PW 8

Poster n° 2

Optimization of Pulmonary Vasculature Tridimensional Phenotyping in Rat Fetus

Emrah Aydin, Brittany Levy, Marc Oria Alonso, Hussam Nachabe, Foong-Yen Lim, Jose L Peiro

The Center for Fetal, Cellular, & Molecular Therapy, Pediatric General and Thoracic Surgery Division, Cincinnati Children's Hospital Medical Center

Aim

Comparative, functional, developmental, and morphological studies on animal anatomy require accurate visualization of three-dimensional structures. Nowadays, few widely applicable methods exist for non-destructive whole-volume imaging of animal tissues. The purpose of this study is to develop a method for quantitative analysis of the total fetal pulmonary vasculature using a technique that could be translated into the human studies.

Methods

Experimental Design
Following approval of IACUC protocol #2016-0068 by the Cincinnati Children's Research Foundation Institutional Animal Care and Use Committee, age-matched Sprague Dawley rats were mated and the date when the vaginal plug is seen was accepted as embryonic day 0 (E0). At E21, all dams were harvested by cesarean section after inhaled general anesthesia. The umbilical cord was tied to prevent any blood loss and pups were euthanized.

Specimen Preparation
Lugol solution, which was prepared from 10g KI and 5g I2 in 100ml H2O and was diluted with de-ionized H2O to create a physiologically isotonic solution, allowing better diffusion and decreased shrinkage of soft tissue, was used as an intravascular contrast. To get a better visualization of the pulmonary tree multiple specimens were prepared including solely lung, upper half body of the fetus, direct contrast injection into the right ventricle, and a whole fetal body with partial peeling of the skin (Figure 1). These preparations were individually imaged for appropriate and adequate diffusion of iodine into the pulmonary vasculature to determine which preparation provided the best catalyst for Lugol penetration.

Figure 1. Partial peeling of the pup skin

Specimen Treatment
Fetuses were fixed overnight in 4% paraformaldehyde (PFA) within phosphate buffered saline (PBS). Then, they were treated with 25% Lugol solution for 72 hours.

µCT Imaging
The µCT scanner used in this study is a MicroCAT II (ImTek, Inc., Knoxville, TN). For the scanner geometry employed here, the usable field of view was slightly less than 3x2 cm. Raw data were acquired over a 55-minute scan time using the following parameters: 80 kVp, 200 µA, 800 projections, 0.25° increment, 3.3 s exposure, with 2-by-2 binning of the 3072 x 2048 element CCD detector. The projection data were corrected for distortion and detector anomalies, and then reconstructed by Feldkamp cone beam filtered back projection, resulting in a 3D image with an isotropic voxel size of 19 µm (isotropic resolution of 21 µm, calculated by the Imtek Resolution Estimator software, based on scanner geometry, source, and camera conditions).

Image analysis
A commercial software, Analyze 12.0 (Rochester, MN) was used for data acquisition and 3D reconstruction of the pulmonary arterial tree from the data gathered by µCT. A 3D reconstruction of the pulmonary vascular tree was achieved with multiple steps. First, the area of interest was named. Then, a threshold segmentation was optimized by the specimen. Seeds were selected on the stained vasculature and the object was extracted by the region grow function. Vessels other than those belongs to the pulmonary system were excluded by the object separator function. The high-resolution 3D renderings of the vascular tree were generated with a 3D volume rendering technique. Tree analysis was done by the generate tree function 5 Lastly, number of branches and generations were calculated automatically by the software.

Histology
Samples stored in Lugol solution up to 180 days after µCT scan. Before H&E analysis, samples were washed using ethanol and were embedded in paraffin. Five µm sections were cut by cryostat and mounted on polylysine slides. Slides were baked a 60°C overnight, stained with hematoxylin and eosin, and mounted. Tile scanned 4X and individual 20x images were obtained using a Nikon Eclipse 80i widefield microscope and Nikon Elements software.

Statistical analysis
Statistical analysis was performed with IBM SPSS Statistics 20.0.0 (Chicago, IL). Data were expressed as means ± standard deviations.

