microCT for Systematic Mouse Phenotyping

Frantisek Spoutil, Michaela Prochazkova, Tereza Michalcikova, Ivana Uramova, Sarah Clewell, Vendula Novosadova and Jan Prochazka
Czech Centre for Phenogenomics, BIOCEV - IMG, Prumyslova 595, Vestec, Czech Republic

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Abstract: Phenotyping of mouse mutants is one of the crucial methods for uncovering genetic network at the level of a whole organism which could help us to understand origin of rare diseases, developmental malformations, but also the process of mammalian evolution. For studying morphological aspects of either embryos or adults, the X-ray computed microtomography (microCT) has become a gold standard within the last years. The three-dimensional (3D) context, availability of data to additional analysis (e.g. volumetric, bone density, or body composition), and *in-vivo* approaches in the case of adults are the main advantages when compared to classic histology and bone morphology. On the other hand, the amount of data is enormous making the data storage and analysis the bottle-neck of the microCT method. To overcome this obstacle, we cooperate with bioinformatics experts to set up automation of the process at maximal possible level. Nevertheless, knowledge and experience of a specialist remain indispensable.

1 INTRODUCTION

The aim to understand and fully annotate mammalian genome led to establishment of the International Mouse Phenotype Consortium (IMPC) for the systematic generation and analysis of all coding sequence mutations in mice. On the basis of IMPC, we have built an advanced phenotyping pipeline using in-vivo microCT scanning technology for adult mutant mouse cohorts. The primary challenge of implementing 3D phenotyping of embryos using microCT technology was to create standardization across all stages of embryonic development. However, the advanced, high resolution microCT scanning allows for detailed morphological analysis of embryos from the earliest developmental stages up to perinatal period. The standardization of the whole process, as established by IMPC, is critical for relevant comparison and reproducibility of data between research centers.

The advantages of 3D data generation compared to more conservative approaches, such as plain X-ray imaging, for adult skeletons or histological analysis of embryos are obvious. While the products of microCT imaging are incredibly detailed, the system requires significant and time-consuming efforts in order to process and further analyse the large 3D datasets. This article will provide an overview of the standardised morphological phenotyping pipeline, as well as outline the data analysis in further detail.

Here we present the contemporary, state-of-the-art high-throughput embryo and bone morphology pipelines of mouse phenotyping used in our department with the help of microCT technology, as well as the obstacles we try to solve to reduce the disadvantages of the method. We hope this approach can make the microCT method more accessible to broader spectrum of researchers, better their results significantly, and reduce amount of animals used in the experiments in the future.

2 METHODS

2.1 Embryology

The standardised embryo phenotyping pipeline contains multiple critical steps for reproducibility and reliability of data: proper and unified embryo breeding, embryo harvest and fixation, contrasting of soft tissues, microCT scanning with metadata recording, data reconstruction and processing, data upload to public open database mousephenotype.org.

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2.1.1 Embryo Phenotyping Workflow

To determine the effects of embryonic lethal gene mutations on development, embryos are harvested from pregnant females a strictly systematic way, leading to identification and characterisation of lethal phenotypes. The initial stage for investigation is E12.5. In case there are no living knockout (KO) embryos at this stage the E9.5 embryos are analysed. If KO embryos are absent at E9.5, earlier developmental stages are then analysed individually. In case there are living KO embryos at E12.5, the subsequent developmental stages (E14.5 and E18.5) are analysed.

After mating, females are visually examined every morning for a presence of the vaginal plug, which indicates embryonic day 0.5 (E0.5) of development.

Gravidity is confirmed by a weight gain or at earlier stages by ultrasonography. During embryo harvest on the desired day of embryonic development, yolk sacks are collected for genotyping and embryos are fixed with 4% paraformaldehyde (PFA).

2.1.2 Embryo Contrasting

High-resolution microCT provides an opportunity to visualize embryos at various stages of development in 3D. Due to weak tissue mineralisation, a contrast agent must be applied to all specimens.

Smaller samples, e.g. E9.5 embryos, are fixed for 24 hours in 4% PFA and stained with 1% PTA (phosphotungstic acid) for up to 2 weeks. Larger samples, e.g. E18.5 embryos, are fixed for 1 week in 4% PFA and stained with Lugol's Iodine solution for 2 weeks or longer. Lugol's stock solution (10g KI and 5g I_2 in 100ml H_2O;) is diluted to 25% working solution in H_2O to achieve neutral osmotic pressure to avoid tissue distortion.

