Summary of the Ph.D. thesis

MULTIMODAL IMAGING OF THE CANINE BRAIN BASED ON STRUCTURAL IMAGING, CRYOSECTIONING AND 3-DIMENSIONAL MODELING

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1. Introduction and aims of the study

There are several ways to visualise macro-anatomical structures. For example, conventional preparations and cross-sections can be made from fresh cadavers; bodies can also be perfused with a fixative agent prior further investigation (Brenner, 2014). Macerated bones and skeletons can be created (King & Birch, 2015; Offele et al., 2007; Simonsen et al., 2011); alternatively, objects can be prepared with corrosion casting (Hirschberg et al., 1999; Krucker et al., 2006; Verli et al., 2007). Moreover, the results of these tissue preparations can also be captured both in photos and video recordings. The aforementioned procedures can be combined with different structural imaging techniques, like computed tomography (CT) or magnetic resonance imaging (MRI), thus specimens can be digitised for three dimensional (3D) analysis.

To study the central nervous system *in situ*, different techniques are available, which either maintain the original colours and/or tissue integrity, or which modify their structural properties. Diagnostic imaging methods create greyscale images, and the quality of the CT and MRI data obtained depends on factors such as the sequence, signal-to-noise ratio, or presence of various artefacts (Goerner & Clarke, 2011; Roe, 2010; Thrall, 2012). As the brain is enclosed in the neurocranium, it cannot be routinely sectioned together with the skull during histological studies, due to the hardness of the bone (therefore, *ex situ* studies are preferred). For this reason, there are two main paths to creating macro-anatomical sections of the entire head: slicing the tissue block into layers with a band saw, or mill the adjusted volume stepwise and photograph the resulting surfaces with a camera. In the first case, slices could be handled individually, and their thickness could vary from centimetres to millimetres.

In contrast, the milling procedure removes a layer from the volume's surface (being a tissue destructive method), and the revealed polished surface is recorded on the consecutive photograph. With this method, the required layer thickness depends only on the applied milling technique and its precision, ranging from millimetres to micrometres. This method is called cryosectioning, or cryomacrotomisation (Park et al., 2014, 2005; Spitzer et al., 1996). There have been human studies in the cryosectioning field (Bergström et al., 1983; Lufkin et al., 1987; Rauschning, 1983), and an initiative by the National Library of Medicine in 1996, the Visible Human Project (carried out in association with the University of Colorado Center for Human Simulation), used cryomacrotomisation to visualise a complete male human body (Spitzer et al., 1996). In recent decades, similar projects have been carried out in China (Chinese Visible Human, Virtual Chinese Human Project) (Tang et al., 2010; Zhang et al., 2003), and in South Korea (Visible Korean Human) (Park et al., 2005).

Cryosectioning of smaller animals, such as mice and rats, has already been performed (Dogdas et al., 2007; Roy et al., 2009; Toga et al., 1995), but to date, there are only a few studies that have used the cryomacrotomisation technique on larger animals. The cryosectioning of an entire dog was first performed in 1999 (Böttcher et al., 1999). Subsequently, the whole body of a one-year-old female beagle was cryomacrotomized by Park et al. (in 2014), and a one-year-old domestic shorthair cat was studied by Chung et al. (2018). Among the most recent anatomical studies using this technique was the cryosectioning of a Rhesus monkey (Chung et al., 2019). While in these studies whole bodies were cryosectioned, we prioritised the proper visualisation of the *in situ* canine brain.

The primary aim of our research was to produce:

- high resolution,
- thin-layered,
- true-coloured,
- macro-anatomical image series from a canine brain,
- with cryosectioning the brain *in situ* in the skull,
- without the need for any previous fixation or decalcination procedure, which would interact with the original colour or alter the composition of the different tissues,
- with the shortest postmortem time possible until freezing and embedding,
- performing both MRI and CT examinations with different imaging sequence protocols,
- generating a software-based volume from the cryosectioned images, to be able to create multiplanar orthogonal view reconstructions,
- where the cryosectioned image volume can be registered in a common coordinate space with the scanning results from the structural imaging techniques,
- using 3D computer graphics software to create models,
- where annotated figures can be made based on the co-registered image volumes and the 3D models, to show the advantages of this technique.

