Research Article

Imaging of Mouse Brain Fixated in Ethanol in Micro-CT

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1. Introduction

Microcomputed tomography (micro-CT) scanning provides nondestructive imaging of tissues and has potential to produce 3D images. Highly mineralized structures, such as bones and teeth, give very good contrast in micro-CT [1]. On the other hand imaging of soft tissues such as nerve, muscle, adipose tissue, or ligaments is very problematic [2]. Interestingly, alcohol fixation works well with the neuronal tissue and specifically with the brain but together with the iodine and phosphotungstic acid [3]. Brain tissue has several distinguishing characteristics compared to other soft tissues. It is composed of white matter that contains relatively high amount of phospholipid molecules forming myelin sheaths around axons of neurons that behave on the micro-CT simply as fat (mostly visualized on micro-CT darker compared to grey matter) and from grey matter containing bodies of neurons (appear on micro-CT lighter compared to white matter bundles) that share basic common cellular characteristics (biophysical, biochemical, and biological) of other soft tissues. Micro-CT studies visualizing neuronal tissue usually focus on peripheral nerves and their lesions [4], overall brain atrophy [5], freeze-dried human acellular nerve allografting (hANA) [6], and brain tumor models in mice [7]. Generally there are more micro-CT studies on pathological neuronal tissues than on the healthy ones.

Behavioral studies on the mice model often require precise analysis of the brain area selected for the experiment. For example, in animal models of ischemia exact place of the neuronal lesion has to be verified and quantified [8]. Evaluation of exact lesion site needs different kinds of structural/histological atlases ([9, 10]; Allen Mouse Brain...
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possibleneuroanatomicalstructuresoncoronal,sagittal,andonvivoimageacquisition,CombiningofMRIandmicro-CT imagingwithphasecontrastoftheethanolfixedratbrainwassuccessfullydescribedin[12],althoughonly
gross neuroanatomical structures were observed. Soaking of the brains in nonionic iodinated contrast agent resulted in
clear differences in signal between the grey matter, the white matter, and the ventricular spaces [13], but without possibility
to distinguish neuroanatomical borders of individual brain nuclei or cortical regions. Diffusible iodine-based contrast-enhancedcomputedtomography(diceCT)infemalemouse
was suggested to be effective for gross differences in the overall brain shape in large numbers of samples [14]. Com-
bined MRI-CT atlases of developing and adult mouse brains fixed with paraformaldehyde and subsequent PBS wash-out
are unique for coregistration of brain areas but without detailed neuroanatomical structures delineation [15]. We
tried to visualize and identify on micro-CT as much as possible neuroanatomical structures on coronal, sagittal, and
horizontal sections of the healthy mouse brain.

2. Materials and Methods

2.1. Tissue Sample Origins. We evaluated 5 brains from C57BL/6 genetically modified male mice (weight 17-20 g)
from the Institute of Experimental Imaging, First Faculty of Medicine, Charles University, Prague, Czech Republic.
This mouse strain was selected because it is commonly used in neurosciences and other research fields [16]. Mice were
euthanized by cervical dislocation and their brains were harvested for purpose of this study; this method did not
affect mice brain distortion at all. Study was approved by Ethical Committee of the Third Faculty of Medicine, Charles
University, Czech Republic.

2.2. Tissue Sample Processing. Brains from 5 mice were carefully extracted from the skulls by the following steps.
Cervical spinal cord and brain stem were released by small
tongs as disruption of cervical vertebrae. Then temporal
bones and vestibulocochlear, oculomotor, optic, and olfac-
tory nerves were dissected. After extraction of the brain from
skull, any remnants of bone fragments on the brain surface
were carefully checked and cleaned before scanning. The
brain samples were put into Eppendorf tubes with ethanol-
soaked gauze at the bottom for the purpose of the scan. The
conical shape of Eppendorf tubes very efficiently supports the samples and avoids undesirable movements. The wet gauze
maintains a saturated gaseous atmosphere preventing further
drying out and shrinkage of sample. After the extraction,
brains were fixated subsequently in 25%, 50%, 75%, and 97%
ethanol for 12 hours. This type of ethanol fixation is also
known as graded dehydration series of ethanol (GEHC) and
has been documented as promising in undistorted soft tissue
fixation [17]. Micro-CT scanning was performed after 168
hours of fixation.

