Exploring MicroCT and its potential to visualise and score a phenotype in neonatal mouse models.

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Abstract
Developmental studies of animal morphology require accurate visualisation of three-dimensional structures. As part of the Deciphering Mechanisms of Developmental Disorders (DMDD) program we use micro computer tomography (MicroCT) to visualize soft tissue structures and anatomy. Here, we describe our Lugol's based staining protocol to enhance MicroCT imaging of soft tissues for comparative morphology of wild-type and transgenic neonatal mouse using MicroCT Skyscan 1172 and available software. We describe how MicroCT represents a powerful imaging system to spot variations in morphology.

Introduction
The Deciphering Mechanisms of Developmental Disorders (DMDD) consortium studies embryonic and perinatal lethal knockouts in mice. Multiple embryos from each line are imaged in 3D at near-histological resolution to allow comprehensive morphological phenotyping. Parallel screens identify placental abnormalities and provide gene expression profiles for each line. All DMDD data is freely available (dmdd.org.uk). As part of this screen we also include imaging by MicroCT of neonatal pups.
The phenotyping efforts of DMDD screen have focused on *in utero* development. However, out of 240 lines which have come through the screen to date 18% are viable at embryonic day E.d.18.5, suggesting lethality through the neonatal period. The embryonic phenotyping screen cannot determine causes of death for many of these lines. By using MicroCT imaging we systematically phenotype perinatal lethal and sub-viable lines in order to obtain greater insight into whether lethality in such lines results from structural and anatomical defects. Together with standardised *in vivo* and *ex vivo* phenotyping tests, the MicroCT is used to further understand and visualise abnormalities of the internal organs. Using X-ray imaging on soft tissues carries consequences, due to low X-ray absorption of non-mineralised tissues. Discrimination between soft and hard tissue relies on differences in photon attenuation levels of these types of tissue. Bone, with its calcium phosphate minerals, attenuates X-rays more intensively than the surrounding soft tissues such as muscles and organs. Soft tissue is mainly composed of low atomic number elements, which results in low contrast level. Therefore, effective heavy metal stain protocol has to be use to enhance the visualization

Materials and methods
Animals:
The majority of the mouse P0 new-born samples used in this study was generated using wild-type animals. Few samples were generated using mouse lines carrying specific gene deletion. All lines were produced and maintained on a C57BL/6N genetic background at the Wellcome Trust Sanger Institute (http://www.mousephenotype.org/) as part of the DMDD project. Use of all animals was in accordance with UK Home Office regulations, the UK Animals (Scientific Procedures) Act of 1986 and was approved by the Wellcome Trust Sanger Institute's Animal

Welfare and Ethical Review Body. Neonatal mouse pups (P0) were generated from timed-matings. Females on average littered at E.d.19.5 days of gestation counting the day of the vaginal plug as E.d.0.5. Pups were culled after phenotypic assessment using 0.05ml Pentobarbital. Before fixation, two scoring cuts were performed on the lower trunk sides, to assure fixative and stain penetration. Samples were fixed in 10% Buffered Formaldehyde for a minimum of 7 days and maximum, as in this experiment, to a year.
Sample process:

Figure 1a

Figure 1b

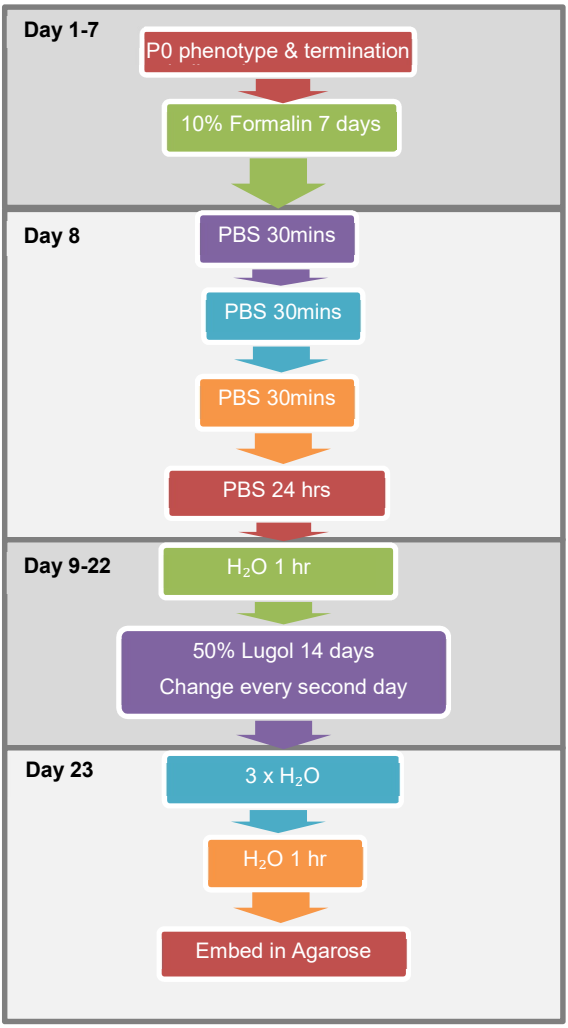

Figure 1c

Figure 1 a) Litter of neonate pups (P0); b) P0 sample embedded in Agarose gel; c) Workflow for staining protocol

After fixation samples are washed in phosphate-buffered saline (PBS) three times 30 minutes and one time for 24 hours. To remove any salt residues, samples are then wash in purified water for one hour and then placed into 50% Lugol staining solution for 14 days. Staining solution is replaced with fresh one every other day. After absorption of Iodine was completed samples were washed in purified water for an hour and embedded in 1% Agarose gel into a 5ml bijoux tube. Samples were scan on the same day to avoid Lugol leaking into Agarose as this will affect data quality.
Scans are performed on SkyScan 1172 version 1.5 on medium resolution, pixel size 9.01um with Copper/Aluminium filter and voltage 74kV. Images reconstruction is performed using NRecon 1.7.0.4. Results are first aligned in DataViewer and subsequently images are adjusted and analysed in CTvox.

Results
The Lugol's stained protocol described here works well on all soft tissues, and stain penetrates neonate samples giving good contrast to distinguish tissues and organs. Initial problem with skin barrier and artefact was overcome by simple skin scoring, which allows better fixation resulting in better quality image. It is possible to stain necrotic tissue with good quality resulting images, however it is recommended to fix samples as soon as possible, due to tissue decomposition as this will result in tissue shrinkage.

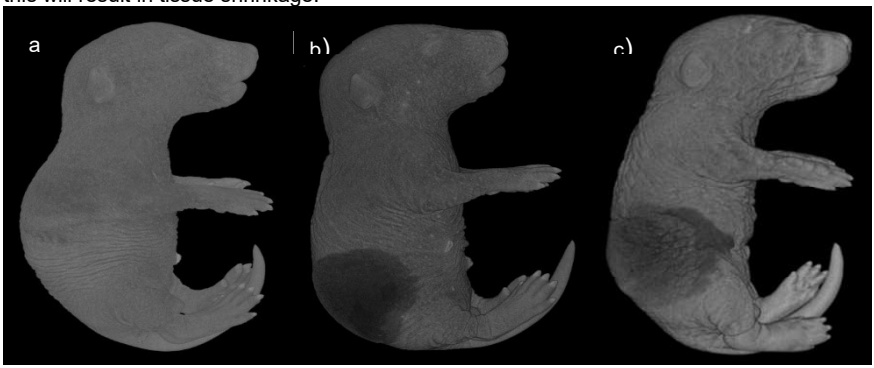

Figure 4.a) Scored neonate with two cuts on the lower sides, b) Unscored neonate showing artefact on the lower part of the trunk (darker spot extending dorsal to ventral) c) Dead neonate displaying similar artefact as b and tissue shrinkage.

