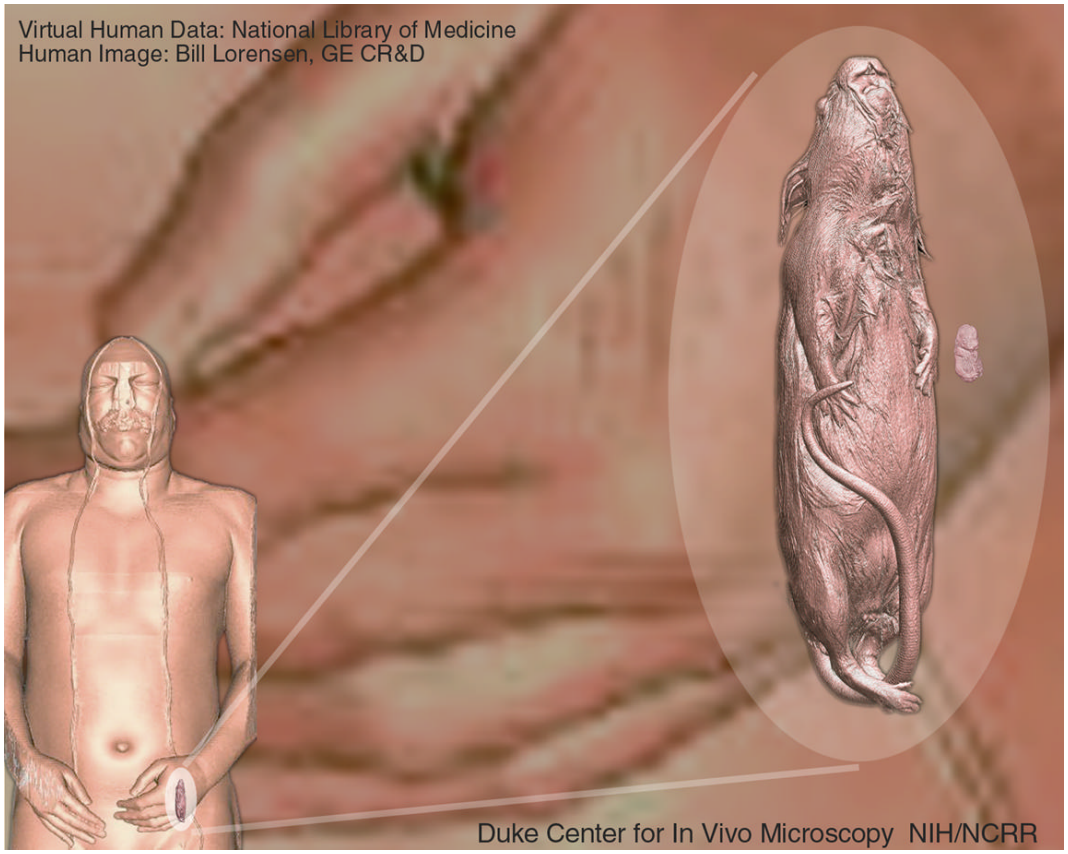
In Vivo Microscopy: Technologies and Applications

A workshop for small animal imaging

March 18 & 19, 1999
Gaithersburg, MD



G. Allan Johnson, Ph.D.
Daniel H. Turnbull, Ph.D.
Elaine G. Fitzsimons, M.S.
Editors

Introduction

Several seminal papers in the early 70's have revolutionized modern medicine through the introduction of advanced imaging technologies. Hounsfield's[1] and Cormack's[2] papers led to a Nobel Prize in medicine for their invention of computed tomography. Lauterbur[3] and Mansfield's[4] papers may eventually lead to a Nobel Prize for the invention of magnetic resonance imaging. Ter-Pogossian's paper laid the foundation for modern nuclear tomography.[5] Ultrasound imaging has older origins,[6] but improvements and new approaches continue to make this image modality an indispensable tool in many clinical areas. An extraordinary body of research in imaging technologies and applications has developed over the last 25 years. The majority of this work has understandably focused on the clinical applications of these technologies. But a number of researchers have recognized the potential for imaging in the basic sciences.

One of the most promising areas of application for imaging in the basic biomedical sciences is the study of small animal models. The mouse, rat, and guinea pig have become ubiquitous participants in most areas of molecular

[1] Hounsfield GN: Computerized transverse axial scanning (tomography). 1. Description of system. British Journal of Radiology 1973; 46(552): 1016-22.
[2] Cormack AM: Reconstruction of densities from their projections, with applications in radiological physics. Physics in Medicine & Biology 1973; 18(2): 195-207.
[3] Lauterbur PC: Image formation by induced local interactions - examples employing nuclear magnetic resonance. Nature 1973; 242: 190-191.
[4] Mansfield P, Grannell PK: Diffraction in microscopy in solids and liquids by NMR. Phys. Rev. B 1975; 12: 3618.
[5] Ter-Pogossian MM, Phelps ME, Mullani NA: A positron-emssion trasnxaial tomograph for nuclear imaging (PETT). Radiology 1975; 114: 89-98.
[6] Wild JJ, Reid JM: application of echo-ranging techniques to the determination of the structure of body tissue. Science 1952; 115: 226-230.

biology, toxicology, and drug discovery research. Well-characterized models have been developed for a wide range of diseases to facilitate more complete understanding of the diseases and provide appropriate vehicles for drug validation. The mouse, in particular, has become a key animal model system to study development and human disease. The ability to manipulate the mouse genome to produce accurate models of many human diseases has resulted in significant progress in understanding these diseases. However, it is clear that the full potential of these new mouse models has not been realized, in part due to the lack of readily available noninvasive imaging methods to investigate disease progression and response to therapeutic agents in mice.

