Virtual Histology of Transgenic Mouse Embryos for High-Throughput Phenotyping

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A bold new effort to disrupt every gene in the mouse genome necessitates systematic, interdisciplinary approaches to analyzing patterning defects in the mouse embryo. We present a novel, rapid, and inexpensive method for obtaining high-resolution virtual histology for phenotypic assessment of mouse embryos. Using osmium tetroxide to differentially stain tissues followed by volumetric X-ray computed tomography to image whole embryos, isometric resolutions of 27 μm or 8 μm were achieved with scan times of 2 h or 12 h, respectively, using mid-gestation E9.5–E12.5 embryos. The datasets generated by this method are immediately amenable to state-of-the-art computational methods of organ patterning analysis. This technique to assess embryo anatomy represents a significant improvement in resolution, time, and expense for the quantitative, three-dimensional analysis of developmental patterning defects attributed to genetically engineered mutations and chemically induced embryotoxicity.

Introduction

Gene targeting in mice allows unprecedented insight into the function of genes and their roles in patterning the mammalian embryo [1]. A full understanding of mammalian development by this means, using the gene-targeting approach for every one of the approximately 25,000 or more mouse genes, may seem like a daunting task. Nevertheless, more than 10% of known mouse genes have already been disrupted by gene targeting. Moreover, the National Institutes of Health is leading an effort to create a collection of mouse lines with disruption of every known gene [2]. The challenge laid before developmental biologists will be to systematically analyze morphological phenotypes and, where possible, determine the quantitative contribution of each gene towards patterning of the embryo. Tools for this type of “phenomic” analysis must include rapid, inexpensive, and accessible high-throughput methods of high-resolution anatomical imaging (addressed here) as well as stage-specific, statistically-averaged wild-type morphological atlases that can be used to discern normal variation from mutant phenotype [3].

The introduction of magnetic resonance microscopy (MRM) represented an important time savings over classical histology in the screening of E6.5–E19 mouse embryos for mutant morphological phenotypes [4] (reviewed by Schneider and Bhattacharya [5] and Tyszka et al. [6]). However, the specialized and expensive equipment required for such high field magnetic resonance scans is not widely available. Furthermore, scans at useful resolutions (12–43 μm, but generally 25 μm) require 9–14 h of instrument time [7,8] at a cost of approximately US$200 per hour.

We introduce a new method of obtaining virtual histology using X-ray microscopic computed tomography (microCT). This technique permits mid-gestation mouse embryos to be scanned at up to 8-μm resolution in comparable or less time, and at a fraction of the expense of MRM. This new method, which employs osmium tetroxide to differentially stain tissues, will facilitate the quantitative, three-dimensional analysis of mouse developmental defects for researchers at a broad range of institutions at relatively little expense—and at resolutions and throughput comparable to or exceeding magnetic resonance methods.

Results

Virtual Histology by Computed Tomography Is a Powerful Method for Imaging Embryo Anatomy

Formalin-fixed wild-type E11.5 embryos whose cell membranes were stained in a 1% solution of osmium tetroxide were imaged by volumetric computed tomography (CT) at 8-μm isometric resolution. External surface features of the scanned embryos were represented as isosurfaces (Figure 1A), demonstrating a level of detail comparable to a dissection microscope. Internal structures could be visualized by a semitransparent maximum intensity projection of the entire embryo, similar to a plain radiograph (Figure 1B). In order to compare the spatial resolution of traditional optical...
Synopsis

Developmental biology is entering the digital age, thanks to advancements in imaging technologies, instrumentation, and software. These advancements are converging with discoveries in developmental biology to deliver unprecedented insight into how human development is impacted by the products we use, the environment we live in, and our genetic composition. Industrial societies are becoming increasingly concerned with the exposure of women and their unborn fetuses to pharmaceuticals and commonplace household chemicals. In addition, understanding genetic causes of birth defects is now possible through the isolation of specific genes, which can be efficiently disrupted in embryos, and subsequently observed for birth defects. Such studies of embryotoxicity typically involve the use of mouse embryos. However, evaluation of mouse embryos in the past has involved expensive and cumbersome external inspection and thin sectioning for view under the microscope. As such, developmental biologists have eagerly anticipated the advent of tools that would allow them to routinely assess the complex and dynamic organization of embryos using techniques that are fast and inexpensive. In this article, the authors introduce a rapid, high-quality, and inexpensive technique for the three-dimensional visualization of mouse embryos using X-ray computed tomography that is ideally suited for researchers in pharmaceutical, industrial, and academic laboratories.

