ×   |
| column of the fornix        | ✓        | ×   |
| dentate gyrus               | ✓        | ×   |
| mamillothalamic tract       | ✓        | ×   |

Figure 4. Visualization of steps during segmentation. (A) Area of the coronal section in the 3D model of the adult mouse brain, (B) Unmarked tomographic section, (C) Tomographic section with manually segmented corpus callosum (pink), myelinated fibres in caudoputamen (light blue) and anterior commissure (green).

3D models was possible thanks to high-resolution tomographic sections in all coronal, sagittal and horizontal planes and also, a fine contrast was implied by the improved staining protocol. Such a precise 3D visualization could be useful in future for variety of structural analyses in developmental biology and pathological studies. Above that, such data can be used for the educational purposes as they may be utilized for a subsequent 3D printing [31].
4 Conclusion

Three-dimensional imaging of the rodent brain plays an important role in modern neurobiology and biomedicine research. This kind of imaging enables a precise analysis of developmental changes, tissue damage and tumor recognition with a focus on fine morphological modifications. We worked with several chemical contrasting protocols and compared their ability to visualize brain tissues by a laboratory micro CT device. Our results proved this method to be a powerful tool for a high-resolution ex vivo 3D imaging of the rodent soft tissues. The state of the art of the laboratory CT systems together with a staining protocol based on iodine solution enabled to reveal an intricate and geometrically complex internal brain structures in a high resolution. Tissue contrast was found to be considerably stronger in the micro CT images in comparison to the commonly used post-mortem MRI. Consequently, CT data enabled an easier segmentation and 3D reconstruction of internal brain structures. Generally, the 24 hours sample processing together with a two-hour-long CT measurement can provide a quick and complete visualization of the brain’s internal and external morphology with a simultaneous and semi-automated identification of the major neural tracks, nuclei and other delicate details. Thanks to all these benefits, the micro CT systems have their place in a routine use in various types of neurobiological experiments.

Figure 5. Structures segmented from micro CT images. (A) Complex of corpus callosum, dorsal hippocampal commissure and alveus (pink), (B) Myelinated fibres in caudoputamen (light blue), (C) Column of the fornix (purple), (D) Mamillothalamic tract (orange), (E) Anterior commissure (green).
Figure 6. Segmented brain structures micro CT images. (A, B) Sagittal view, (C, D) dorsal view, (E, F) ventral views on dorsal hippocampal commissure and alveus (pink), column of the fornix (purple), mamillothalmic tract (orange), myelinated fibres in caudoputamen (light blue), anterior comissure (green) and complex of corpus callosum, dorsal hippocampal commissure and alveus (pink), in context of the whole adult mouse brain.

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