Extension of modern imaging techniques to the small animal presents some fascinating challenges and opportunities. It is important to recognize that tomographic imaging, i.e., imaging involving definition of a "slice" of the patient or specimen under study, is best characterized by a volumetric resolution. Each pixel in the image represents the signal from a voxel of tissue. The figure on the cover of this document demonstrates this effect dramatically by comparing volume-rendered images acquired using MRI and MR microscopy. The volume-rendered image of the “visible human” is depicted next to the “visible mouse.” The images are scaled relatively, showing the mouse in relation to the human hand, and the embryo, in turn, in relation to the mouse. Note the embryo is much smaller than the human finger in the background. The increase in resolution required to go from a 200-kg man to a 1-g mouse embryo while maintaining the relative organ definition is more than five orders of magnitude. The biological, physical, and engineering challenges in achieving this sort of resolution improvement are enormous. But the opportunities for new science in a broad range of biomedical research suggest that these challenges should be addressed. The goals of this two- day conference on small animal imaging held at Gaithersburg, MD on March 18–19, 1999 were a) to explore the state of the art in small animal imaging research; b) identify the major challenges facing the field; c) list some of the most immediate applications of small animal imaging; and d) define some specific directions for federal support.

State of the Art in Small Animal Imaging

The conference was divided into two sessions: Technologies and Applications. Five specific imaging technologies were identified: computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT), and ultrasound. Two additional emerging technologies were identified: small animal physiologic support and monitoring, crucial to the maintenance of animal models in all the imaging research, and new imaging markers that promise to enhance both sensitivity and specificity through novel targeting. There are undoubtedly additional imaging technologies not covered, for example, infrared imaging, optical techniques, microwave imaging, etc. Given the limited time of the conference, we chose to focus on these five "main line" modalities because they are all reasonably well developed and have a broad base in their clinical applications. We include below the summaries of each of the speakers.

Technologies

MR Microscopy

G. Allan Johnson, Ph.D.
Center for *In Vivo* Microscopy
Duke University Medical Center

Magnetic resonance microscopy (MRM) was first demonstrated by Johnson et al. and Eccles and Callaghan in 1986.[7,8] The fundamental principles are the same as clinical magnetic resonance imaging (MRI), but MRM is substantially more than high-resolution MRI. The challenge to achieve spatial resolution in a small animal where the anatomy is scaled at the same level relative to that in clinical imaging is substantial. Resolution is limited by motion, by the limited signal from the small voxels, by magnetic susceptibility, and by diffusion.

The Center for *In Vivo* Microscopy, an NIH / NCRR National Resource, is committed to the development of MRM and its application in the widest range of biomedical research. The Center has taken an interdisciplinary approach with four core activities: 1) development of physiologic support and monitoring to limit the consequences of motion, 2) construction of special purpose radiofrequency (RF) coils to optimize sensitivity, 3) creation of novel new encoding strategies to enhance both sensitivity and contrast at the ultrahigh magnetic fields (up to 9.4 T) required for MRM, and 4) development of an extensive computer infrastructure to support acquisition, reconstruction, analysis, and archival of very large (up to 1 GB) image arrays.

The interdisciplinary nature of our approach is best demonstrated by example. The first example is that of MRM in small animal models of pulmonary disease. The lung is particularly challenging. Cardiac and respiratory motion require careful synchronization of these physiologic activities with the acquisition strategy. A second barrier is the limited signal from the lung. Since the density of lung is ~ 10% of that of other tissues, one must expect a comparably lower signal. For proton imaging, the situation is exacerbated by the physics of the microscopic environment of the lung. Small variations in local magnetic susceptibility contribute to an extraordinarily rapid signal decay. Using a novel projection encoding strategy, we have reduced the delay between excitation and reception of the signal (the “echo time”) from the 5–10 ms common in traditional encoding strategies to < 1 ms, thereby capturing the very weak signal as effectively as possible. Figure 1a shows a representative axial proton image of a live rat imaged using a projection encoding sequence synchronized with the animal’s ventilation.

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[7] Johnson GA, Thompson MB, Gewalt SL, Hayes CE: Nuclear magnetic resonance imaging at microscopic resolution. Journal of Magnetic Resonance 1986; 68: 129-137.
[8] Eccles CD, Callaghan PT: High resolution imaging: The NMR microscope. Journal of Magnetic Resonance 1986; 68: 393-398.

