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Animals In Synchrotrons: Overcoming Challenges For High-Resolution, Live, Small-Animal Imaging

Martin Donnelley[1], David Parsons[1,2,3,4], Kaye Morgan[5], Karen Siu[5,6]

[1]*Respiratory and Sleep Medicine, Women's and Children's Hospital, Adelaide, South Australia.*
[2]*Women's and Children's Health Research Institute, Adelaide, South Australia.*
[3]*Department of Paediatrics and Reproductive Health and* [4]*Centre for Stem Cell Research, University of Adelaide, South Australia.*
[5]*School of Physics and* [6]*Monash Centre for Synchrotron Science, Monash University, Melbourne, Victoria.*

Abstract. Physiological studies in small animals can be complicated, but the complexity is increased dramatically when performing live-animal synchrotron X-ray imaging studies. Our group has extensive experience in high-resolution live-animal imaging at the Japanese SPring-8 synchrotron, primarily examining airways in two-dimensions. These experiments normally image an area of 1.8mm x 1.2mm at a pixel resolution of 0.45µm and are performed with live, intact, anaesthetized mice.

There are unique challenges in this experimental setting. Importantly, experiments must be performed in an isolated imaging hutch not specifically designed for small-animal imaging. This requires equipment adapted to remotely monitor animals, maintain their anesthesia, and deliver test substances while collecting images. The horizontal synchrotron X-ray beam has a fixed location and orientation that limits experimental flexibility. The extremely high resolution makes locating anatomical regions-of-interest slow and can result in a high radiation dose, and at this level of magnification small animal movements produce motion-artifacts that can render acquired images unusable. Here we describe our experimental techniques and how we have overcome several challenges involved in performing live mouse synchrotron imaging.

Experiments have tested different mouse strains, with hairless strains minimizing overlying skin and hair artifacts. Different anesthetics have also be trialed due to the limited choices available at SPring-8. Tracheal-intubation methods have been refined and controlled-ventilation is now possible using a specialized small-animal ventilator. With appropriate animal restraint and respiratory-gating, motion-artifacts have been minimized. The animal orientation (supine vs. head-high) also appears to affect animal physiology, and can alter image quality. Our techniques and image quality at SPring-8 have dramatically improved and in the near future we plan to translate this experience to the Imaging and Medical Beamline at the Australian Synchrotron.

Overcoming these challenges has permitted increasingly sophisticated imaging of animals with synchrotron X-rays, and we expect a bright future for these techniques.

Keywords: Synchrotron, X-ray, phase contrast imaging, mouse, lung, airway.
PACS: 87.85.Pq

INTRODUCTION

There is little scientific literature available about how to perform live animal imaging using synchrotron radiation. Our group has extensive experience in animal model studies at the Women's and Children's Hospital in Adelaide, where we are researching gene therapy treatments for cystic fibrosis airway disease[1, 2]. We have transferred and adapted these animal handling techniques to high-resolution live-animal imaging at the Japanese SPring-8 synchrotron. We typically perform 2D longitudinal imaging studies of airways or air containing structures using a field of view of 1.8mm x 1.2mm at a pixel resolution of 0.45µm. In this paper we describe our experimental techniques, indentify areas that limit or alter standard experimental approaches, and describe some of the ways we have overcome the challenges involved in imaging the airways of live mice using a synchrotron.

There are many advantages to using synchrotron X-rays, including the ability to acquire very high-resolution images, as well as to utilize techniques such

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as phase contrast imaging[3, 4]. However there are also a number of challenges involved in utilizing this unique imaging modality. Firstly, imaging is confined to a specialized imaging hutch, a lead-lined experimental room attached to the end of a synchrotron beamline. The hutch is essential for containing the intense radiation produced by the high flux source. This means that when performing live-animal imaging it is necessary to perform remote animal monitoring, maintain stable anesthesia and remotely deliver any test substances or pharmaceuticals. Secondly, the fixed beam location and orientation limits experimental flexibility in terms of animal restraint and positioning. Finally, the high imaging magnification produces its own set of challenges including the significant time and radiation dose required to locate a small region of interest within complex anatomy, minimizing motion artifacts produced by respiratory, cardiac and skeletal muscle movements, and eliminating the image artifacts produced by the animals fur.