2.1.3 Embryo Scanning and Reconstruction

Stained specimens are removed from the contrast agent, rinsed with PBS and embedded in 2.5% low-gelling temperature agarose in tubes. Tubes of various sizes are used, depending on embryo size, (single 0.2ml PCR microtubes, 2ml microtubes with caps or 15ml falcon tubes cut to desired length). All specimens have to be wrapped in Parafilm to prevent evaporation.

Depending on the embryo size, SkyScan 1272 high-resolution microCT (Bruker, Belgium) is set up for voxel size 0.2 - 7µm, and 0.5 or 1 mm Al filter. 360° scan with 0.200° rotation step and 3 frames averaging setup is used for scanning. Scanning takes from 5 to 20 hours per one sample, depending on size. Automated scanning of multiple samples is acquired by using sample carousel.

InstaRecon CBR Premium software (InstaRecon, USA) is used for reconstruction. The setup of reconstruction parameters such as smoothing, ring artefacts correction, beam hardening and intensities depends on the embryonic stage.

2.1.4 Embryo Phenotyping

Within the embryonic lethal screen, three knockout embryos and one littermate, wild-type embryo are scanned and their phenotypes are evaluated. Gross morphology (growth retardation, development of limbs, formation of orofacial area, etc.) is assessed in whole-mount images and defects of inner organs (positioning and size of organs, tooth development, presence of cleft palate, etc.) are examined in the virtual sections.

2.2 Adult Morphology

For standard morphological phenotyping, a cohort of 28 mice (14 wild types and 14 mutants, composed from 7 males and 7 females each) at the age of 13 weeks is scanned *in-vivo*.

2.2.1 Adult Morphology Workflow

Each mouse is anesthetized by Zoletile injection, arranged in natural position and scanned in SkyScan 1176 in-vivo microCT (Bruker, Belgium). After scanning, the mouse is weighed and some basic body measurements are obtained with digital dial calliper.

Two reconstructions are produced from the primary data: i) for skeletal morphology and bone mineral density (BMD), and ii) for body composition analysis.

While the bone morphology is evaluated directly from its reconstruction files, for body composition analysis the core body is selected first before entering the analysis. Volume of interest (VOI) from body composition analysis is used also for BMD analysis excluding skull, tail and distal limbs.

The whole process takes about 34 working hours and 0.314 TB per cohort. See Figure 1 for a detail.

2.2.2 Mouse Scanning and Reconstruction

SkyScan 1176 in-vivo microCT (Bruker, Belgium) is set up for voxel size 35 µm, voltage of 50 kV, current of 160 µA, and 0.5 mm aluminium filter with 180° rotation. When using these parameters, scanning one

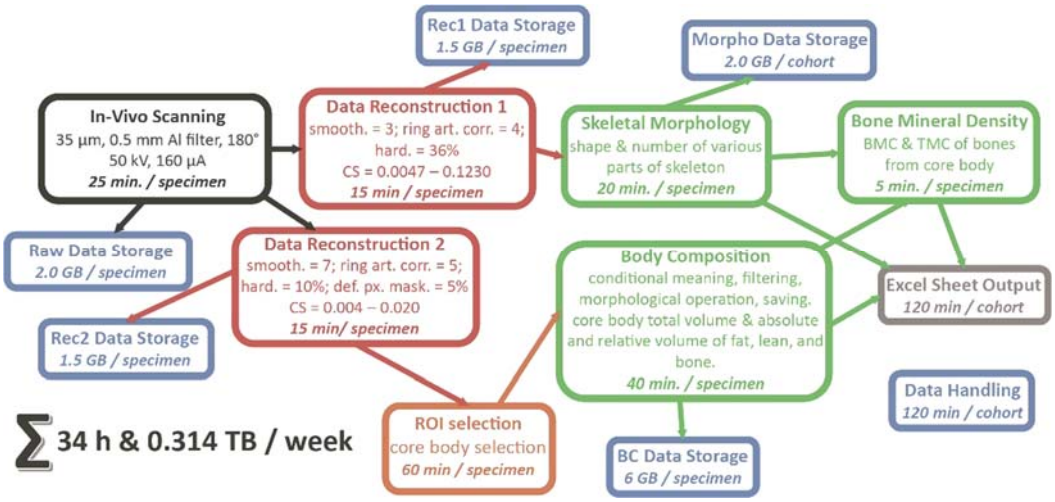

Figure 1: Scheme of adult mouse morphology and body composition workflow. Black: data acquisition; Red: data reconstruction; Orange: data preparation; Green: analysis; Blue: data storage; Grey: Excel output. smooth. = smoothing; ring art. corr. = ring artifact correction; hard. = beam hardening; def. px. mask = defect pixel masking; CS = border intensities for reconstruction.

mouse takes about 16 minutes and the mouse takes about 55.4 mGy/min.

