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Imaging System for Creating 3D Block-Face Cryo-Images Of Whole Mice

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Abstract

We developed a cryomicrotome/imaging system that provides high resolution, high sensitivity block-face images of whole mice or excised organs, and applied it to a variety of biological applications. With this cryo-imaging system, we sectioned cryo-preserved tissues at 2–40 μm thickness and acquired high resolution brightfield and fluorescence images with microscopic in-plane resolution (as good as 1.2 μm). Brightfield images of normal and pathological anatomy show exquisite detail, especially in the abdominal cavity. Multi-planar reformatting and 3D renderings allow one to interrogate 3D structures. In this report, we present brightfield images of mouse anatomy, as well as 3D renderings of organs. For BPK mice model of polycystic kidney disease, we compared brightfield cryo-images and kidney volumes to MRI. The color images provided greater contrast and resolution of cysts as compared to *in vivo* MRI. We note that color cryo-images are closer to what a researcher sees in dissection, making it easier for them to interpret image data. The combination of field of view, depth of field, ultra high resolution and color/fluorescence contrast enables cryo-image volumes to provide details that cannot be found through *in vivo* imaging or other *ex vivo* optical imaging approaches. We believe that this novel imaging system will have applications that include identification of mouse phenotypes, characterization of diseases like blood vessel disease, kidney disease, and cancer, assessment of drug and gene therapy delivery and efficacy and validation of other imaging modalities.

Keywords

small animal imaging; block-face imaging; cryosection; brightfield microscopy; fluorescence microscopy; histology

1. INTRODUCTION

Imaging of small animals, particularly mice has become an important tool for studies of biological processes, pathologies and therapeutics. The genetic and physiological similarity of mice to humans, speed of reproduction, and the ability to manipulate the mouse genome creating various human disease models make the mouse an ideal choice for biological investigation. Genetic manipulation of mice started more than a century ago¹ and have since become an extraordinary tool in biology. Researchers are producing an extraordinary number of genetic modifications, using a variety of techniques^{2,3}. This has been paralleled

by a development of imaging techniques specifically targeted to the small animal. Techniques include MRI, PET, SPECT, CT, *in vivo* fluorescence, *in vivo* bioluminescence, and intravital imaging⁴⁻⁹. New imaging agents and reporter gene approaches are enabling researchers to characterize biological processes at the cellular and subcellular level *in vivo*⁴. Each of these methods has advantages and disadvantages. All are limited either with respect to resolution, contrast mechanisms, or volume of view.

We are developing a new cryo-imaging system with advantages over existing mouse imaging techniques. Briefly, the system consists of a modified mouse-sized, cryomicrotome that includes a microscopic imaging system. With this system, we alternatively slice tissue and image the block face. This system is capable of producing ultra-high resolution color/fluorescence 3D images with excellent morphological details. Unlike every other imaging technique it allows one to image at microscopic resolution (as good as 1.2 μm) over a volume of view that includes the entire mouse. Hence, as compared to existing imaging systems, our system provides a unique combination of field of view, depth of field, resolution and color/fluorescence contrast. With fluorescence imaging, one can exploit a variety of imaging agents and reporter gene techniques. Another benefit is the ability to collect sections for histology.

Earlier work concerning systems for episcopic imaging of the block-face has been made most famous by the visible human projects in the USA and elsewhere¹⁰⁻¹⁴. These projects have provided extraordinary new information about human anatomy, and data have been used for a variety of applications. There have also been reports of episcopic block-face imaging of small animals and organs¹⁵⁻¹⁹. Over the years, there have been research papers on 3D reconstruction from serial histology sections^{20,21} for a variety of biomedical applications²²⁻²⁴. In many cases, researchers had to deal with the difficulties presented by incomplete, torn, and/or otherwise spatially distorted sections. It is especially difficult to obtain histology sections at fixed slicing intervals with a large tissue volume, where multiple paraffin blocks are usually used. A cryo-imaging system alleviates many of these difficulties.

Our current system has been developed with expertise gained from a block-face imaging methodology that was developed earlier in our laboratory to validate MR image data from interventional MRI radiofrequency ablation experiments^{25,26}. In this predecessor system, color block-face images of fixed, wax-embedded tissues were obtained following the acquisition of each relatively thick slice about 3 mm.

In the next section we describe the basic system and an overview of the methods used to embed, section and image a specimen. In subsequent sections we present images acquired in various experiments to highlight the resolution, detail and color contrast.

2. CRYO-IMAGING SYSTEM & METHODS

The cryo-imaging system is shown in Figure 1. It consists of a modified cryomicrotome with an integrated microscopic imaging system. The imaging system includes a stereo microscope, filters for fluorescence imaging, low light camera and an illumination system with fiber optic light guides. The camera is connected to the camera/video port of the

microscope and controlled through an imaging workstation. The system provides a wide range of magnifications allowing one to image a mouse-sized field of view and pixels as small as 1.2 μm .