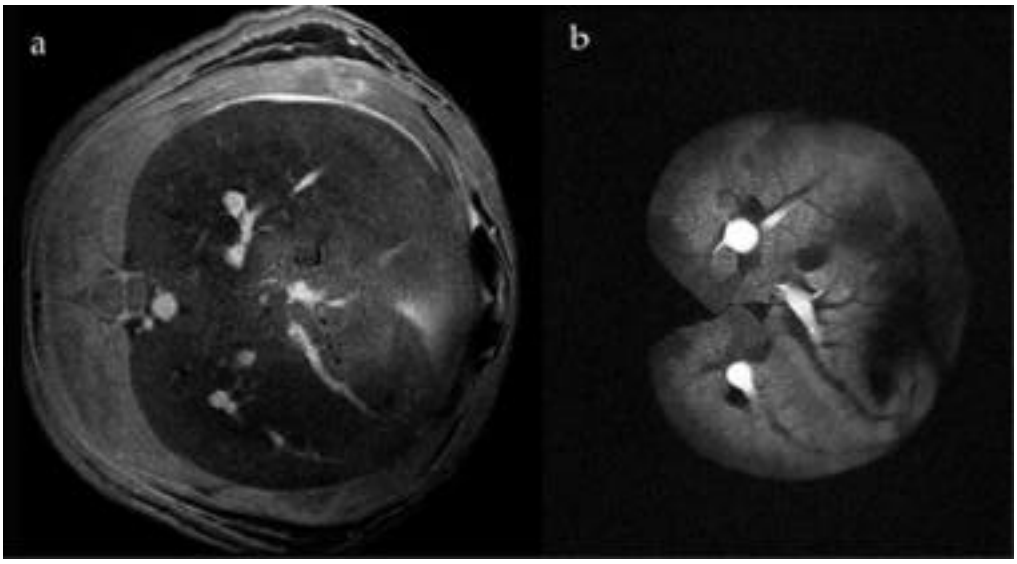

Figure 1: MRM image of a live rat. (a) A proton image using a projection encoding sequence synchronized with the animal's ventilation. (b) The same animal imaged using hyperpolarized helium as a signal source.

Figure 1b shows a comparable level of the same animal imaged with a novel new strategy relying on the results obtained from fundamental studies in optical pumping. ^{3}He has been polarized via laser polarization to produce an extraordinary new signal source. Instead of the water protons in tissues, the image signal source is the polarized 3He in the gas spaces. The complementary nature of the ^{1}H and ^{3}He images is evident: blood vessels are bright in the ^{1}H image and dark in the 3He image, airways are bright in the ^{3}He image and dark in the ^{1}H image.

Our second example is the demonstration of a new approach to rapid phenotyping using MR microscopy. Figure 2 shows a composite image from a 3D data set of a fixed mouse. The specimen has been carefully perfused with formalin containing Gd-EDTA. The Gd-EDTA preferentially reduces the tissues' spin lattice relaxation time in a fashion probably related to the local interstitial spaces. By reducing the spin lattice relaxation time of all the tissues, we are able to acquire data using a very short TR (100 ms). We have expanded our encoding, reconstruction, archive, and display technologies to accommodate 3D arrays of up to 1024 x 1024 x 1024. This particular data set at 256 x 256 x 1024 is sampled at 100 x 100 x 100 µm. Since the data is truly isotropic, it can be viewed along any plane without loss of resolution. In this composite image, we have magnified three specific regions. At 100 µm resolution in-plane with a 100 µm thick slice, there is no evidence of pixelation, even in the magnified image. MR histology (MRH) provides three unique opportunities over conventional optical techniques: 1) MRH is nondestructive; 2) MRH permits one to employ unique "proton stains", in this case the Gd to produce altered T1; and 3) MRH is inherently 3D, as demonstrated in Fig. 2c. The potential of this tool as a rapid phenotyping tool for molecular biologists should be obvious.