Virtual Histology is Most Amenable to Mid-Gestation Embryos

Unlike MRM, which does not necessarily require staining, the osmium tetroxide stain for microCT-based virtual histology is best suited to gestational ages with limited epidermal layers (unless the epidermis is manually removed with a #2 forceps or protease digested prior to staining [9]). In our experience, mid-gestation whole embryos (E8–E13.5) that lack significant epidermal development are most appropriate for this method, although skinned embryos up to E19 can be satisfactorily stained and imaged as well (unpublished data). Figure 2 demonstrates isosurfaces (Figure 2A–2D) and sagittal cross-sections (Figure 2E–2H) of a time series of E9.5, E10.5, E11.5, and E12.5 embryos, respectively, scanned at 8-μm isometric resolution. At these resolutions, features such as the developing brain vesicles, neural tube, heart chambers, and liver can be clearly delineated. Due to the increased lipid content of the liver, attenuation of osmium-stained hepatic tissue results in the highest opacity and brightness.

Rapid 27-μm Resolution Scans Can Be Performed on a Small Animal CT Scanner

Lower spatial resolutions, comparable to most typical magnetic resonance methods (25–27 μm) can be achieved with only a 2-h acquisition time on a small animal CT scanner. These live-animal scanners are more likely to be available at academic imaging core facilities. Employing a GE eXplore RS small animal scanner, we performed scans of wild-type E12.5 embryos at 27-μm isometric resolution in approximately 2 h. A comparison of 8-μm and 27-μm scans of the same embryo is shown in Figure 3. Although 8-μm sections (Figure 3A–3D) display considerably higher spatial resolution, the 27-μm sections (Figure 3E–3H) were nonetheless adequate to distinguish features such as the semicircular canal, the neural tube central canal, and the cardiac chambers. From the perspective of high-throughput phenotyping, the resolution of these 27-μm microCT scans were in the range of MRM, but at a nearly 6-fold time savings and a 300-fold cost savings. Furthermore, these 2 h, 27-μm resolution scans were adequate to perform high-quality segmentation analysis of major organ compartments, a prerequisite for computer-based, automated phenotyping (Figure 3M–3P). A caveat is that the small lumens within some organs, such as the right atrium of the heart, are less well segmented in the rapid scan than by the higher resolution scan (Figure 3I–3L). However, as shown in this figure, the same osmium-stained embryo scanned at 27-μm resolution...
can be scanned at 8-\(\mu\)m resolution when increased definition of smaller structures is necessary.

Rapid 27-\(\mu\)m Scans Are Sufficient for Segmentation and Screening for Developmental Defects

To test the value of microCT virtual histology for high-throughput phenotyping for major organ compartments and tissue structures of younger embryos, we utilized Pax3:Fkhr transgenic mouse embryos known to have complex rostral neural tube malformations [10,11]. These embryos express the Pax3:Fkhr fusion oncogene in place of the Pax3 gene in the dorsal neural tube and the dermomyotome, resulting in partial failure of neural tube closure. Wild-type and Pax3:Fkhr mutant E11.5 embryos were scanned at 27-\(\mu\)m resolution, then renderings with segmentation were performed to visualize the cephalic forebrain, midbrain, and hindbrain vesicles, the heart wall and cardiac ventricles, and the liver (Figure 4A–4J). With these renderings, one can appreciate failure of neural tube closure at the level of the hindbrain and midbrain, overgrowth of the midbrain mesenchyme, as well as the hypotrophy of the telencephalic vesicles. Although these findings would have been apparent with real histology derived from paraffin-embedded specimens, the complex global three-dimensional organization of the mutant forebrain, midbrain, and hindbrain vesicles would not have been. The cardiac ventricular wall (blue) is essentially the same between wild type and mutant at this age, with no appreciable difference in the volume or patterning of the common ventricle. The liver also appears to be patterned normally in both wild type and the mutant. Using individual 27-\(\mu\)m planes, an additional, more subtle defect was detected in the neural tube at the level of the forelimbs (Figure 4K and 4L). Mutant embryos exhibit mispatterning (dysmorphology) of the dorsal neural tube, which was confirmed by confocal microscopy and immunohistochemical detection of the dorsal neural tube marker, Pax7 (Figure 4M and 4N). From the point of view of a semi- or fully automated high-throughput screen for developmental patterning defects, the rapid 27-\(\mu\)m scan represents a feasible method of morphological typing of both complex gross and relatively subtle morphological features.