Prior to cryo-imaging study, animals are euthanized in a method approved by Case Animal Resource Center (ARC) which consists of either inhalation of carbon dioxide delivered from tank or anesthetized using an agent such as pentobarbital, at a dose prescribed by the Case ARC. Animals are covered under various IACUC-approved projects. Euthanized mice are then covered with Optimal Cutting Temperature (OCT) solution (Tissue-Tek, Terrance, CA). This step ensures that the carcass is wet and no air bubbles are formed in the next step of embedding. The mouse is then embedded in OCT inside an aluminum foil mould. The entire mould is snap-frozen in liquid nitrogen. Following this, we remove the mould assembly from liquid nitrogen bath and place it inside the cryomicrotome chamber for equalizing the specimen temperature to the cryomicrotome temperature. After few hours, the mould is removed and the frozen specimen is mounted on the microtome stage using more OCT. After initial “facing” (continuous slicing at maximum thickness to reach animal proper), the desired slice thickness (typically 10 μm) is set. The imaging system is readied, and the system is used to alternately slice and image the block-face. The system provides for two modes of cutting – a single cut mode which is normally used when images are acquired after every slice and a continuous cut mode which takes a series of continuous slices before an image is acquired.

Following acquisition of 2D images on the imaging workstation we perform image processing, analysis and visualization on a high-end workstation using customized Matlab code (Mathworks Inc., Natick, MA) and AMIRA software (Mercury Computer Systems, San Diego, CA). Processing steps include illumination intensity compensation, fine image alignment, removal of subsurface image signal, and, optionally, image interpolation to create isotropically distributed samples. The 3D image data sets can be resliced using multi-planar reformatting techniques. Structures of interest can be manually or semi-automatically segmented. Segmented 3D models can be rendered with surface rendering in three dimensions. We can also take quantitative measurements like distance, area, volume etc. For transgenic mice with polycystic kidney disease, we compared cryo-image volumes to MRI images. Quantitative measurements like kidney volume, cyst diameters and volumes were computed.

3. RESULTS

We have acquired cryo-images from a variety of wild type (WT) and genetically modified specimens (Figures 2-8). In Figure 2, we show a wide field of view sagittal image of a WT mouse which shows the anatomy in exquisite detail. We can clearly identify the cerebral hemisphere, medulla oblongata, vertebral column, sternum, iliocostal muscle, heart, liver, lung, gastro-intestinal system and the testis. We obtained high resolution images of individual organs such as the heart (Figure 3). In this image of an ≈ 5 mm heart, details like the four chambers of the heart, the tricuspid and the mitral valves, the interventricular septum, the trabeculae carnae in the ventricle, the chordae tendannae and the pulmonary trunk are all visible.

Figure 4 is an image of a parabiosis mouse model obtained in recent experiments where we have extended the system capabilities to include fluorescence imaging. In this experiment, a Green Fluorescent Protein (GFP) positive mouse was surgically conjoined with a WT recipient. We are using this mouse model to investigate healing process of injury sites and the effects of a conjoined circulatory system.

The cryo-imaging system is an effective tool for studying diseases. An interdisciplinary team at our institution is investigating the genetics of autosomal recessive polycystic kidney disease (ARPKD) in mice and potential new drug therapies^{27,28}. PKD is one of the most common human genetic diseases and affects over 500,000 people in the US²⁹. The disease is characterized by excessive proliferation of renal tubular epithelial cells which form fluid-filled cysts that eventually replace most of the normal kidney tissue. Consequently, PKD leads to severe enlargement of the kidneys, and renal failure. The cryo-imaging system is ideally suited for reconstruction of large multicellular cysts (50–2,000 μm) and dilated tubules (1–3 mm). Figure 5 shows sections through the kidney of a 19-day old WT mouse and its cystic littermate (BPK +/+). As compared to WT, BPK mouse has an enlarged kidney and multiple cysts, some of which are $< 50 \mu\text{m}$ in diameter. 3D reconstructions provide anatomical details (Figure 5d) of great biological interest and volumes as small as 0.2 mm^3 . Unlike cryo-images, MRI images obtained in a clinical scanner show little detail but many artifacts (Figure 5c).

From serial section cryo-images, specific organs can be reconstructed after segmentation. As an example, Figure 6 shows 3D reconstruction of the kidney of a wild type mouse, after manual segmentation. Thresholding was applied to obtain the renal vasculature, consisting of blood vessels $< 100 \mu\text{m}$ in diameter.

In Figure 7, we demonstrate the usefulness of color contrast. Serial sections are obtained through the heart and lungs of a WT and its genetically modified knockout (KO) littermate used for studying heart and lung disfunctions. The KO mouse image shows an enlarged heart and affected lungs. We computed volume estimates after manual segmentation. Epicardial volumes were 852 mm^3 and 926 mm^3 for WT and KO, respectively, while average ventricular wall thickness was similar at 0.6 mm. The cryo-images of the lung show morphological details as well as extraordinary color differences, not observable in CT or MR.