For reconstruction InstaRecon CBR Premium software (InstaRecon, USA) is used. In the case of bone morphology, the reconstruction parameters, as recommended by Bruker microCT (Belgium) are set up as follows: smoothing = 3, ring artefact correction = 4, beam hardening = 36%, intensities = 0.0047 – 0.1230. For body composition analysis the values are changed to: smoothing = 7, ring artefacts correction = = 5, beam hardening = 10%, defect pixel masking = 5%, intensities = 0.0040 – 0.0200.

2.2.3 Bone Morphology Phenotyping

CTvox software (Bruker microCT, Belgium) is the basic tool for bone morphology evaluation used. We record 53 qualitative and numerical variables describing axial, brachial, and cranial skeleton to localize effect of the mutation. 8 standardized views on the whole skeleton or its selected parts (cranium, limbs) are taken for each sample, as well as details of malformations if they occur.

2.2.4 Body Composition Analysis

CT analyzer (CTan: Bruker microCT, Belgium) and Batch Manager (BatMan: Bruker microCT, Belgium) software are used for VOI selection as well as analysis itself.

The analysis is based on different density of bones (and teeth), lean, and fat (with lungs) which is then distinguishable by X-rays. Primary data includes absolute and relative volume of all three parts, which are recalculated to absolute and relative mass assumptions of lean and fat.

Machine learning-based procedure is developed and tested at our centre to automatize the VOI selection.

2.2.5 Bone Mineral Density

The same software is used also for evaluation of bone mineral density (BMD) and tissue mineral density (TMD) within the VOI of body composition analysis.

The values are calculated based on results from calibrated hydroxyapatite phantoms scanned and reconstructed under the same conditions.

Two secondary variables are computed from the basic ones: bone mineral content (BMC), and BMC per body mass.

2.3 Bioinformation Solution

As one of the primary goals in phenotyping is to implement a high-throughput approach, maximum automation of the whole procedure with the help of machine-learning is optimal for our methodological development, now and in the future. Although we want to end up with total automation, we start step by step by solving the most time-demanding problems, which are i) VOI selection and ii) extraction of analysis results, for body composition analysis.

Manual selection in CTan was originally used for VOI definition. The biggest challenge for automation is how to train the software to delineate what is the