Discussion

We present a rapid and inexpensive screening method for anatomical phenotyping of mid-gestation embryos using osmium tetroxide staining and microCT-based imaging. MicroCT-based virtual histology matches or exceeds the tissue contrast achieved by more time- and cost-intensive MRM, while delivering more than 2-fold higher resolution [3] (up to 8 \(\mu\)m for microCT). At lower microCT resolutions (27 \(\mu\)m), as many as 120 embryos can be simultaneously scanned in approximately 2 h with adequate quality for post-imaging segmentation analysis allowing the recognition of gross and subtle mutant phenotypes (Figure 4; Table 1). For increased detail of abnormalities suspected on the low-cost 27-\(\mu\)m scans, the same osmium-stained specimens can later be rescanned at 8-\(\mu\)m resolution for unprecedented detail of organ subcompartments and fine tissue structures. We believe that this technique will be most useful as a first-line screen of embryonic defects, from which investigators could then perform traditional histological/immunohistochemical analysis of regions of interest. This technique could also be valuable in the high-throughput evaluation of teratogenic effects of medications and chemicals (e.g., embryotoxicity studies), evaluation of tissues from adult animals, and neocapillary mapping for tumor biopsies of patients undergoing anti-angiogenesis therapies. For increased throughput...
of these types of studies, multiple samples can be scanned simultaneously in the same field of view (e.g., six to 120 embryos can be scanned at once). This high-throughput technology may be especially applicable to Tier 1 Phenotyping in the National Institutes of Health Mouse Knockout Project [2]. The expected higher sensitivity of this embryotoxicity analysis tool also has the potential to streamline the protocols for evaluating chemical- and drug-related reproductive safety in rodents, thereby reducing the number of animals required for testing, a growing concern in the European Union and elsewhere [12].

A practical consideration is whether mid-gestation is an appropriate interval for the detection of birth defects and embryotoxicity; however, in one forward genetic screen for recessive mutations, 58 of 83 (70%) of murine birth defects evaluated before and after birth had arisen prior to E13 and could be detected at E9–E13 by routine examination and microscopy [13,14]. Using microCT-based virtual histology, the sensitivity may be even higher than 70%.

We expect that an entire field of microCT-based virtual histology methods will soon emerge given the recent advances in high-resolution microCT instrumentation and the exploration of existing and new electron-dense stains. In preliminary experiments on a newly available commercially scanner, we have achieved isometric spatial resolutions as high as 6 μm in pilot studies. Our other preliminary experiments suggest that
a combination of osmium and cis-platinum (or ethidium bromide) may allow for differential staining of cell membranes and nuclei, respectively, so that the staining characteristics of organs and tissues can be further differentiated (unpublished data). Furthermore, our preliminary experience and studies by Bentley (see [15]) demonstrate that osmium-stained tissue, with or without counterstains, can be later sectioned for true histological staining. The multiple uses of osmium-stained

Table 1. Comparison of Embryo Phenotyping Screening Methodologies and Costs

| Method          | Sample Handling | Acquisition | Resolution | Information Content          | Embryos/Scan | Cost/Scan       |
|-----------------|-----------------|-------------|------------|------------------------------|--------------|----------------|
| EFIC            | Fixation and embedding | 3–4 h &l#5; | 1.5 μm     | 3D morphology               | 1            | Not available |
| OPT             | Fixation and embedding | 12 h     | 5–10 μm   | 3D morphology               | 1            | Not available |
| MRM             | Fixation/none     | 12 h      | 25 μm     | In situ molecular markers   | ≤6           | US$2,400 (US$600/embryo)³ |
| MicroCT (standard) | Fixation and staining | 12 h     | 6–8 μm   | 3D morphology               | ≤6           | US$480 (US$80/embryo)⁴ |
| MicroCT (rapid) | Fixation and staining | 2 h      | 27 μm     | 3D morphology               | ≥120         | US$80 (US$1.50/embryo)⁴ |

³Diffusion tensor imaging allows the diffusion of water along tissue planes to differentiate similar-density tissues.

⁴Cost estimates are based upon an academic rate of US$200/hour for MRM and US$40/hour for microCT.