2.3. Tissue Sample Scanning. Brains were left prior to scan-
ing on the gauze for 40 minutes in air temperature 23°C.
This allowed vaporization of redundant ethanol from the
whole brain, including the ventricles and other cavities. After
the period of drying, brains were positioned in the special
plastic holder with an ethanol reservoir, which made an
atmosphere of gas, which prevented structural changes of
the brains during scanning [18, 19]. Two different scanning
techniques were performed. First, just X-ray radiography
was performed followed by a microtomography and final
3D reconstruction [18, 19]. The data were reconstructed into
the final 3D dataset using Voxel reconstruction software and
visualized using program CTVox in standard PC [20]. On
the sagittal projection some processing artifacts are often
seen: flattening of the whole brain craniocaudally, artificial
space between the hippocampal formation and thalamus,
imbria fornixis separated from the white matter nearby stria
terminalis and ventriculus lateralis, and cerebellar fissure
behind inferior colliculi.

2.4. Micro-CT Apparatus. The used micro-CT set-up was
described in detail in our previous publications [18, 19, 21].
Briefly, apparatus consisted of two different custom-built
micro-CT systems; routine detection system was equipped
with a Kevex™ PXS-11 X-ray tube and Timepix detector in
Quad configuration (four read-out chips with a common
silicon sensor providing sensitive area 28 × 28 mm, 512 × 512 pixels, 55 µm pixel pitch). The highest achievable
spatial resolution was approximately 28 µm. Presented 2D
microradiographic images were acquired with the introduced
setup. The other high-resolution system was equipped with
a large area photon counting detector WidePIX10×5-. WidePIX
is a recently introduced technology for tiling of large area
PCD arrays from individual Timepix chips [22]. Specifically,
detector WidePIX10×5 is composed of 50 Timepix tiles and
offers approximately 140 x 70 mm field view (2560 × 1280 pix-
els). High quality microfocus X-ray tube Hamamatsu L8601-
01 enables spatial resolution down to 5 micrometers [23].

2.5. Scan Parameters. The high-resolution setup was used for
the presented micro-CT scans. The data were acquired with
an emphasis on high CNR. The acquisition time was adjusted
in order to reach at least 10³ or 10⁴ detected photons per pixel
in the background of the object in microradiography or a
micro-CT projection, respectively. CT reconstructions
were done by Voxel reconstruction engine (courtesy of Fraunhofer
IIS and Technology, Germany).

The detector as well as the whole CT scan was controlled
using Pixelman software [24]. The CT scan was carried out
with 4.4 µm EPS. The total number of 848 projections was
acquired with 0.38 degree angle step. The acquisition time
was 3.6 seconds per projection. The tube voltage was set
to 60 kVp and it was operated with 6 W of output power.
The projections were processed using a dedicated beam-
hardening correction [25] and the slight image distortions
Figure 1: Micro-CT slices (program CTVox) of the 5 coronal sections of the mouse brain with labeled grey and white matter structures. Brain sections from top down are taken 1x at the level of anterior commissure, 2x at the dorsal hippocampus, 1x at the ventral hippocampus, and 1x at the brain stem (superior colliculi). Section orientation: L – left, R – right.

coming from the tiled detector construction were corrected [26]. The CT reconstruction was carried out using Volex reconstruction engine.

2.6. Gray and White Matter Labelling. For the frontal and sagittal sections Allen Mouse Atlas was used as reference [27]. For the horizontal sections C57BL/6J Atlas was used as reference (The Mouse Brain Library, http://www.nml.org). Anatomical structures of digitalized brain sections were labeled in the environment of freeware program Xnview (https://www.xnview.com/en/). All depicted pictures of the labelled mouse brain are from one specimen only so that structures correspond between exactly three planes (horizontal, sagittal, and coronal), because of tiny morphological differences between various mouse brains.

3. Results

We identified 42 white matter and 53 grey matter brain structures (see Abbreviations) in five coronal (Figure 1), four sagittal (Figure 2), and three horizontal (Figure 3) brain sections of ex vivo healthy mouse brain using micro-CT. All structures were identified manually by two experienced neuroanatomists and registered in micro-CT scans using online Adult Mouse Brain Atlas [27] for coronal and sagittal sections and the online Mouse Brain Library (C57BL/6J Atlas) for horizontal sections.