To achieve these objectives, we had to conduct several pilot experiments before the final study could took place. With these pilot cryosectioning and visualisation studies, we could clarify that crucial elements (e.g., proper settings of the milling system, thermoregulation, photography and image post-processing) which were decisive for the outcome of the project.

2. Materials and methods

Pilot cryosectioning studies

Five pilot studies were performed to find the optimal settings used in the final study. Cadavers for the pilot experiments were obtained from owners who donated the body of their deceased animals to us in accordance with the Hungarian law. These trial sectioning and milling studies comprised:

(a) different specimens (dogs, cats, pig);
(b) various cutting and milling machines;
(c) individually designed embedding boxes;
(d) improved cooling and thermoregulation methods;
(e) improved surface care and photography.

Study I: Cryosectioning a complete pig cadaver

We started our cryosectioning experiments with a full-body sectioning of a domestic pig (*Sus scrofa domestica*), using an electric band saw (Biodur Products, Heidelberg, Germany). The body was embedded into polyurethane foam and was frozen to -80°C. During the sectioning procedure, the block was placed on a table where the cut surface was in contact with a continuously dry-ice-cooled (-78.5°C) plate. Sectioning interval was set to be 7 mm, and each of the cut surfaces were recorded with a Nikon D800 digital single lens reflex (DSLR) camera, using macro lens, polarised filters and colour checker. A total of 166 pictures were recorded from the full body. While the quality of the images was good, we also realized that sectioning with a band saw cannot guarantee the required small layer thickness (due to unintentional side-movements of the blade) and the even distance between the slices. Therefore, in subsequent studies we used milling devices.

Study II: Cryosectioning the head block of a dog

The second cryosectioning was performed with a JAFO FWD-32U universal milling machine (JAFO Jarocin Machine Tool Factory, Jarocin, Poland). First, red polyurethane rubber was injected postmortem into the head of an adult mongrel dog (*Canis familiaris*). Thereafter, the head block was embedded into a wooden box filled with polyurethane foam, and it was kept at -80°C until the cryomacrotomisation. We used a Canon EOS 7D DSLR camera with macro lens and polarising filters to capture 238 images. The slice thickness was 500 µm during the process. The main conclusions of this pilot test were that proper thermoregulation has to be maintained on the surface and around the block, as low temperature causes rapid re-freezing (and as a consequence, decrease in image quality), and the warmer tissues tend to cause smearing. We also found freezing artefacts and surface contamination.

Study III: Cryosectioning the complete head of a cat

The arteries of the head of an adult domestic shorthair cat (*Felis catus*) was filled with polyurethane rubber postmortem, and we performed MRI of the head using a 3T Magnetom scanner (Siemens AG, Erlangen, Germany) and a Siemens Somatom Perspective 128 slice CT (Siemens AG, Berlin and München 2013). Afterwards the head was embedded into a custom-made plexiglass box, which we filled with gelatin solution and froze at -80°C. Cryosectioning was carried out with a Dufour G230 universal milling machine (Gaston Dufour, Montreuil, France), with a milling interval of 400 µm. As a result, 260 images were recorded with a Canon EOS 5D Mark II DSLR camera. The gelatin embedding agent proved to be a good material, but the loss of the surrounding dry ice was extensive due to the poor thermoregulation, and we could observe circular milling stripes on the block surface.

Study IV: Cryosectioning the head block of a cat

In the fourth pilot we used a custom-made 3-axis high-speed computer numerical control (CNC) milling machine with a two-flute end mill. A head block was created from the cadaver of an adult domestic shorthair cat (*Felis catus*), and it was embedded in gelatin solution and kept at -80°C. We tested different rotational speeds and feed rates to assess the quality of the milling. Layer thickness was set to 200 µm. A Canon EOS 7D DSLR camera was used to capture images from a total of 17 layers. The CNC system was very effective in the automatisation of the process, but the small contact cutter area created stripes on the surface, scattered the tissue particles onto the block, and one milling step took longer time to proceed compared to the previous cryosectioning systems.