EFIC, episcopic fluorescence image capturing; OPT, optical projection tomography; 3D, three-dimensional.

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tissues will therefore speed the transition from microCT-based screens to episcopic [16] and microscopic histological verification of suspected morphological phenotypes. In order to achieve high-throughput phenotyping, or photonics, the computing methods for analysis must be semi-automated or fully automated. Magnetic resonance imaging of postnatal mouse brains (as well as human brains) has led to significant advances in semi- or fully automated deformable shape mapping whereby specimens of different sizes can be compared for the eventual detection of statistically different features of morphology [17]. MicroCT-based virtual histology datasets are ideally suited to this type of analysis. MicroCT-based virtual histology is not intended to replace the generally more versatile magnetic resonance methods (for a review see references [3,5,7]), but is instead a useful adjunct for anatomical imaging. A comparison of CT and magnetic resonance methods, applications, and costs are given in Table 1. Included for comparison are the low-throughput but exceptional resolution methods of episodic fluorescence image capturing [16,18] and optical projection tomography [19]. Magnetic resonance imaging allows the analysis of a variety of tissue properties to be interrogated, depending upon the pulse sequence, and post-processing parameters such as diffusion tensors [17] allow the organization of tissues to visualized and modeled in unprecedented ways. Instead, microCT-based virtual histology offers a potentially higher resolution mode of morphometrics that is simple to implement, relatively inexpensive, and more rapid than comparable methods of phenotyping embryo anatomy.

**Materials and Methods**

**Sample preparation.** The Pax3:Fkhr transgenic mice have been previously described [10,11]. Embryos were harvested at E9.5–E12.5 gestational ages, then fixed in 10% buffered formalin overnight at 4°C. Hematoxylin and eosin–stained paraffin sections were prepared using established methods [10], then visualized at 2x magnification on a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan). Immunohistochemistry with the Pax7 monoclonal antibody (Developmental Hybridomas Studies Bank, Iowa City, Iowa, United States) was performed as previously described [10]. For microCT-based virtual histology, formalin-fixed embryos were stained using a beta version of the manufacturer’s proprietary EVSBeacon. The 600 MB three-dimensional dataset and the raw data for the scan were transferred to a single DVD (approximate total, 4.5 GB). Preliminary visualizations (unpublished data) and virtual histology sections were generated in real time with the freely-available MicroView program (http://micrreview.sf.net).

**Isosurface generation.** Isosurfaces renderings of the CT datasets were generated using a combination of the open-source Teem utilities [20] and the open-source, platform-independent BiomeImage [21]. Both sets of software are available online (http://software.scil.uitala.edu). Sub-sampling of the microCT volumes, which are significantly larger than the available memory on current graphics cards, was also performed using the Teem libraries. The final subsampled volumes were 255 × 255 × 255 mm and equally spaced using a 0.2-mm aluminum. At 8-mm resolution, the field of view of this instrument is 15 × 15 × 15 mm. Each scan took approximately 12 h. Cost of this method is approximate US$40 per hour (US$480 per scan). Images were reconstructed with the manufacturer’s proprietary EVSBeacon software.

More rapid volumetric CT scans of embryos performed at 27-μm³ isometric voxel resolution using an EnSolve Locus RS small animal MicroCT scanner (GE Healthcare). Like the specimen scanner, this live-animal volumetric scanner employs a 3,500 × 1,750 CCD detector for Feldkamp cone-beam reconstruction and is similar in performance to other commercially available in vivo scanners under $300,000 that are commonly operated at regional core facilities (http://ccti.uthscsa.edu/ImagingFacility.html). In this study, the platform-independent parameters of current, voltage, and exposure time were kept constant at 150 mA, 80 kVp, and 2000 ms, respectively. Faster, 450 evenly spaced views were averaged from six frames/view. At 27-μm resolution, the field of view of this instrument is 45 × 45 × 45 mm. Each scan took 2 h and 4 min. Cost of this method is also approximate US$40 per hour (US$80 per scan). Images were reconstructed in the manufacturer’s proprietary EVSBeacon. The 600 MB three-dimensional dataset and the raw data for the scan were transferred to a single DVD (approximate total, 4.5 GB). Preliminary visualizations (unpublished data) and virtual histology sections were generated in real time with the freely-available MicroView program (http://micrview.sf.net).