3.1. Frontal Sections of the Mouse Brain. The positions of the five coronal sections of the mouse brain were selected because of their relevance to experimental neuropsychological studies in animal model. Infrontodorsal order the sections were taken (a) in the frontal lobe at the level of the anterior commissure, (b) at the ventral part of the dorsal dentate gyrus, dorsal hippocampus, and the third ventricle, (c) at the dorsal part of the dorsal dentate gyrus, dorsal hippocampus, and the paraventricular nucleus of the thalamus, (d) at the level of the ventral dentate gyrus, ventral hippocampus, and midbrain reticular nucleus, and (e) at the level of the brain stem with superior colliculi and dorsal raphe nucleus. We were unable to identify hyperintensity in the brain stem between trigeminal nuclear complex, lateral lemniscal nuclei, and medial cerebellar peduncle. Another poorly visible area is located below superior and inferior colliculi, towards thalamic nuclei. Similarly, resolution of the bed nucleus striae terminalis and substantia innominata is poor. Opposite, there is good resolution for both zona incerta and reticular part of the substantia nigra. Although there is relatively big trigeminal nuclear complex that is easy to identify, separate subnuclei of the complex are hard to differentiate. The caudatoputamen is
a very well visible structure and inside is rich system of the hypointensities that could be either white matter of the internal capsule or the Virchow-Robins spaces formed by capillary bed stream of thalamostralateral artery. Hypothalamic subparts are more difficult to discern compared to thalamic subnuclear groups. Stria medullaris thalami is normally found on the superior part of the thalamus; however here it is detached from it and bound to dorsal hippocampal commissure on the caudal surface of the fornix. While most of the thalamic nuclei are hyperdense, most midbrain structures (reticular nucleus, periaqueductal gray, etc.) are hypodense.

3.2. Sagittal Sections of the Mouse Brain. Within hippocampal formation dentate gyrus, CA1, CA2, and CA3 subfields and subiculum were identified. Sagittal projection offers better visibility over frontal projection. Above cerebellar peduncle zona incerta, substantia nigra, and ventrally stria terminalis were visible. Within brain stem trigeminal nucleus, dorsal vagal nucleus, and nucleus interpeduncularis were visible. On the other hand, we have not seen well borders of the amygdaloid complex; it had the same gray color as nearby structures (olfactory tubercle or nucleus accumbens).

3.3. Horizontal Sections of the Mouse Brain. We identified in the ventral part of the sections clearly visible medial and lateral septum. Callosal body in contrast to online atlas was very well visible in all three sections and in front of it was well preserved medial frontal and/or orbital cortex. Also frontal part of the lateral ventricles clearly separated caudatoputamen from septal nuclei and internal capsule. Penetrations of the internal capsule into caudatoputamen are, especially, well visible. Dorsally to septal area structures of the thalamus (paraventricular nucleus and laterodorsal complex of nuclei) were located, and also stria medullaris thalami and lateral geniculate complex. On the other hand, detailed inner structure of the hippocampal formation (ventral part) was not very well visible as in the frontal sections. Besides reticular, pontine, or parvocellular nuclei, other brain stem nuclei were not visible compared to the frontal sections.

4. Discussion

Micro-CT imaging in mouse is often limited to the whole body scans, including skeleton, organs and blood vessels [28, 29], and brain blood supply changes in various experimental pathological conditions [30, 31], or to the brain tumors (for example glioblastoma) [32]. Scanning of the mouse brain gives better results when it is extracted from the skull. The reason is that the skull induces beam-hardening artifacts to adjacent soft tissue [15]. Our work provides comparable results as reported by [3]; nevertheless, in our case, any high-Z contrast agent was needed.

High-resolution MRI three-dimensional atlas of the mouse brain shows sixty-two structures at the resolution 32 𝜇m with the habenular nuclear complex being the smallest visible structure [33]. In comparison our micro-CT ethanol fixated brain scans showed more than fifty structures within only 5 representative coronal sections. It seems that pure GEHC ethanol brain fixation shows better differences between white and gray matters on micro-CT compared to MRI. We identified small white matter structures like cingulum bundle, medial lemniscus, crossed tectospinal pathway, and stria terminalis which is a better result compared to the MRI.

Frontal and sagittal sections atlases of the mouse brain [27] are easier to find in the literature compared to the horizontal ones (for example MRI atlas of C57BL/6, DBA/2, or A/J mouse from The Mouse Brain Library). Some MRI atlases are without grey and white brain matter labels, for example, 8-week-old 129S1/SvImJ male mice atlas [34].

We did not attempt yet to create the whole atlas of the mouse brain on the micro-CT. Our goal was to visualize clinically important brain structures like hippocampal formation and its subfields, thalamic nuclei, fornix, medial and lateral septal nuclei, and others. We suggest as the next step manual or semiautomatic reconstruction of the whole mouse brain micro-CT atlas. Histological staining (Nissl) and optical microscopy are mostly used for brain lesion evaluation in experimental studies (insertions of cannulas, electrolesions, chemical lesions, electrodepo-positions, polymer substance delivery, ischemia after carotid arteries ligations, etc.). The disadvantage of these approaches is altered brain tissue that cannot be used afterwards for other staining (for example immunostaining) or at the cost of complicated
protocols for sections storing and handling. With the micro-
CT lesion verification, we can use intact brain tissue for
further processing and thus replacing classical histological
verification with virtual visual evaluation. Moreover, micro-
CT lesion visualization can be enhanced by computer pro-
cessing leading to volume rendering or providing virtual
dissection of the brain in unorthodox planes unavailable in
classical histology. Level of details in high-resolution micro-
CT almost corresponds to the classical histology sections.
Within destructive methodologies, it seems to be a choice for
immunohistochemistry since the brains are processed only in
ethanol.