SYNCHROTRON IMAGING SETUP

Experiments are typically performed on the BL20XU undulator beamline at the SPring-8 synchrotron radiation facility in Japan. Here a 25keV monochromatic beam is used for synchrotron phase contrast X-ray imaging (PCXI). PCXI provides enhanced image contrast by utilizing X-ray refraction in addition to conventional absorption and is particularly useful for achieving soft tissue contrast where the absorption differences are small. Tissue boundaries are enhanced by the phase changes induced by differences in their X-ray refractive indices, provided the X-ray beam has sufficient spatial coherence and the sample to detector distance is sufficiently long[5, 6], characteristics that are achievable using a synchrotron source. We have demonstrated the use of PCXI for novel non-invasive airspace imaging in small animals[4, 7], and for non-invasive particulate detection in live mouse nasal airways[3]. Throughout these experiments we have been able to improve image quality in a number of ways.

Ethical Treatment Of Animals

All studies were approved by the Animal Ethics Committees of the Women's and Children's Hospital and SPring-8 Synchrotron. Protocols are designed to ensure that experimental animals do not suffer pain, discomfort or distress, and that they remain sufficiently anaesthetized throughout the experiments. While the animals are isolated in the closed imaging hutch they are closely monitored using video surveillance via two PTZ IP cameras (Panasonic BB-HCM580). These real-time video feeds allow the level of anesthesia to be visually verified to be adequate at all times, from any computer on the local network. Visual impression are always supplemented with monitoring of physiological parameters such as the ventilator respiratory pressures, ECG and body temperature (SCIREQ flexiVent). Although it would be valuable to measure oxygen saturation, mouse-specific equipment reliable enough for primary use has not yet been located.

Animal Strain

When performing high-resolution PCXI the mouse fur can cause image artifacts as it produces strong phase effects. Two strategies have been used to eliminate these effects. Firstly, using nude strains such as Crl:CD1-*Foxn1*^{nu}, an athymic and T-cell deficient immunodeficient mouse, or HOS:HR-1 a commercial hairless mouse available in Japan, was found to be an apparently simple solution. While this allowed us to easily acquire images without fur artifacts, we are unsure whether these strains exhibit other physiological differences compared to normal mice that may affect our respiratory studies. In addition, using only hairless strains precludes the imaging of other useful strains such as the transgenic cystic fibrosis mice that are the focus of much of our non-synchrotron research efforts. The second strategy is removing fur from the imaging area (e.g. the trachea) of normal C57BL/6 mice using depilatory cream (e.g. Nair, Church & Dwight, Australia). Provided the area to be imaged is small and the cream is not vigorously rubbed into the skin there appears to be little adverse reaction, and the images are free from fur artifacts.

Airway Access

For some imaging studies there are advantages to having airway access via tracheotomy or intubation, including the ability to perform mechanical ventilation, pulmonary function testing and pharmaceutical delivery. In early experiments tracheotomies were performed to gain airway access, but this was a relatively slow and invasive procedure and could significantly alter airway biology (including potentially allowing blood to enter the trachea). In considering our future need for repeat-imaging studies, tracheal intubations are now performed via the mouth, since these can be rapid, minimally invasive and readily repeatable.

We have adapted an intubation method described by MacDonald[8], in which we use a 0.5mm plastic fiber optic guide as an introducer, and a 20Ga i.v. catheter (Insyte, Becton Dickinson, Utah, USA) as the

endotracheal (ET) tube (See Figure 1). The end of the fiber is attached to a bright fiber-light source so that the tip, which extends ~5mm past the end of the ET tube, provides good direct illumination to visualize the vocal cords and trachea for ET tube placement.

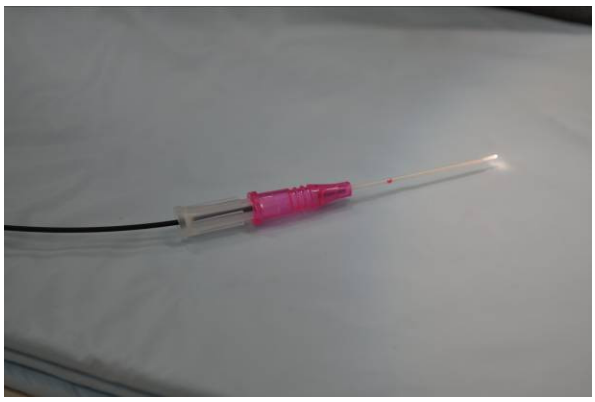

FIGURE 1. The 20Ga. Insyte intubation cannula and plastic fiber optic introducer.