**Segmentation.** Image volume segmentations were done using a Watershed algorithm [22] provided in the National Library of Medicine Insight Segmentation and Registration Toolkit (ITK; http://www.itk.org). Watershed segmentation is a region-growing algorithm in which user-defined seed points are positioned in areas of interest, and a statistical analysis is made of the gradient magnitude in the local neighborhood to find the standard deviation. This structure is then found as the boundary of the segmentation extend through the continuous surrounding area, without crossing points wherein the gradient magnitude is beyond one standard deviation of the mean. The resulting structures are saved to disk as separate volumes. Segmentations were done on a computer equipped with a 2.5 GHz Intel Pentium III processor and 2 GB of RAM. The resulting volumes were then combined into one volume by marking each segmentation as a unique value, and then merging it into the original again, such that the original value of existing segmentation is overwritten with the new marked value.

**Volume renderings.** The volume renderings of wild-type and Pax3:Fkhr mutant embryo datasets were created using direct volume rendering techniques using the in-house software package, Nenners [23]. Nenners reads a volume and casts its geometric rays through the volume per pixel. For each ray that intersects with the volume, equally spaced samples of length Δ are acquired along the respective ray. To ensure that renderings capture the fine detail of these high-resolution datasets Δ ≤ |v - n - |, where n is the position of i-th voxel, the data are sampled at least once per cubic voxel. Samples are acquired by convolution using separable kernels (page 197 in [24]). Cubic splines were used to interpolate both the CT and gradient [23]. Color and opacity were defined at each sample point using a two-dimensional transfer function, with gradient magnitude and X-ray density defining the domain [20,27]. This was done in a front-to-back fashion, such that when the opacity of a ray becomes equal to or greater than 1, the ray is terminated early and the next ray is computed. Bounding volumes were also used to speed this process such that rays that have not yet or will not hit the volume were not sampled. The gradient was also used to approximate the Phong shading models used [28]. For Figure 3, a curvature-based transfer function [28] and depth cueing was also applied to the volume renderings. The transfer function is based on the dot product of the angle of an incident ray with the surface normal. If the dot product...
was less than some constant \( k, 0 < k < 1 \), then the surface was marked black to emphasize surfaces perpendicular to the viewing direction. Furthermore, a depth queue to shade interpolated samples was used to give a greater feeling of depth and to separate foreground from background. Parameters of camera position, viewing direction, and lighting relative to the specimen were also passed to define the view of the rendering. The software was run on an SGI Onyx 3800 (Silicon Graphics, Mountain View, California, United States) at 80 s per rendering.

**Supporting Information**

**Video S1.** Sagittal Sections of a Wild-Type E12.5 Embryo at 8-µm Resolution

Found at DOI: 10.1371/journal.pgen.0020061.sv001 (1.2 MB WMV).

**Video S2.** Coronal Sections of a Wild-Type E12.5 Embryo at 8-µm Resolution

Found at DOI: 10.1371/journal.pgen.0020061.sv002 (1.3 MB WMV).

**Video S3.** Axial Sections of a Wild-Type E12.5 Embryo at 8-µm Resolution

Found at DOI: 10.1371/journal.pgen.0020061.sv003 (1.7 MB WMV).

**Video S4.** Rendering of a Segmented Pax3:Fkhr Mutant E11.5 Embryo

This embryo corresponds to Figure 4F–4J. Found at DOI: 10.1371/journal.pgen.0020061.sv004 (3.6 MB MOV).

**Video S5.** Rendering of a Segmented Wild-Type E11.5 Embryo

This embryo corresponds to Figure 4A–4E. Found at DOI: 10.1371/journal.pgen.0020061.sv005 (3.6 MB MOV).

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**Accession Numbers**

The National Center for Biototechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) accession number for the Pax3:Fkhr fusion oncogene is AY743299, and for Pax7 is NM_0010139.

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**Author contributions.** CK conceived and designed the experiments. MSH, IW, and CK performed the experiments. JTT, LJH, GMJ, MRC, and CK analyzed the data. CRJ and GMJ contributed reagents/materials/analysis tools. JTT and CK wrote the paper.

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**Competing interests.** CK is the co-founder of a biotechnology company named Numira Biosciences, which may pursue the commercialization of the patent-pending technology in this manuscript. The intent of our manuscript, however, is to communicate this method so that it may be reproduced and utilized freely at academic institutions.

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