5. Conclusion

We show that micro-CT could be used in neuroresearch
alongside classical histology or magnetic resonance imaging.
Besides higher price and lower resolution of the magnetic
resonance imaging, it is not always available to all laboratories
and micro-CT is easier to get access to. Even if one does not
have micro-CT in the laboratory, it is possible to use fixation
of the brain specimen and send it to micro-CT for analysis.
This is not so simple for magnetic resonance imaging; we
cannot use fixation or it would not be visualized properly.
Fixation of the brain tissue should be done as soon as possible
or the brain would decompose. Magnetic resonance imaging
is better for living organisms while micro-CT for fixed brain
tissue. Laboratories with micro-CT could offer services for
others (sending fixated brain specimen) since acquisition
time for micro-CT scanning is relatively short compared
to magnetic resonance imaging. Immunohistochemistry or
general staining histological protocols could then follow in
a short time. The disadvantage of the micro-CT is still the
relatively small Timepix detector area but with time we could
expect an increase in its size. Ex vivo ethanol fixation of the
brain tissue grants sufficient tissue contrast, but we trend to
the situation where brain rotation could be highly contrasted
even during in vivo scanning.

Abbreviations

ACB: Nucleus accumbens
AHA: Anterior hypothalamic area
alv: Alveus
Amy: Amygdala
APN: Anterior pretectal nucleus
aq: Cerebral aqueduct
arb: Arbor vitae
aud: Auditory cortex
av: Anteroventral nucleus of the thalamus
bnst: Bed nucleus striae terminalis
bsc: Brachium of superior colliculus
bsRN: Brain stem reticular nuclei
ca: Anterior commissure
CA1: Field of CA1
CA3: Field of CA3
cb: Cell bridges of ventral striatum
cbx: Cerebellar cortex
cc: Corpus callosum
cing: Cingulum bundle
cn: Cuneiform nucleus
CP: Caudatoputamen
cpd: Cerebral peduncl
cst: Corticospinal tract
d3v: Dorsal third ventricle
dco: Dorsal cochlear nucleus
dG: Dentate gyrus
dhc: Dorsal hippocampal commissure
dr: Dorsal nucleus raphe
dscp: Superior cerebellar peduncle decussation
ect: Ectothalmic cortex
em: External medullary lamina of the thalamus
ent: Entorhinal area
EW: Edinger-Westphal nucleus
fi: Fimbria fornici
flm: Medial longitudinal fascicle
fn: Fastigial nucleus
fx: Fornix
Gpi: Globus pallidus, internal segment
GRN: Gigantocellular reticular nucleus
hf: Hippocampal fimbria
hippo: Hippocampal formation
HY: Hypothalamus
chpl: Choroid plexus
IC: Inferior colliculus
icp: Inferior cerebellar peduncle
III: Oculomotor nucleus
inc: Interstitial nucleus of Cajal
int: Internal capsule
IP: Interposed nucleus of cerebellum
IPC: Interpeduncular nucleus
iv: Trochlear nucleus
LAV: Lateral vestibular nucleus
LD: Lateral dorsal nucleus of the thalamus
ldt: Laterodorsal tegmental nucleus
lgc: Lateral geniculate complex
lh: Lateral habenula
LHA: Lateral hypothalamic area
ll: Lateral lemniscus
lo: Lateral orbital cortex
LP: Lateral posterior nucleus of the thalamus
LPO: Lateral preoptic area
ls: Lateral septal nucleus
MARN: Magnocellular reticular nucleus
mcp: Middle cerebellar peduncle
MD: Mediodorsal nucleus of the thalamus
mfb: Medial forebrain bundle
MGC/mgc: Medial geniculate complex
mh: Medial habenula
ml: Medial lemniscus
mo: Somatomotor cortical areas
MOB: Main olfactory bulb
mOR: Medial orbital cortex
mpe: Medial cerebellar peduncle
MRN: Midbrain reticular nucleus
ms: Medial septal nucleus
mtt: Mammillothalamic tract
MV: Medial vestibular nucleus
Data Availability

Pictures of the mouse brain are available on request in our laboratory. The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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