The ET tube is inserted into the trachea to a fixed depth of 22.5mm from the nose tip (as marked in Figure 1) to avoid physical perturbation of the more distal imaging region in the trachea. For lung imaging studies this depth may not be so critical and the ET tube could be inserted deeper. The catheter needle-hub is immediately cut off to minimize respiratory dead-space, and so that the ET tube is ready for connection to the ventilator circuit.

Anesthesia

Due to Japanese government regulations only two anesthetics are available at the SPring-8 synchrotron. We use pentobarbital (~72 mg/kg, i.p.) for anesthetic induction as well as for maintenance of anesthesia in non-ventilated animals. The limitations of pentobarbital include the need for continuous injection to maintain anesthesia, the induction of unpredictable leg "kick" movements in some mice despite deep anesthesia, and the potential for overdose. Thus, wherever possible we use the inhalable anesthetic isoflurane (2% in oxygen) delivered by an isoflurane vaporizer (Univentor U400, Malta). Advantages of isoflurane are that the concentration can be easily set and adjusted from outside the hutch, it does not produce leg movements as with pentobarbital, and its wide therapeutic index means there is a far lower potential for overdose. Thus, for all free-breathing experiments we use pentobarbital with maintenance doses delivered by syringe pump (World Precision Instruments, UMP2) to a 30Ga needle placed i.p. For all experiments requiring mechanical ventilation we induce anesthesia with pentobarbital and then switch to isoflurane for maintenance. In a typical lung airway imaging study the isoflurane and oxygen mix is passively humidified by bubbling it through 10cm of water prior to delivery to the ventilator inspiratory circuit.

Mechanical Ventilation

Wherever possible mice are mechanically ventilated using a flexiVent small animal ventilator (SCIREQ, Canada) when performing synchrotron imaging studies. This type of system has three significant advantages. Firstly, it allows respiratory system mechanics to be measured throughout an experiment to determine the physiological effects of a treatment while also simultaneously acquiring images. Secondly, it allows for the coordinated delivery of aerosols for pharmaceutical or test substance delivery. Thirdly, it enables respiratory-gated image acquisition so that images can be captured at corresponding points within the respiratory cycle and within a breath hold to minimize respiratory movements.

Ventilation is normally set at 80 breaths/min with a tidal volume of 20 ml/kg (minute ventilation of approximately 1.6 ml/g), and ~3 cmH2O of PEEP. The almost continuous chest motion present during normal breathing provides a technically challenging experimental setting, especially at the high image magnifications we use. With a requirement for a 75ms exposure length the ventilatory profile is configured with $T_{inspiration}$ = 0.25 sec, T_{pause} = 0.1 sec and $T_{expiration}$ = 0.4 sec, providing a sufficient end-inspiratory pause to allow relatively motion-artifact-free image capture.

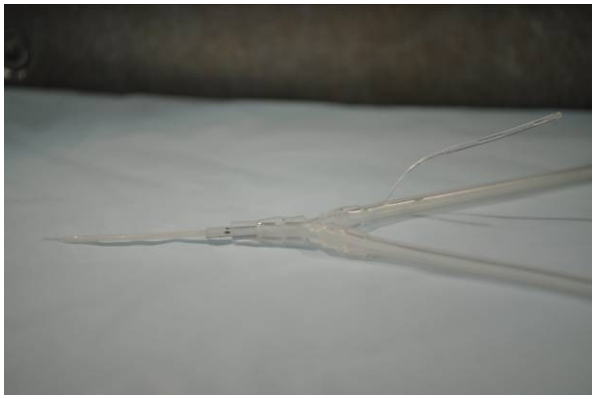

FIGURE 2. The ET tube (left), Y-connector, inspiratory and expiratory tubes (right) that connection to the ventilator, and the fine delivery cannula.

The i.v. catheter ET tube is easily connected to the ventilator circuit as shown in Figure 2. The diameter of the tubing between the tip of the ET tube and the Y-

piece is small so that the respiratory dead-space is minimized. The diameter and composition of the inspiratory and expiratory tubes does not appear to be critical, but nonetheless the shortest possible lengths are used. In addition, for some studies a length of heat-thinned PE10 tubing is fed through the wall of the inspiratory tube to the tip of the ET tube to allow test substances or pharmaceuticals to be delivered to the trachea or lung airways. The diameter of this tube is sufficient for liquid delivery, but not so large that it perturbs airflow through the ET tube. Liquid delivery is typically performed using a remote control syringe pump (World Precision Instruments, UMP2) connected to the PE10 cannula via a 30Ga needle.