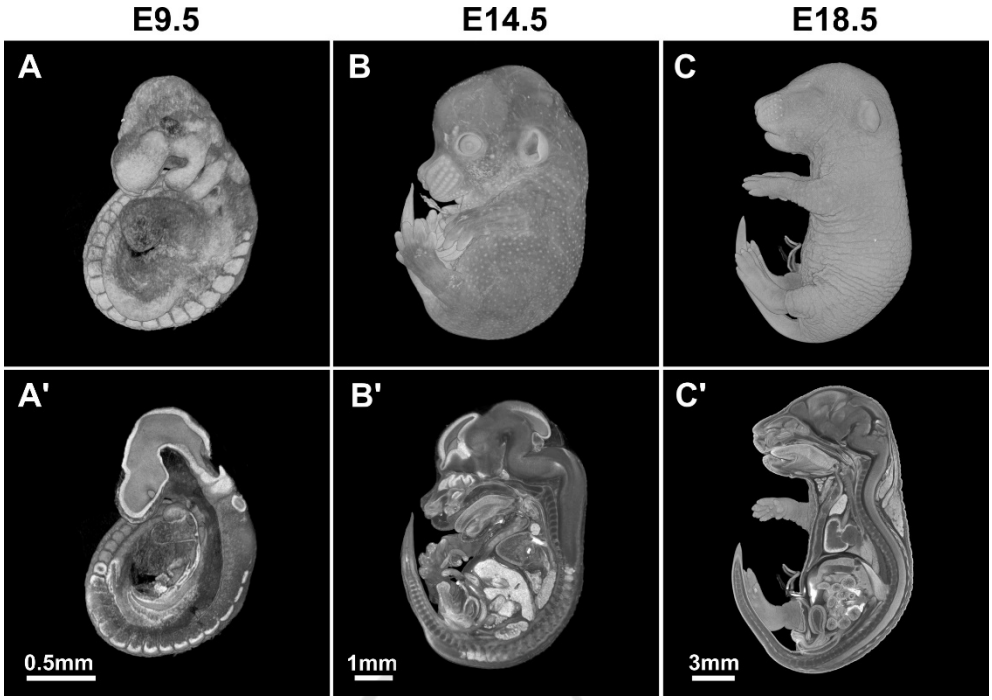

Figure 2: MicroCT scans of wild type murine embryos at different stages of development. A-C - whole-mount views, A'-C' - virtual sagittal sections.

relevant specimen (i.e. mouse), as the size, shape, and even orientation of some parts (e.g. limbs) have significant variability between samples and slices. To overcome this huge variability, machine-learning using U-net deep neural network in PyTorch (Paszke et al., 2017) with principal component analysis based reorientation in R (R Core Team, 2015) and RStudio (RStudio Team, 2016) with EBImage (Pau et al., 2010), pixmaps (Bivand et al., 2015), and magick (Ooms, 2018) libraries are applied based on previous manual selection. Although the algorithm is still under development and results need to be check by a specialist, it has already increased the efficiency of VOI selection 15:1 hour per whole mouse cohort (i.e. 28 mice). The data for VOI selection is rotated first with principal component analysis (PCA), the starting virtual section image for VOI is selected based on the shape similarity in the cervical area, and then the software crops all images section by section, i.e. in 2D to keep core body only. The accuracy of selecting the 1st virtual slice is very high and differs a few slides from selection of specialist, especially in animals with less standard position (e.g. when shoulders are moved more cranially). We were testing also VOI selection based on 3D model, but the results were comparable to 2D model approach, but computation time was longer. Moreover, 2D approach enables better correction of results by specialist. While training the algorithm for automatic selection of the areas of interest, we used 100 mice (more than 60 thousands virtual sections), which were manually corrected. The final algorithm has been now used for more than 1000 mice, and will be retrained to achieve increasingly higher accuracy.

CTan saves results of every analysis to .CSV table format with all procedures data acquired. The ideal final stage of automation would connect the ROI selection and data extraction with CTan's macro for body composition analysis along with its sophisticated methods for smoothing, noise reduction and separation.

However, the real challenge is automation of bone morphology. We want the software to be able to distinguish and identify individual bones or parts of the skeleton (e.g. spine, skull, paws), to compare them with standard shape variability of baseline, and highlight any differences worthy of attention of a morphologist. Although some similar approaches already exist (e.g. Baiker et al., 2010, Wise et al., 2013), their results are not fully applicable for our demands and their optimization or finding of original solution will be needed.

3 RESULTS & DISCUSSION

3.1 Embryo Morphology

Within the embryology lethal screen, we have at the moment phenotyped 12 lineages. One of the lineages was lethal before E9.5, four were perinatal lethal and two were identified as “subviable” lineages. The rest of strains were lethal between E9.5 and birth. See Figure 2 for examples of scans and virtual sections of wild type embryos at different developmental stages. For embryos older than E9.5, we used the Lugol’s solution as contrast agent. Noteworthy, dilution of working solution in water and not PBS turned out to be methodologically crucial as this approach does not cause tissue shrinkage.

We have observed various pathologies, such as growth retardation, short face, and heart and intestinal dysmorphology in the embryos. We could evaluate the latter mentioned phenotypes thanks to the high resolution microCT scanning. It would be very difficult or even impossible to get this data from classical histology, especially in cases where we assessed the length and shape of the inner organs (e.g. in case of embryonic intestine).

Our next goal in embryo screen is the adoption of an atlas-based approach of organ recognition shared among the IMPC centers (eg. Brown et al., 2018), which will point out even slighter differences in organ shape and position to a researcher, and quantify the volume.