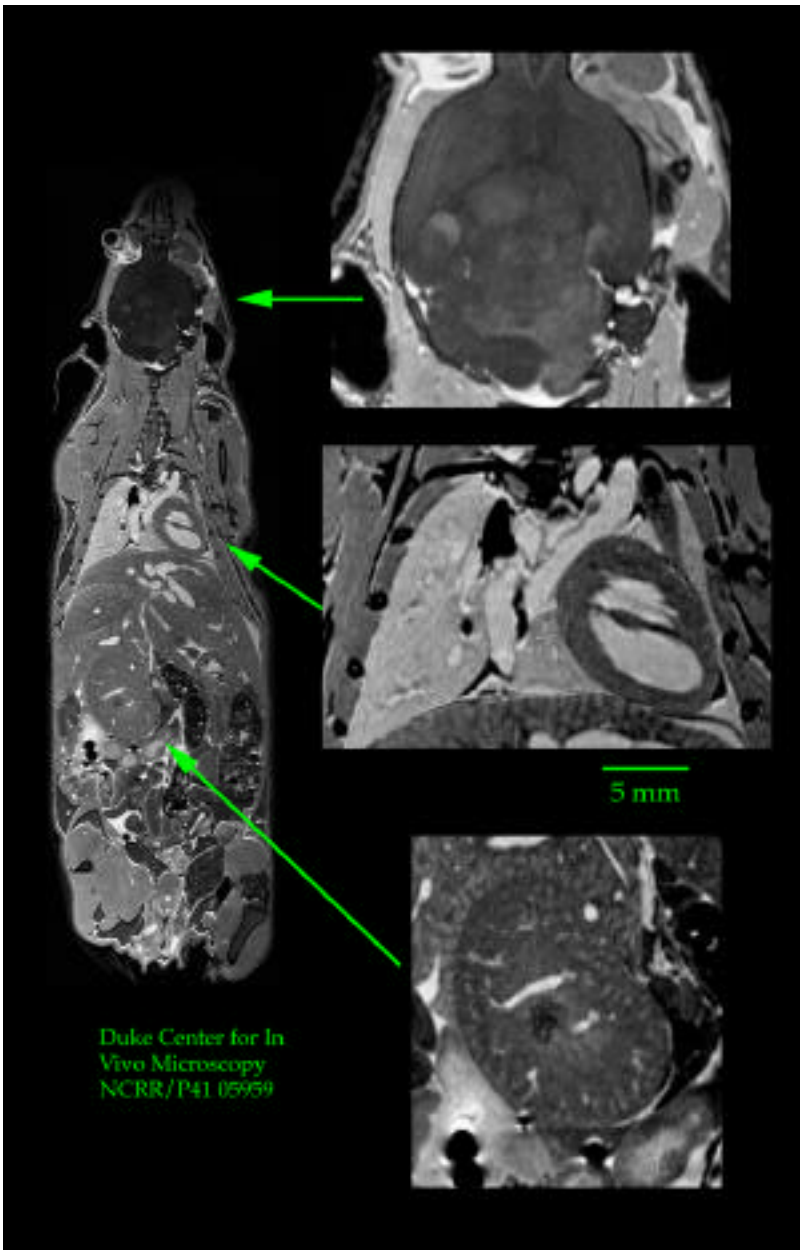

Figure 2: A 3D dataset of a fixed mouse with isotropic resolution of 100 x 100 x 100 µm.

The utility of MRM has already been demonstrated in a wide range of small animal applications. The soft tissue discrimination is superb. And the resolution available allows anatomical definition of anatomy at resolutions of more than 5 orders of magnitude higher than that of routine clinical MRI. The technology continues to improve, and as it does, so will its many applications.

Micro X-ray Computed Tomography (MicroCAT) for Mouse Phenotype Screening

Michael Paulus, Ph.D.
Instrumentation and Controls Division
Oak Ridge National Laboratory

A new small-animal x-ray computed tomography system (MicroCAT) has been developed to screen mutagenized mice in the Oak Ridge National Laboratory Mammalian Genetics Research Facility. The MicroCAT hardware consists of a high-resolution phosphor screen / CCD detector, a low-energy x-ray tube, several precision-motion translation stages and a Windows-NT workstation. Data sets for animal screening are typically acquired in seven minutes and produce reconstructed images with resolutions of approximately 150 microns. High-resolution data sets are typically acquired in 15–20 minutes and produce reconstructed images with resolutions of approximately 50 microns. Data from several recent scans including images of tumor models, obesity models, and mice with skeletal abnormalities are presented (Fig. 3). A new "deformable contours" algorithm for automatically identifying and evaluating organs in image space has been developed and validated through the study of a population of normal mice and mice with polycystic kidney disorder (Fig. 4).

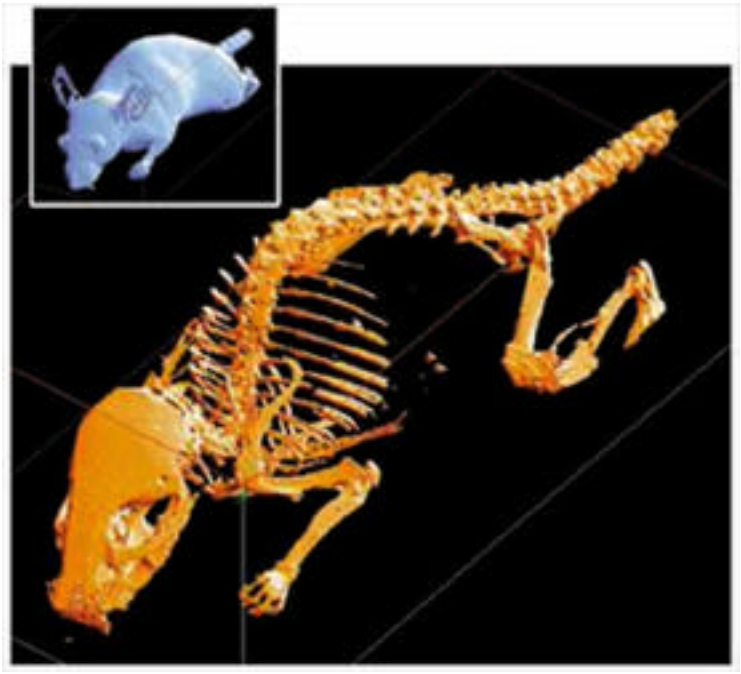

Figure 3: Three-dimensional volume renderings of high-resolution x-ray computed tomography data acquired using the ORNL MicroCAT. The subject is a 25-g wild-type mouse.