One important limitation of the flexiVent software is its inability to perform data logging. Although it can display the cylinder displacement, pressure, ECG and temperature onscreen, those signals cannot be recorded for later analysis. Thus, for applications where these signals are required a separate data logger and sensors must be used.

Animal Positioning

The fixed location and orientation of the X-ray beam limits experimental flexibility in terms of animal restraint and positioning. For anterior-posterior (AP) imaging it is necessary to mount the animals in a head-high orientation, but for lateral imaging they can be mounted head-high or supine (although the non-uniform shape of the X-ray beam can limit setup choices).

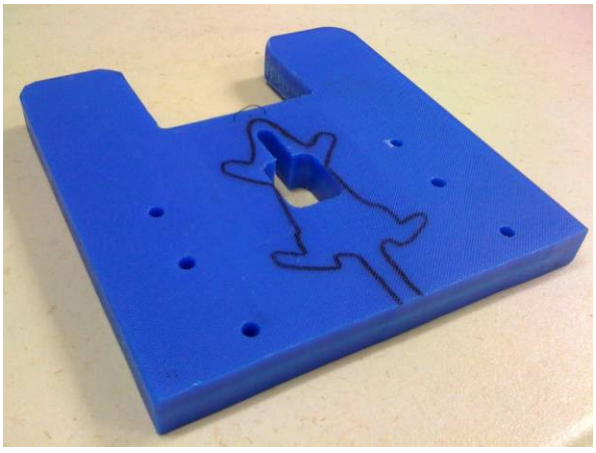

FIGURE 3. The mouse imaging board including a wire loop for the teeth, 6 holes for mounting to the X-Y-rotation stage in the hutch, and a slot and cutout for AP imaging.

In early experiments a stereotactic frame that attached at the incisors and ears was used to hold the mice securely in the beam in a head-high position. The frame was difficult to set up and did not adequately prevent movement. This was a severe limitation because at the high magnifications at which we image, even small amounts of movement can severely degrade the images. This setup has since been redesigned to use a flat imaging board cut from an 18mm thick polyethylene kitchen cutting-board. The board contains a wire loop to support the teeth, and the mouse body is tethered using surgical tape (Micropore, 3M Corporation) onto the skin. The board (See Figure 3) contains a cutout to allow AP imaging of the nasal airways and lung, and mounting holes to directly attach it to the X-Y-rotation stage in the hutch. In practice this setup was substantially more effective at minimizing small body movements during imaging.

For technical simplicity our previous lung studies have oriented the imaging board and mouse in a head-high orientation. Despite being well anesthetized, after approximately 25-30 minutes of imaging some mice appeared unsettled and displayed uncontrollable and unpredictable respiratory excursions that degraded image quality, limiting usable imaging time to less than 30 minutes. Changing to a supine imaging position prevented these movements from occurring, and we speculate that loading on the diaphragm in the head-high position was causing muscle fiber shortening, ventilatory loading and potential hypoxemia and hypercapnia. In addition, venous return problems when vertical may have also reduced cardiac output and potentially metabolism, further exacerbating the problem.

When possible we now utilize a supine imaging position to minimize respiratory movements and image artifacts due to movement.

Dose Minimisation

Several strategies have been implemented to minimize the radiation dose delivered during imaging. A fast imaging shutter that is only opened during the image exposure is used so that at all other times the mouse does not receive any radiation. The delivered dose is measured using an ion chamber located downstream of this shutter. During recent experiments the dose rate was calculated to be 0.44Gy/sec. The exposure times are also reduced wherever possible, thus trading image quality for dose. Finally, laser alignment is used in the hutch prior to beam activation, so that the anatomical region of interest can be rapidly located and the amount of radiation delivered prior to an experiment actually commencing can be reduced.

Although we attempted to limit the radiation dose by reducing the size of the imaging area and limiting the exposure times, at present it remains too intense to

consider animal recovery, or repeat-imaging experimental designs.

CONCLUSIONS

We propose that PCXI is a valuable technique for studying the airways of live mouse models, and that despite its limitations there are currently no other imaging modalities with these capabilities. The rapid development of imaging and synchrotron technologies has and will continue to produce improvements in light sensitivity and image resolution that could allow shorter exposure times to minimize motion artifact and produce desired reductions in radiation dose.

Imaging live mice using synchrotron X-rays has many challenges, but overcoming these challenges has permitted increasingly sophisticated imaging of animals with synchrotron X-rays. We expect a bright future for these techniques.

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