Results

Staining Results
Different methods to introduce the iodine were tried to improve the adequacy of iodine stain penetration into the pulmonary tree. Whole lung extraction from the fixed fetus, with Lugol solution exposure, yielded no visible vessels on the CT scan. Direct injection of the contrast material into the right ventricle, on the other hand, was an unexpected failure, in which, almost none of the vasculature was illuminated, however significant contrast pooled in the heart. When a fetal half body was fixed and placed in the Lugol solution, there was only partial penetration of the iodine stain, therefore the complete pulmonary tree could not be displayed. Conversely, in the pups with partially peeled skin (Figure 1), Lugol diffused homogenously throughout the vessels, providing detailed imaging of the lung vasculature. (Figure 2)

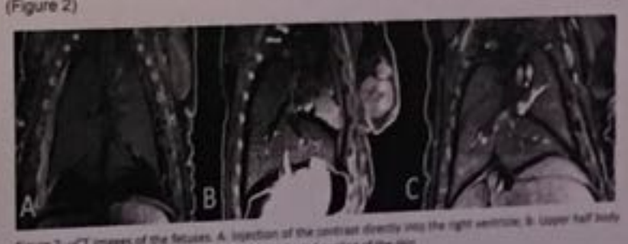

Figure 2. µCT images of the fetuses. A: Injection of the contrast directly into the right ventricle, B: Upper half body of the fetus embedded into the lugol solution, C: Partial peeling of the skin

Gross Results
Four rat fetuses were scanned in each group. The partially peeled skin technique was the only successful preparation that proved the microarchitecture. Therefore, using this technique both the side branches of the high generation vessels and 3D interconnectivity of the whole lung prenatally were revealed. Iodine stains only the blood intensely, and so, any bleeding in pups impaired filling of whole pulmonary vascular tree down to the level of alveolar capillaries. In these cases, a clear visualization of the pulmonary microcirculation could not be achieved. The 3D images gathered by µCT and processed by the software showed pulmonary artery, pulmonary vein, and micro vessels distributed through the lung on E21 of a rat fetus. (Figure 2) The pulmonary vascular tree was representative of the shape and structure of the lungs.

Morphometric Results
The morphometric results of the µCT scan were comparable with the gross imaging findings. The mean number of vessels segmented in the pulmonary tree was 900 ± 24 with a mean diameter of 134.13µ (range 40.72-265.89µ). Comparison of the measurements between arteries and veins were summarized in Table 1. While up to the 30th generation of vessels were able to be segmented both in arteries and veins, majority of the branches were between the 11th and 20th generations (Figure 3).

	RPA	RPV	LPA	LPV
Area of vessel	56.29 ± 28.94	53.46 ± 42.81	48.64 ± 25.03	42.91 ± 34.34
Vessel length	21.02 ± 14.51	25.04 ± 18.8	18.5 ± 13.04	22.28 ± 13.91
Diameter of the vessel	122.97 ± 46.07	145.78 ± 65.19	121.34 ± 47.46	137.68 ± 61.72
Circumference of the vessel	386.72 ±	457.95 ±	381.05 ±	432.73 ±
L/D ratio	0.20 ± 0.07	0.18 ± 0.14	0.18 ± 0.14	0.21 ± 0.16

Table 1: The morphometric results per vessel type. (RPA: Right pulmonary artery, RPV: Right pulmonary vein, LPA: Left pulmonary artery, LPV: Left pulmonary vein, L/D ratio: Vessel length to diameter ratio.)

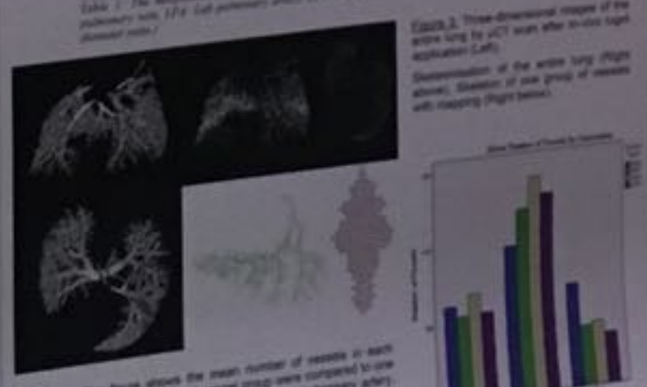

Figure 3. Three-dimensional images of the entire lung by µCT scan after in-vivo lugol application (Left).
Segmentation of the entire lung (Right above). Division of one group of vessels with mapping (Right below).

Figure 4. The figure shows the mean number of vessels in each vessel type. The means of each vessel group were compared to one another within the generation group. (RPA: Right pulmonary artery, RPV: Right pulmonary vein, LPA: Left pulmonary artery, LPV: Left pulmonary vein, L/D ratio: Vessel length to diameter ratio.)

Conclusion

µCT and iodine staining procedures has allowed for analysis of embryologic development at multiple time points. With proper staining and preparation techniques vasculature patterns can be visualized using µCT in any vascular organ throughout fetal development. Further, this early analysis of lung anatomic growth has application for early detection of abnormalities in embryologic and fetal development. The gross and morphometric findings in this study is comparable with the studies published in the literature with other methods.