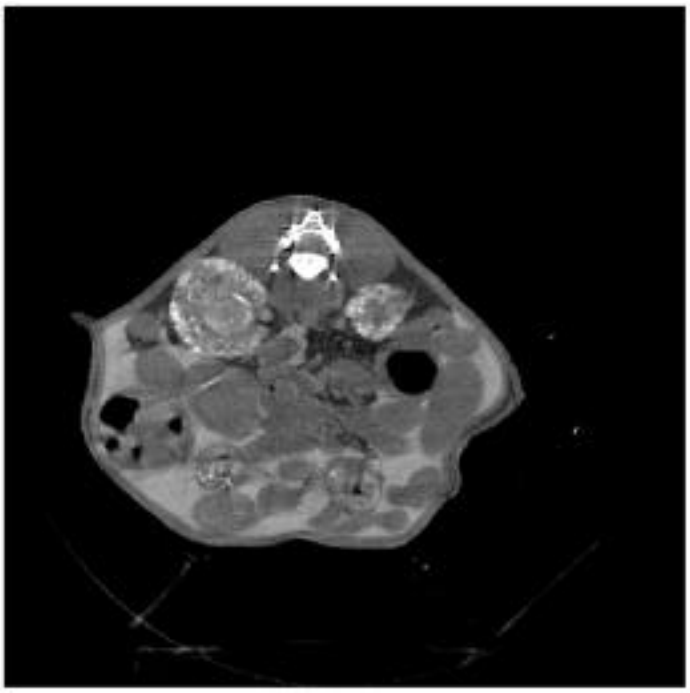

Figure 4: ORNL "orpk" polycystic kidney disorder insertional mutation in the Tg737 gene. Mouse Model PKD resembles that seen in human autosomal recessive PKD patients. Specimen received IP contrast media injection 30 minutes prior to scan. Cysts lead to uneven distribution of contrast media uptake in the kidneys.

High Resolution Pet Instrumentation for Small Animal Imaging

Simon Cherry, Ph.D.
Crump Institute for Biological Imaging
UCLA School of Medicine

Positron Emission Tomography (PET) is an in vivo analog of autoradiography, which gives it the potential to become a powerful new tool in imaging biological processes in small laboratory animals. PET allows individual animals to be studied repeatedly, an advantage for longitudinal studies (for example, in the study of development) and for assessing the effects of intervention (be that surgical, pharmacological, or genetic manipulation), as each animal can serve as its own control. With the importance of studying human disease models in small laboratory animals, particularly in the mouse, the potential for high-resolution PET technology to contribute valuable information has become apparent to many. While the attractions are obvious, the challenges are also significant. A new generation of very high-resolution, high-sensitivity, and inexpensive PET scanners need to be developed for these demanding but exciting applications.

A number of research groups throughout the world have risen to this challenge and there are now several working prototype PET scanners dedicated to imaging small laboratory animals, with many others existing in various stages

of development. The microPET scanner developed at UCLA consists of 30 high-resolution fiber-optically coupled lutetium oxyorthosilicate scintillation detectors arranged in a 17-cm-diameter ring. Each detector is readout by a 64 element photomultiplier tube. The detectors and electronics are mounted inside a gantry measuring 90 cm wide by 140 cm high. The animal port is 16 cm in diameter. There is also a computer-controlled bed (which has a built-in wobble motion to improve spatial sampling) and a laser positioning system. The transverse field of view is 11 cm and the axial field of view is 1.8 cm. MicroPET is a fully 3D imaging system with no slice-defining septa.

Figure 5: The microPET scanner developed at UCLA. This scanner is designed for mice and rats and has an isotropic reconstructed resolution of 1.8 mm.

MicroPET has a reconstructed image resolution of 1.9 mm in all three axes (volumetric resolution = 0.007 cc) and an absolute sensitivity of 5600 cps / MBq (250 keV lower energy threshold) at the center of the field-of-view. When the built-in wobble motion is used, the volumetric resolution improves to 0.005 cc. This is more than an order of magnitude better than state-of-the art clinical PET systems. MicroPET became fully functional in summer 1997 and over 1000 studies in mice, rats and small primates have been successfully completed using a range of tracers including [F-18]-FDG, [C-11]WIN35,428 and [F-18]-FESP.