Study V: Cryosectioning the head block of a dog

The head of an adult American Staffordshire terrier cadaver (*Canis familiaris*) was frozen to -30°C, and a neurocranium block was created from it, which was embedded into a gelatin solution and kept at -80°C until the milling took place. A Kondia NCT B-640 precision CNC milling machine (NCT Industrial Electronics Ltd., Budapest, Hungary) was tested. We made cryosections with different thickness settings (varying between 50 and 400 µm), and 16 images were recorded with a Canon EOS 7D DSLR camera. Based on our evaluation, this milling system provided convenient technical parameters (cutting diameter was large enough for a neurocranium block to proceed in one turn, system was programmable), and the best image quality among the pilot studies (no milling and freezing artefacts occurred, and we did not experience contamination, as the debris was rolled off from the milled surface, due to the high rotational speed). As a result of the evaluation, we chose this CNC system to use it in our final cryosectioning study.

Pilot visualisation studies

In parallel with the pilot cryomacrotomisation tests, various software packages were assessed to gain in-depth knowledge of the visualisation, and to practice the image post-processing.

Study VI: Making the osseo-vascular 3D model of a canine head

An adult male French bulldog cadaver was used for the first 3D visualisation study. The arteries of the head and neck were filled with red polymethyl-methacrylate resin, and then this separated block was put into a container filler with biological activator (Septifos Vigor) and placed into a thermostat at 39°C. Following the maceration, the corrosion cast was scanned with an YXLON Precision microfocus CT (YXLON, Hamburg, Germany). Medical images from the scanning were imported into the Thermo Scientific Amira software, and vascular and osseous 3D stereolithography (STL) models were created with semi-automatic segmentation. After refining the 3D models with Autodesk MeshMixer, they were 3D-printed in actual size.

Study VII: Making the 3D model of an equine petrosal bone

The left petrosal bone of a horse skull was scanned with an YXLON Precision microfocus CT. Following the images being segmented in Amira, STL models were created for the petrosal bone, the auditory ossicles (malleus, incus, os lenticulare, stapes), the inner ear and for the facial canal. By taking photographs from the real bone in different angles, a direct comparison was achieved between the real and the virtual bones, by decreasing the opacity of the digital model and in this way visualizing the inner structures. Using the Blender software publicly available animations were created, and with the help of 3D-printing technology, the osseous inner ear and the auditory ossicles were printed in 3D and colorized.

Study VIII: Multimodal visualisation of a feline head

Using the imaging data from Study III, the cryosectioned, CT, and MR images were all imported into the same project view of the Amira program. The red, green and blue (RGB) images were aligned and converted to one volume, and then the CT and the MR data were co-registered to this image volume. Due to the small cryomacrotomisation slice interval, a multiplanar reconstruction with good resolution was obtained from the original dataset, together with the corresponding diagnostic images. Arteries of the cat head were modelled in detail, and a flip-book was also published based on the image series.

Study IX: Creating the MRI label map of a canine brain

This study was associated to an ongoing research series at the Eötvös Loránd University (ELTE, Budapest). Researchers at the Family Dog Project trained privately owned dogs to lay awake and motionless during functional MRI (fMRI) studies. To aid the fMRI analysis, an individual template brain was chosen, and a detailed MRI label map was created. Structural scans from 22 dogs of various breeds were produced with a Philips Ingenia 3T whole-body MR machine (Philips Medical Systems, Best, The Netherlands). Following anatomical evaluation of the shape, size, and gyral pattern of the brains in Amira, a template volume was selected, that showed the most typical mesaticephalic canine brain conformation. Subsequently, using the ITK Snap software 86 different label masks were made with manual segmentation of the main cortical and subcortical areas. As a part of the quality control, our individual template was co-registered to an averaged-based dog brain template (Nitzsche et al., 2018). Comparison of the two datasets confirmed a proper placement. In addition, to support the comparative MRI analysis across dog breeds, a normalization protocol was also developed.

