"The future belongs to those who see possibilities before they become obvious"
J. Sculley

Chapter 7. Summary and Future Direction

7.1 Summary and Future Direction

In the preceding chapters, a framework for imaging lung structure, function and pathology from *in vivo* imaging modalities down to the "ground truth" histopathology has been developed. Many technical limitations of current imaging modalities and techniques have been overcome through the development of novel imaging approaches.

These developments included a Large Image Microscope Array (LIMA), a 3D pathology system for acquisition of histology through a refined process enabling accurate registration of non-invasive three-dimensional modalities such as CT, MRI and PET down to "ground truth" histopathology. The system construction included several milestones including the development of a dual frequency large scale vibratome, motorization of a large scale microtome including implementation of a photo lock mechanism, and finally complete computer automation of the sectioning and imaging process. The utility of the LIMA system was established through several examples including a fixed sheep lung and whole mouse lung, imaged, sectioned and registered with micro-CT and histology.

An evaluation of imaging artifacts and noise associated with a Siemens micro-CT scanner was undertaken in the fourth chapter. Current limitations and pitfalls were

discussed and several image-processing algorithms were implemented in order to increase the resolution and repeatability of the pre and post reconstructed images. A novel *in vivo* Intermittent Iso-pressure Breath Hold (IIBH) technique was developed to reduce motion artifacts in live micro-CT imaging. Included in this achievement was development of animal handling, anesthesia and surgical protocols along with hardware and software development of the respiratory ventilation and triggering system. The breath-hold technique was evaluated for repeatability and accuracy against standard spontaneous and respiratory-gating techniques. Through this investigation, it was concluded that the IIBH technique produced superior images when compared to standard respiratory gating approaches in small animals.

In the fifth chapter, we focused on two confocal microscopy techniques developed for imaging the lung. A novel laser scanning confocal microscopy technique was created for imaging freshly excised whole mouse lungs through the development of a water and air tight imaging chamber. This system provided high-resolution imaging of the lung parenchyma in two, three and four-dimensions. Using this imaging technique, a study on alveolar mechanics during respiration was undertaken. For the first time, high-resolution cross-sectional images of the alveoli were obtained over sequential inflation and deflation pressures within the same lung. Automated imaging algorithms were developed to extract alveolar metrics from these images. Quantitative results confirmed for the first time direct evidence of alveolar recruitment, a long-standing question in lung structure and function. From the empirical results found in this study, a new theory on alveolar mechanics was proposed. In the second section of this chapter, a flexible fiber optic Catheter Based Confocal Microscopy (CBCM) system was described and developed. A series of image processing and analysis steps were described, providing accurate quantitative analysis of alveolar structure obtained from the CBCM images. A high-speed CBCM system was then used for the first time to observe recruitment of alveoli in live mice lungs.

Using the developed techniques and approaches discussed throughout this thesis a longitudinal mouse lung cancer study was undertaken and described in Chapter 6. A group of mice was imaged using the micro-CT breath-hold technique over a span of 6-months. Several animal preparation protocols were developed in order to maintain

mouse vital signs during micro-CT imaging and ensure longevity over the span of the study, including an ultra micro-bronchoscope to aid in fast and efficient intubation of mice for micro-CT imaging. As a pilot study, a subset of mice was also imaged at the 6-month point using a micro-MRI, and 6 & 9-months using micro-PET imaging, for both additional structural and functional information. *In vivo* CBCM imaging of lung tumors at the end-points was performed for evaluating the ability of this modality for tumor classification at the cellular level. Lungs were excised and re-imaged using the LSCM imaging chamber for high-resolution cellular imaging. Excised lungs were fixed using the Heitzman technique and further embedded for *ex vivo* micro-CT imaging. Foam embedded lungs were imaged and sectioned on the LIMA system and further processed for H&E histology.

From this longitudinal lung cancer study, it was found that lesions as small as 0.11mm could be identified and tracked using the *in vivo* micro-CT imaging system. Lesions were identified across time points within the same mice, and data on tumor size, density, location and number was extracted. Tumor growth, on average, was found to increase at a linear pace in the first 2 months and reduced in growth rate at month 4 and 6, but was expected to increase with a faster rate once the transformation to an adenocarcinoma had occurred post 6 months. In addition, on average, the aggregate rate of tumor growth was similar across mice, an established finding using standard sacrificial studies; however, there was considerable variation in individual tumor growth within mice. This intra-mouse tumor growth rate heterogeneity could only be found using the non-invasive imaging approach implemented in this study. The number of identified tumors increased dramatically in the first few months and took on a slower rate of increase from the third to sixth month. The distribution of tumors was found to be proportional to the lobe volumes, with the exception of the right diaphragmatic lobe, which had an average of 20% more tumors, a finding that is currently unexplained and requires further investigation. There were at least two tumor growth rates identified; one group included smaller tumors growing at a steady slow pace, while a second smaller group of larger tumors grew at a significantly faster more aggressive pace. Further investigation with respect to tumor growth rates and anatomical structures such as vessels, airways and pleural boundaries needs to be undertaken. The pilot micro-PET imaging study revealed that no activity was detectible until 9 months at which

time tumors had transformed into adenocarcinomas from their benign adenoma phase.

CBCM and LSCM imaging revealed that underlying cellular changes were apparent between the parenchyma of normal A/J control mice and "normal" Urethane tumor bearing mice. Notably, the alveolar septa were thicker and there was a prevalence of nuclear stain bound to ssDNA in the Urethane mice as opposed to the normal A/J mice. In suspicious and tumor regions the difference was dramatic, and identification of nuclei and surrounding tissue structures was possible. Further investigation between benign adenomas and malignant adenocarcinomas must be undertaken to identify whether this imaging approach can accurately distinguish between these two critical phases.

Registration of H&E histology back to the *in vivo* imaging modalities was successfully implemented, and tumor growth rates were accurately traced back to their underlying cellular structure. All of the tumors identified in the micro-CT scans in this 6-month longitudinal study were classified as benign adenomas. It was then difficult to make strong conclusions regarding tumor growth rates and underlying cellular progression based on the H&E phenotype. Further investigation with the addition of immunohistochemical staining and genetic expression evaluation may provide stronger clues. Finally, tumor tracking and correlative histology for Urethane mice lungs between 6 to 12 months would also make for a beneficial augmentation to the current data and may provide further clues on their relationship with respect to transformation of adenomas into adenocarcinomas.

In addition, it is envisioned that a publicly accessible website will be developed to house the comprehensive mouse lung cancer micro-CT, micro-MRI, micro-PET, LIMA and histology datasets acquired in the study described in Chapter 6. It is hoped that this dataset will provide a source of information for other groups, providing a comprehensive dataset for both biological studies and image processing and analysis experiments. Additional studies described above would also be added over time to augment the already comprehensive dataset.

Our Understanding of human lung cancer is poor. Lung nodules are now detected in the human lung at a size of 2-3mm, which is equivalent to the adenomas found in this mouse lung study. Viewing these small tumors with catheter based confocal microscopy techniques appears achievable, and may be useful in understanding the transition from benign adenomatous hyperplasia to the malignant phenotype. Further investigation using mouse models is required for this to be realized.

In conclusion, an array of new small animal pulmonary imaging methods to augment previous modalities and development of new modalities has been described in this thesis for assessment of pulmonary structure, function and pathology in mice. Naturally, over the span of this work, many new hypotheses have been generated, and many more are yet to be found.

"Until governments take the problem of global tobacco control seriously, the golden leaf of the Americas will remain the world's leading cause of preventable death, and a black mark on our ability to turn knowledge into a force for human health and well-being" [85]