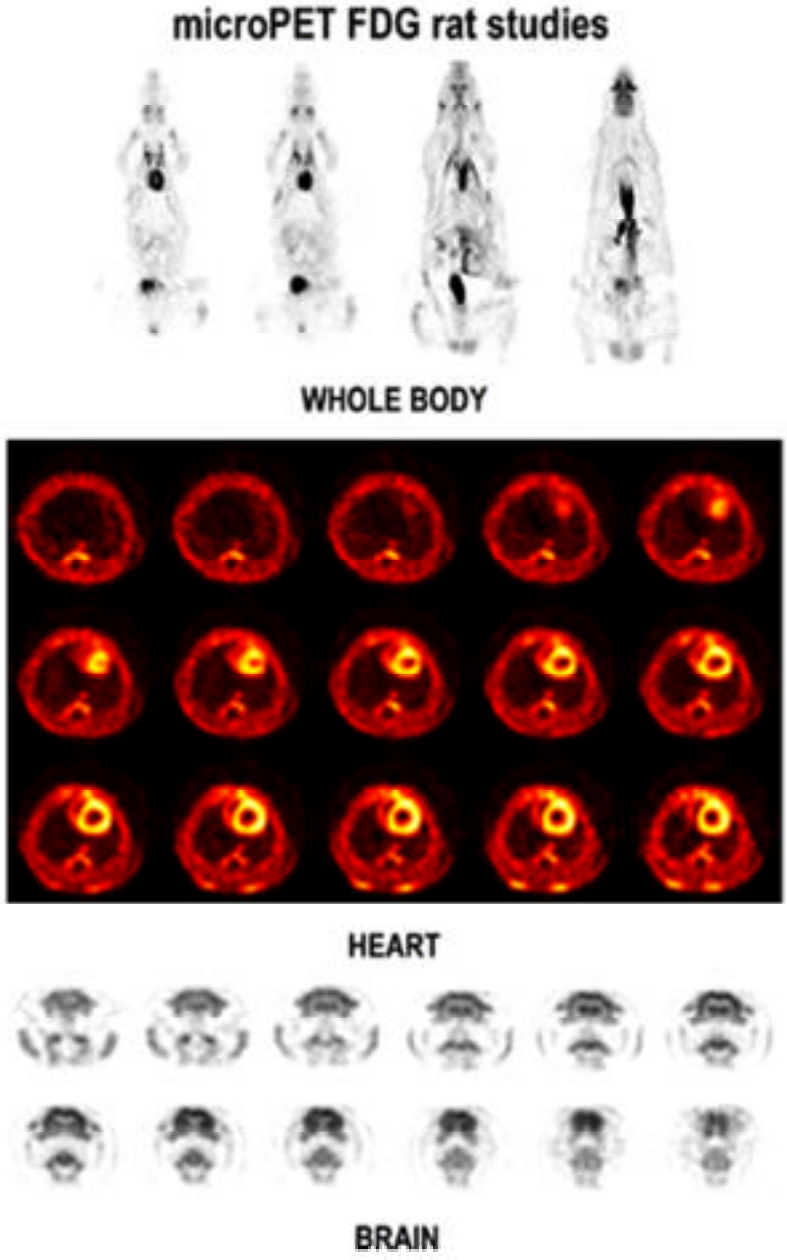

Figure 6: MicroPET rat images, all using 18F-FDG in normal rats. Top: coronal cross-sections through the whole body of a rat. Middle: transverse sections through the level of the heart, clearly showing the myocardium. Bottom: coronal brain sections showing ability to separate cortex, thalamus, and striata. In all cases, injected FDG dose was around 2 mCi and imaging times varied from 90 minutes (top) to 40 minutes (middle and bottom).

High Resolution Ultrasound Techniques

Katherine W. Ferrara, Ph.D.
University of California, Davis

New opportunities exist for the imaging of small animal models using ultrasound. Frequencies significantly higher that those used clinically can be used to improve spatial and velocity resolution for targets near the transducer. High-frequency ultrasound (40–60 MHz) can be used to image small animals and embryos using M-mode or B-mode techniques and can map blood flow in vessels as small as 40 microns. It can provide spatial resolution on the order of 40 microns for targets within 5 mm of the transducer at typical diagnostic power levels. In addition, at higher frequencies the scattered intensity from blood increases with respect to that from tissue, and therefore for a fixed dynamic range, smaller blood vessels can be detected with high-frequency ultrasound. While the Doppler frequency from moving red blood cells also scales with frequency, its relative magnitude compared with clutter from moving tissue remains constant. Due to the improved frame rate and "bench-top" nature of the instrumentation in comparison with other imaging modalities, high-frequency ultrasound can also be used to guide interventions.