The final study

Based on the experiences formed in the pilot studies, we devised the final cryomacrotomisation project. To ensure that our results would be comparable with other studies that show normal anatomical variations (Palazzi, 2011; Park et al., 2014) and to maintain comparability with previous widely accepted laboratory model species, we chose to use a beagle as a subject animal in our study in accordance with the replacement, reduction, refinement (3Rs) principles (Griffin et al., 2014). All husbandry and experimental procedures were approved by the Institutional Ethics Committee and the Hungarian Directorate for Food Chain Safety and Animal Health (PEI/001/956-4/2013).

Data acquisition. The subject of the study was a two-year-old female beagle dog. First, MR imaging was obtained using a 3T Magnetom TIM Trio whole-body MRI scanner (Siemens AG, Erlangen, Germany) with a 12-channel phased array head coil. Under the same anaesthetic episode and immediately following the MRI, CT scans were obtained using a Siemens Somatom Perspective 128 slice CT (Siemens AG, Erlangen, Germany). The arteries of the head were filled with red polyurethane rubber through the common carotid arteries postmortem, and the cadaver was placed into a -80°C deep freezer. The frozen head block was scanned with a YXLON Precision microfocus CT. A neurocranial block was made from the head, and we embedded it into a custom-made thermoregulated plexiglass box, using the previously tested gelatin solution as embedding material. The cryomacrotomisation was performed with the same Kondia NCT B-640 precision milling machine what we used in Study V. The milling interval was set to 100 µm, and overall 1112 RGB photographs were recorded from the milled surfaces with a Nikon D800 DSLR camera and macro lens with polarising filters.

Post-processing. Data from the cryosectioning and diagnostic imaging methods were imported into various software:

- first an RGB volume was generated from the cryosectioned slices in Amira, then it was co-registered with the CT and MR volumes, and also with the individual template from Study IX and its labels as to be in the same global coordinate system;

- the high-resolution CT image series (which was obtained from the microfocus scanning) was opened with 3D Slicer, and the endocranial volume was segmented from the skull. The resulting endocranial cast (endocast) was converted to Adobe 3D PDF-format, where the model was annotated;

- using a combined action series with Adobe Photoshop CS3, the main arteries and veins were filtered out from the RGB images. This resulted in two greyscale-converted image series, which were imported to the same Amira project view where the main image volumes were already present;

- a semi-automatic segmentation was performed with Amira, and 3D models were generated from the segmentations' outcome that comprised the skull, brain, arteries and veins. The STL models were refined with Autodesk Meshmixer;

- utilizing the different 3D visualisation modules of Amira, multimodal comparisons were made, and based on the high resolution RGB data multiplanar reconstructions were created in the other orthogonal (dorsal and sagittal) planes;

- the endocast was 3D-printed in actual size using polyamide powder with selective laser sintering (SLS) technology.

3. Results and discussion

The CNC milling system we used provided optimal workflow and decreased artefact-formation. Together with applying high-resolution DSLR photography, the beagle brain and the surrounding structures were visualised with a high level of clarity and detail. The resolution and the quality of the images were demonstrated in the dissertation with close-up views, showing the fine details. Despite the fact that we did not use tissue staining (in order to preserve the original colours), the boundary between the grey and white matter and the location of the major subcortical nuclei could be clearly identified.

The relatively small (100 μm) slice interval made it also possible to reconstruct the other orthogonal (dorsal and sagittal) planes without losing the details of the individual structures. This means that even at higher magnifications, the structures of the computer-reconstructed (CORE) slices appear sharp, detailed and uninterrupted, as if the cryosectioning had occurred along that plane. Registering the CT and MR image series to the cryosectioned volume resulted in a nearly perfect comparison between the different imaging modalities.

As a result of the digital endocasting, another tool was provided to study the morphology of the brain: due to its detailed surface, not only the impressions of the gyri and sulci, but the placement of some intracranial blood vessels was also recognizable. The vascular 3D modelling showed the arteries and the veins inside and around the neurocranium, and it was combined with the data from the structural imaging and cryosectioning techniques. Finally, to put all the information together, labelled neuro-anatomical illustrations were created, which show the original and CORE images with MR, CT, and extensive annotations.