3.2 Adult Morphology

In-vivo microCT use in adult mouse morphology brings numerous advantages compared to standard, 2D X-ray imaging: the level of detail is much higher, we are able to select appropriate angle and section of view to show a structure of interest without compromise from X-ray shielding by surrounding tissues. In that way we are able to observe structures like rib rudiments on cervical vertebra, even slight opening of skull sutures or dorsal arches of vertebrae, occurrence of *baculum* in females, or ossification in tendons of mouse. See Figure 3. This results in higher probability an effect of mutation will be uncovered (especially in heterozygotes) and that we will be able to separate it from general genetic background of the mouse strain. We have scanned and analysed 27,825 WT mice of both sexes so far, which serves as baseline of comparison for the relevance of abnormal morphology findings in KO mutant mice. There were 784 WT mice (2.74 %) with some abnormal morphology findings. This number is almost two times lower than in KO mutant mice (all mutations together), where some abnormality was recorded in 1680 animals from 33,263 (4.81 %).

The greatest advantage of microCT though, is the possibility to reuse the data of spatially different X-ray absorption repeatedly. This quality is used also for body composition analysis, where double-energy X-ray analysis (DXA) is standard for IMPC. In the case of DXA the amount of fat and lean is computed from differences in absorption of X-rays of two energies. MicroCT brings another quality: spatial distribution of fat and lean, which is important especially for fat tissue, as differences there are clinical differences, whether the fat is stored more subcutaneously or viscerally. In our department, significant differences in baseline WT body composition were found in 17 of 46 KO mutant mouse gene cohorts with this analysis so far.

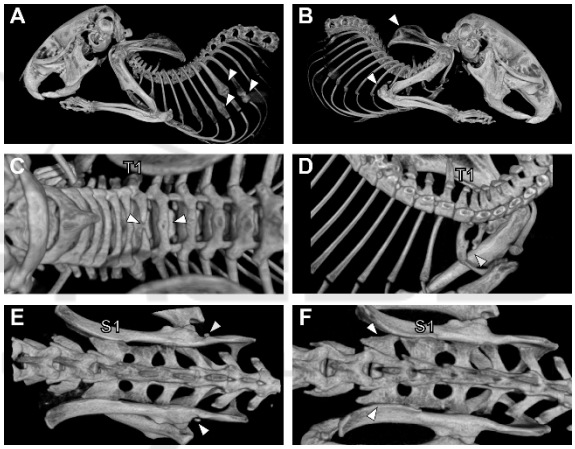

Figure 3: Examples of skeletal dysmorphology. A) Rib osteoneogenesis due to injuries in mouse mutant (compare to B and D). B) Malformation of scapula and ulna in mouse mutant (compare to A). C) Malformation of the 1st and 2nd thoracic vertebra in wild type mouse: 1st one without fusion of dorsal arch, 2nd one without prolongation of spinal process (compare to D). D) Cervical rib of 7th cervical vertebra causing malformation of the 1st thoracic rib in mouse mutant. E) Extra ossification in pelvic region of mouse mutant (compare to F). F) 6th lumbar vertebra with morphology of the 1st sacral one in mouse mutant (compare to E). S1: 1st sacral vertebra; T1: 1st thoracic vertebra; arrowheads: pointing to mentioned malformation. Figures not in the same scale.

Magnetic resonance imaging (MRI) can be used for body composition and even for general skeletal morphology too. Its results are even much better for soft tissue morphology. However, the crucial disadvantage (besides much higher working costs compared to microCT) is, that it isn’t able to quantify bone density.

In the case of microCT, its computational and time requirements are as summarized in Figure 1. Increasing of computational power and machine learning programming, which we are working on and which was summarised elsewhere (Spoutil et al., 2018), we will be able to push usability of microCT for standard phenotyping procedure to broaden the spectrum of usage and users. In the case of body composition, the next goal is to teach the software to differentiate hard particles of food from bone and remove them from sections, plus smooth artificially-increased intensities in their surroundings causing star-like artefacts, which can distort real borders of fat and lean, and thus their estimated volume. In the case of bone morphology, we are planning to use a 3D atlas-based approach similar to embryo screen (e.g. Baiker et al., 2010) able to highlight significant changes from mean morphology of individual bones, as well as sections of skeleton.

We have clearly demonstrated that the data quality of our approach is equal or higher than in the standard 2D methods used in descriptive morphology and anatomy of embryos and adults of mice due to lower tissue deformation, full 3D spatial context, re-usability of data etc. Replacing the work of specialists with machine-learning and automation of the procedure is the way to overcome the biggest disadvantage of the method time demands. Its application brought us first significant time savings. Nevertheless, we still believe, the main role of the computers in this process should be to help researchers to focus more on data of their interest, instead of fully automatic analysis. This is the way we want to direct our future development of our procedure.

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