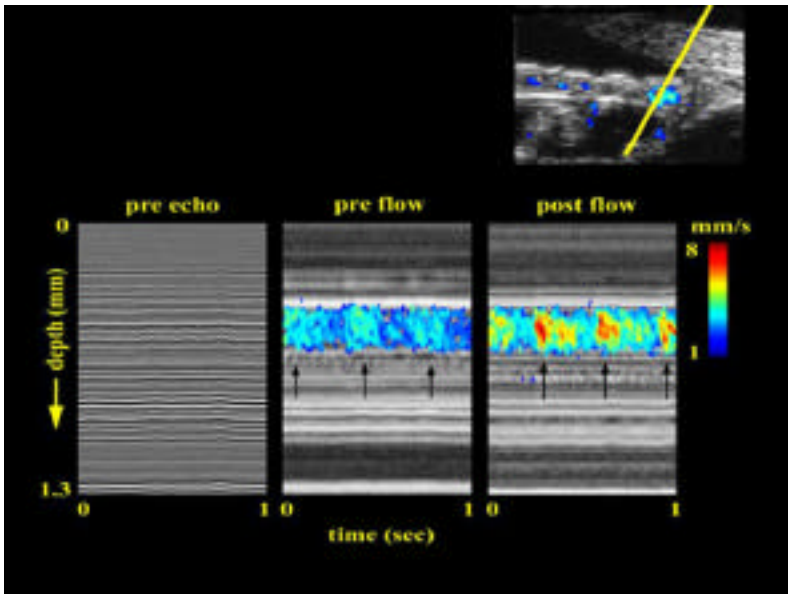

Figure 7: Upper right corner: 2D image showing the orientation of the ultrasound transducer to common anatomical landmarks of the eye (includes sclera, iris and ciliary processes of the eye). To evaluate changes in blood flow over the cardiac cycle, flow along one line-of-sight can be continually monitored. A line is identified in the 2D image as containing the major arterial circle in the iris, and this region is then interrogated further, as shown in the lower M-Mode data. On left: raw M-mode data from the major arterial circle. Stationary structures are horizontal, slanted structures indicate moving red blood cells. The velocity of flow is estimated and encoded in color in the next images. Center: colorflow M-mode data acquired before the application of topical atropine. Right: colorflow M-mode data acquired 15 minutes after the application of two drops of 1% atropine sulfate. The peak systolic blood flow velocity was found to increase 72% after the application and the diastolic velocity increased 46%. Additionally, cardiac cycle pulsatility was found to be 27% pre-atropine and 38% post-atropine.

In order to map capillary density and flow rate, ultrasound contrast agents can be used to increase the sensitivity of ultrasound detection. These agents consist of a gas core encapsulated by a shell of albumin or lipid, and are intra-vascular agents. The microbubbles have a diameter on the order of 2-10 microns and a resonant frequency between 1–14 MHz. Echoes produced from these agents are sufficiently strong that an individual microbubble can be detected by typical ultrasound instrumentation. When insonified at their resonant frequency, these microbubbles produce echoes at this resonant frequency and harmonic frequency multiples. For the imaging of small animals, a transmission frequency of 4 MHz and a center frequency near 8 MHz for reception may produce spatial resolution on the order of 300 microns. Although individual capillaries are not resolved at this frequency, the density of microbubbles within the region is estimated and assumed to reflect the blood volume within the region.

Promising new methods to detect contrast agents include harmonic power Doppler and pulse inversion techniques, which both take advantage of the nonlinear behavior of microbubbles. The resulting images from these techniques allow the differentiation of tumors from normal tissue and the identification of ischemic cardiac tissues. It has also been shown that ultrasound can destroy contrast agents in a small sample volume with a single pulse. The time required for contrast agents to return to this region can then be determined and microvascular flow rate estimated, resulting in a very promising technique to locally evaluate tissue perfusion.

The mechanical effects of contrast agents *in vitro* and in small animal models may also yield new applications for ultrasound mediated interventions. Recent studies using contrast agent insonation within the rat endothelial wall have shown that it is possible to deliver colloidal particle distances on the order of one hundred microns into the interstitium. It has been hypothesized that microstreaming (moving fluid) due to pulsating microbubbles may result in changes in cell permeability and that microstreaming or cavitation may be responsible for enhanced transfection and drug delivery observed *in vitro* and *in vivo*.