When comparing our work to other projects that have aimed to visualize the dog brain, advantages and novelties contain:

- only one specimen was used to obtain all the images (3Rs);
- both ante- and postmortem CT and MR scanning was done;
- no formalin fixation or histological dyes were used;
- there was no need to remove the brain from the skull;
- cryosectioning focused on just the neurocranium and brain;
- the cryosectioning interval was only 100 μm;
- images were captured with a high level of detail (24-bit colour depth, 300 DPI, 7360×4912 pixels, 19.5×19.5 μm resolution);
- originally 1112 transverse sections were captured, but due to the small voxel size detailed images in any other orthogonal or oblique plane could be created with a proper 3D software;
- co-registering the cryosectioned volume to the MR and CT datasets provided directly comparable multimodal images;
- based on the imaging data, segmentation was performed to create various 3D models (e.g., skull, endocast, vasculature).

Moreover, the knowledge derived from our studies already resulted in practical benefits. We illustrated different books, a cooperation was formed with the Semmelweis University to carry out human cryosectioning studies, and recently we started working with an information technology group which develops mixed reality technology. The normalization procedure and the MRI label map were used in canine fMRI studies, and with a veterinarian colleague we performed multiple brain surgeries in Hungary to treat small animals having neurological disorders.

To conclude, the applied cryosectioning technique proved to be a unique complementary tool for studying cross-sectional anatomy and to create volumetric data. We saw advantageous outcomes concerning the neuroanatomical know-how and the visualisation abilities in various academic and clinical fields.

4. New scientific results

Ad 1. Providing a high-resolution, multimodal, macro-anatomical image series from a canine brain.

The image set we recorded in the final study has the highest level of detail among the macro-anatomical canine brain studies conducted to date. The high-resolution digital photography and the small milling interval enabled us to create different multiplanar reconstructions, annotated illustrations and 3D models, which could be directly compared with the CT and MR imaging datasets of the same animal.

Ad 2. Developing a custom-made cryosectioning workflow.

The cryomacrotomisation procedure up to this point had only been used in a couple of research facilities across the world, and it requires dedicated teams and specially designed cryosectioning devices. We developed our own method by testing various milling systems and freezing settings. An optimal setup proved to be feasible for carrying out high-quality studies with a small team and an engineering CNC machine.

Ad 3. Creating a canine brain's MRI label map for research.

To provide an aid in the fMRI analysis and to complete the cryosectioning study with a digital neuroanatomical guide, an individual brain template was chosen and an MRI label map was created which comprises the main cortical and subcortical structures of a dog.

Ad 4. Composing publicly accessible 3D models from complex anatomical structures.

During the visualisation studies different 3D models and publicly available videos and images were created, like an osseo-vascular model of a French bulldog head and neck demonstrating the blood supply; a transparent digital equine petrosal showing the various intraosseous structures on images and animations; and arteries with different details of a cat head.

Ad 5. Associated advances in canine neuroscience and small animal neurosurgery.

The achievements in these fields are connected to our main project as the results of its direct scientific and clinical utilisation; with the fMRI analysis we identified the functional resting-state network system of awake dogs, and the knowledge in neuroanatomy and surgical planning allowed us to perform brain surgeries in dogs on a regular basis, thereby enabling a new clinical field in veterinary medicine in Hungary.

5. Scientific publications

Publications in peer-reviewed journals related to the thesis

Czeibert, K., Baksa, G., Grimm, A., Nagy, Sz. A., Kubinyi, E., Petneházy, Ö.: **MRI, CT and high resolution macro-anatomical images with cryosectioning of a Beagle brain: Creating the base of a multimodal imaging atlas**, PLOS ONE, 14. e0213458, 2019.

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Books, book chapters

Authorship: Czeibert, K., Baksa, G., Grimm, A., Nagy, Sz. A., Horák, D., Petneházy, Ö.: **FlipCat: Cat's head seen on MRI and cryosections**, Budapest: Műszaki Könyvkiadó Kft., 2015.

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Czeibert, K., Baksa, G., Grimm, A., Nagy, Sz. A., Kubinyi, E., Petneházy, Ö.: **Comparative imaging anatomy of the canine brain with MRI, CT, cryosectioning and 3D-modeling**. *65th Meeting of the American Association of Veterinary Anatomists (AAVA).* Banff, 26-29 July 2019.

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