We utilized genetically modified mice used to study choroidal neovascularization (CNV) – a cause of vision loss with age-related macular degeneration. It has been demonstrated that Tissue Inhibitor of Metalloproteinase-3 (TIMP-3) gene is a potent inhibitor of angiogenesis^{30,31}. As shown in Figure 8, the 7-week old TIMP-3 deficient mouse shows extensive vasculature, which was perfused with India ink under pressure to aid contrast. With cryo-imaging, we were able to reconstruct blood vessels $\approx 5 \mu\text{m}$ in diameter (Figure 9). In this example, a single vasculature branch from 50 brain slices was processed. Note that such reconstructions can be done over extended volumes.

4. DISCUSSION

Cryo-imaging provides a combination of field of view, depth of field, resolution and color and fluorescence contrast mechanisms not possible with *in vivo* imaging or with traditional *ex vivo* microscope imaging approaches such as excising tissue samples and performing confocal microscopy and/or histology. We have demonstrated through a variety of projects how this system can be of value. For example, the ARPKD images show 3D cyst details not visible in typical MRI scans. The mouse-sized field of view allows study of disease models and tracking of tagged cells over multiple organs. The cryo-system does not have the depth of field limitation of confocal microscopy and can be adapted for collection of slices for histology as well. Potential applications for cryoimaging include visualizing the morphologic progression of cyst formation in the intact kidney, morphometric analysis of collecting duct cysts, phenotyping mice, angiogenesis study with regard to CNV, and micro-architecture of tumor.

With almost every new biomedical imaging technique or imaging agent, validation is a crucial step that is often difficult to do rigorously. The cryo-imaging system can be a critical tool for such validation studies. In a separate communication³², we describe the use of cryo-imaging system to validate MR imaging of blood vessel disease. Vessel segments were excised from cadaver and brightfield and autofluorescence cryo-images were used to characterize the tissues of atheroma. Three-dimensional cryo-image data was registered to images from MR for a voxel-based validation.

We have presented a new imaging system for creating block-face cryo-image volume of small animals and excised organs and demonstrated example images from a variety of applications. This novel imaging system has advantages of resolution, contrast and depth of field and along with the plethora of other tools available to researchers in small animal imaging, would provide a new insight into disease and cure.

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Figure 1.

The present cryo-imaging system. The imaging system consists of microscope and digital camera mounted on a stand. The system is lowered into the chamber and positioned above the specimen stage (inset) with the lens parallel to the tissue slicing plane. An imaging workstation connected to the camera enables image acquisition and a pendant controller (arrow) is used for manually controlling the slicing motion and specimen stage traversal speed. Cryostat cooling controls are integral to the machine.

Figure 2.

Cryo-image of sagittal section of a male wild type mouse. We can clearly identify the cerebral hemisphere, medulla oblongata, vertebral column, sternum, iliocostal muscle, heart, liver, lung, gastro-intestinal system and the testis.

Figure 3. Heart of a two week old wild type mouse. Details include four chambers of the heart, tricuspid and the mitral valves, interventricular septum, trabeculae carnae in the ventricle, the chordae tendannae, and the pulmonary trunk.

Figure 4. Parabiosis mouse model experiments. A GFP-positive mouse was surgically conjoined with a recipient wild type. This mouse model is being used to investigate conjoined circulatory system and its role in injury healing.

Figure 5.

Sections through the kidney of a 19-day old WT (a) and its littermate, a BPK mouse (b), a model for autosomal recessive polycystic kidney disease (ARPKD). The BPK mouse has an enlarged kidney as compared to WT (note the scale bars) due to the multiple cysts. Cysts are quite visible including very small ones measuring $< 70 \mu\text{m}$. In comparison, MR image (c) of the kidney of a PKD mouse shows enlargement but no cyst details. 3D reconstruction (d) of a few cysts seen through the kidney. Individual cyst volumes were $\sim 0.2 \text{ mm}^3$.

Figure 6.
3D reconstruction of the kidney and the renal vasculature from episcopic image volume of a wild type mouse. Blood vessels are $< 100 \mu\text{m}$ in diameter.

Figure 7.

Heart and lungs of a WT and a KO from the same litter. The manually outlined heart in the KO (b) is larger than the wild type (a). The KO lung (d) shows significant morphological and color difference as compared to WT (c). Unlike other modalities like μ MRI, color cryo-images offer not only higher spatial resolution, but tissues to be differentiated on texture and color.

Figure 8.

Eye of TIMP-3 deficient mouse perfused with India ink to study angiogenesis. We see blood vessels $<5\ \mu\text{m}$ in diameter.

Figure 9.
3D reconstruction of a single vasculature branch from 50 brain slices of the TIMP-3 deficient mouse, depicting vessel size of the order of 5 microns.