Closer examination of internal structures, in some samples, revealed penetration issues (Fig. 3aG). Those were resolved by scoring the dorsal trunk skin and by fixing the sample immediately after collection. In some examples blood residues in organs may give an overexposed image, but those can be adjusted using primary setting and later parameters in the software. (Fig. 3aH)

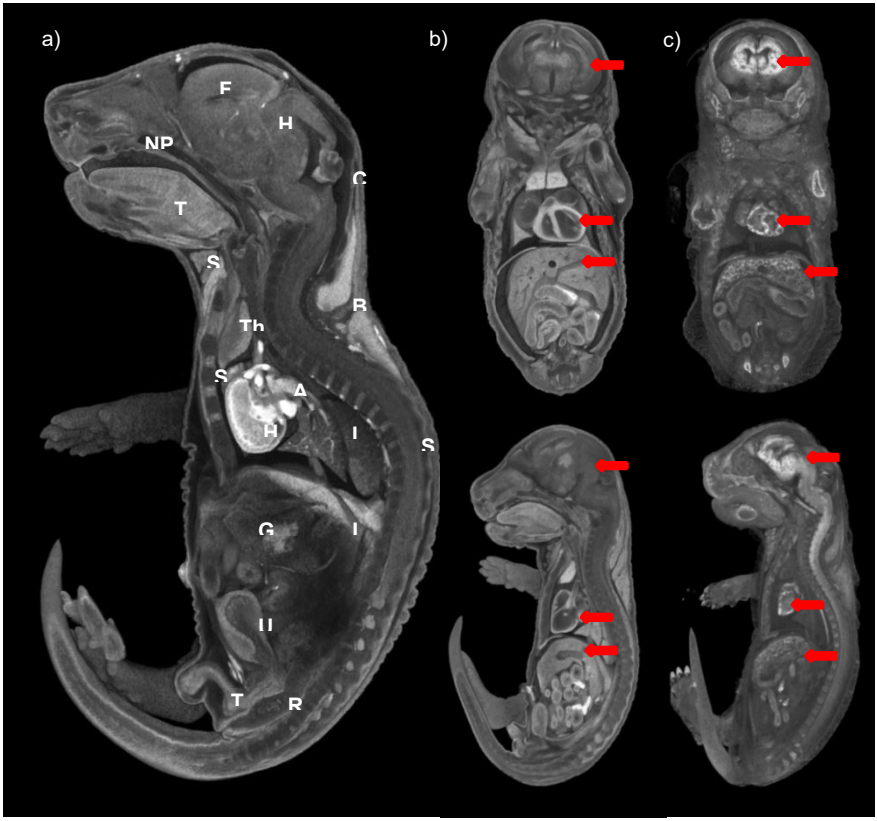

Figure 5.a) saggital section of scored neonate, clearly showing internal strudctures and recognisable organs and their morphology. Fb – forebrain, Hb – hindbrain, C – cerebellum, T – toungue, SG – submaxillary gland, Th – thymus, Br – brown adipose, S – sternum, H – heart, A – aorta, L – lungs, SC – spinal cord, G – gut, Li – liver, U – urinary bladder, Te – testi, R – rectum; b) and c) transverse and saggital sections of two heterozygous neonates showing differences in organ shape and morphology; red arrows indicate brain, heart and liver with clear scan and organ proportions on b) and abnormal tissue structure and tissue shrinkage on c)

Conclusions

The protocol described here has an amazing potential for the scientific community to become a tool for visualizing whole body internal structures for mouse new-borns. The protocol can be further refined to target distinct structures. The high-quality images acquire with this method constitutes a powerful resource, as it allows generating a substantial amount of data from a single sample that can be analysed.

We have experienced minor problems with visibility of tissues in the intestinal area, due to already developed skin barrier. That resulted in poor visibility of this area and was overcome by

skin scoring before fixation. There is room for improvement of the Lugol's protocol and enhancement of a tissue fixation and staining.
Using the method we described we have now generate a wildtype baseline that will be used to compare 3D reconstruction of wild-type versus genetically modify mouse pups, to allow comprehensive morphological phenotyping. We are currently looking into quantified software, like 3D segmented mouse atlas that can measure and recognise organ volume differences between wildtype and knockout mice. It also can automatically measure population average based on the wildtype base line and help with phenotypic measurements for the assessment of mutant mouse phenotype in high-throughput pipelines. Our images resource together with a more advance quantified software could make MicroCT scanning a reliable source of information about dysmorphologies carried by specific gene mutation.

References

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