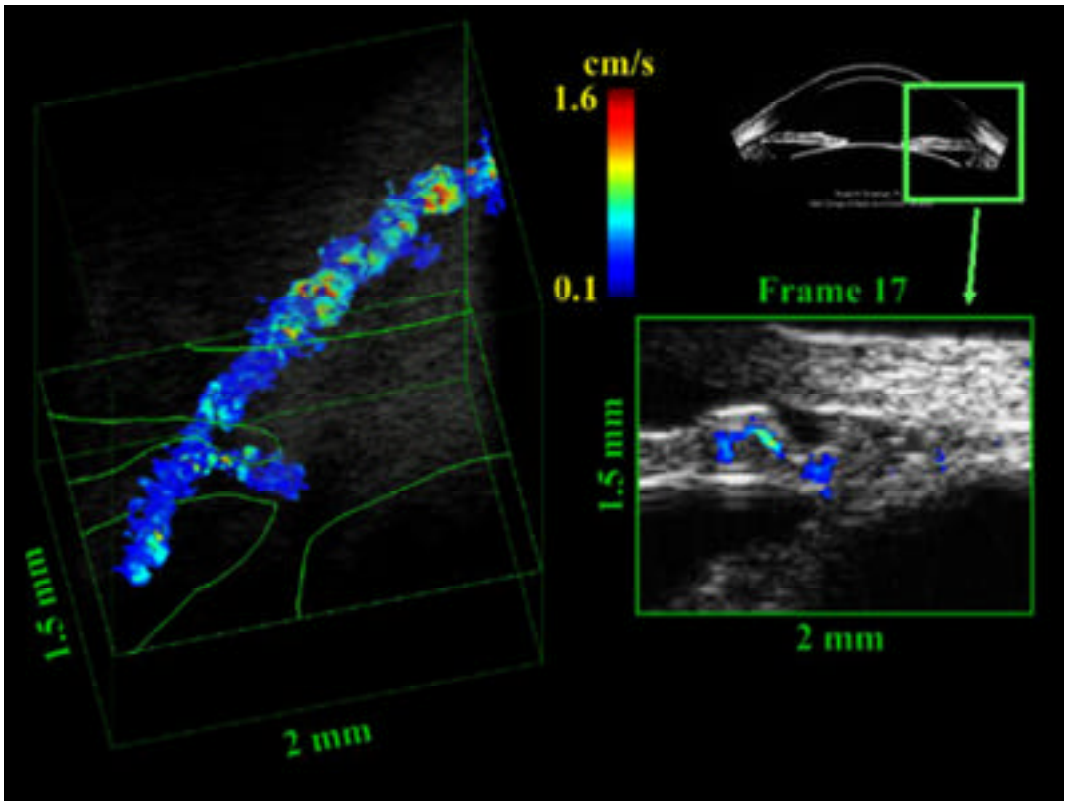

Figure 8: One significant opportunity with high frequency ultrasound is to estimate blood velocity in the microvasculature in two and three dimensions. An example of this capability in ophthalmology is presented here. In the upper right hand corner is a high-frequency ultrasound image of the eye. The area enclosed in the box is then imaged at higher magnification in two and three dimensions to produce the lower color flow images. In color flow imaging, blood velocity is estimated in small volumes and the resulting value encoded in color, with the echogenicity of the surrounding tissue encoded in gray. The image on the left depicts a 3-D reconstruction of 64 color flow scan planes that bisect the major arterial circle in the rabbit eye. The grayscale corresponding to tissue was rendered transparent to avoid hiding the vessel. The image on the right, which is labeled Frame 17, is one frame out of the 64 used to form this 3D image. The anatomical boundaries in this image (edge of the sclera, iris and ciliary process) were outlined and the corresponding outline appears in the 3D image on the left. The small vessel branching from the major artery towards the center of the 3D image is a 40-micron arteriole that appears to feed into the base of a large ciliary process.

In summary, the development of high-frequency ultrasound systems for imaging and blood flow estimation as well as the development of contrast-assisted ultrasound may have a significant impact in the imaging of small animals. The potential to enhance transfection or drug delivery in small animals may also be improved by the use of ultrasound with contrast agents.

Quantification of Organ Function in Small Animals with PET, SPECT and Planar Imaging

Michael V. Green, M.S.
Chief, Imaging Physics Section
Nuclear Medicine Department, CC, NIH

Instrumentation state-of-the-art

PET, SPECT, and their planar imaging variants have achieved a spatial resolution of the order of 2 mm, suitable for quantifying whole organ radioactivity in animals the size of normal mice and rats. However, significant applications-limiting differences exist between these methods in sensitivity that require matching the method to the specific experimental problem; for example, rotational pinhole SPECT can usually only be employed for visualizing static tracer distributions whereas (non-tomographic) planar imaging can quantify rapidly changing activity distributions, albeit with less accuracy.
PET scanners have been developed specifically for small animal imaging and are the most technologically advanced radiotracer imaging systems (spatial resolution better than 2 mm). Conversely, relatively little work has been done to create equally advanced, dedicated single photon SPECT, and planar imaging systems (e.g., multiple, stationary pinhole gamma cameras surrounding the animal, etc.).

Enabling Technologies

Advances in small animal PET will depend on parallel technological improvements in detector module design (e.g., faster, high light output scintillators), improved light collection with new photonic or semi-conductor detectors, the development of advanced iterative, resolution recovery reconstruction algorithms (e.g., 3D OSEM, MAP, and the computational devices and methods that will allow these algorithms to be used routinely), and on a concerted effort to overcome significant infrastructure and equipment costs. Improvements in single photon imaging systems will depend, at least initially, on support of R and D to dramatically improve the sensitivity of these systems while maintaining high resolution. In addition, all of these methods require reliable and reproducible schemes for accessing the vascular systems of small animals, already a very difficult problem in small (20 gram) mice.

Current Applications

Applications of these radiotracer imaging methods (at the NIH) include: quantifying organ structure and function in knockout and transgenic mice, evaluation of new radiopharmaceutical for diagnostic efficacy in animal models of human cancer, and evaluation of new receptor ligands (PET) in the mouse and rat.

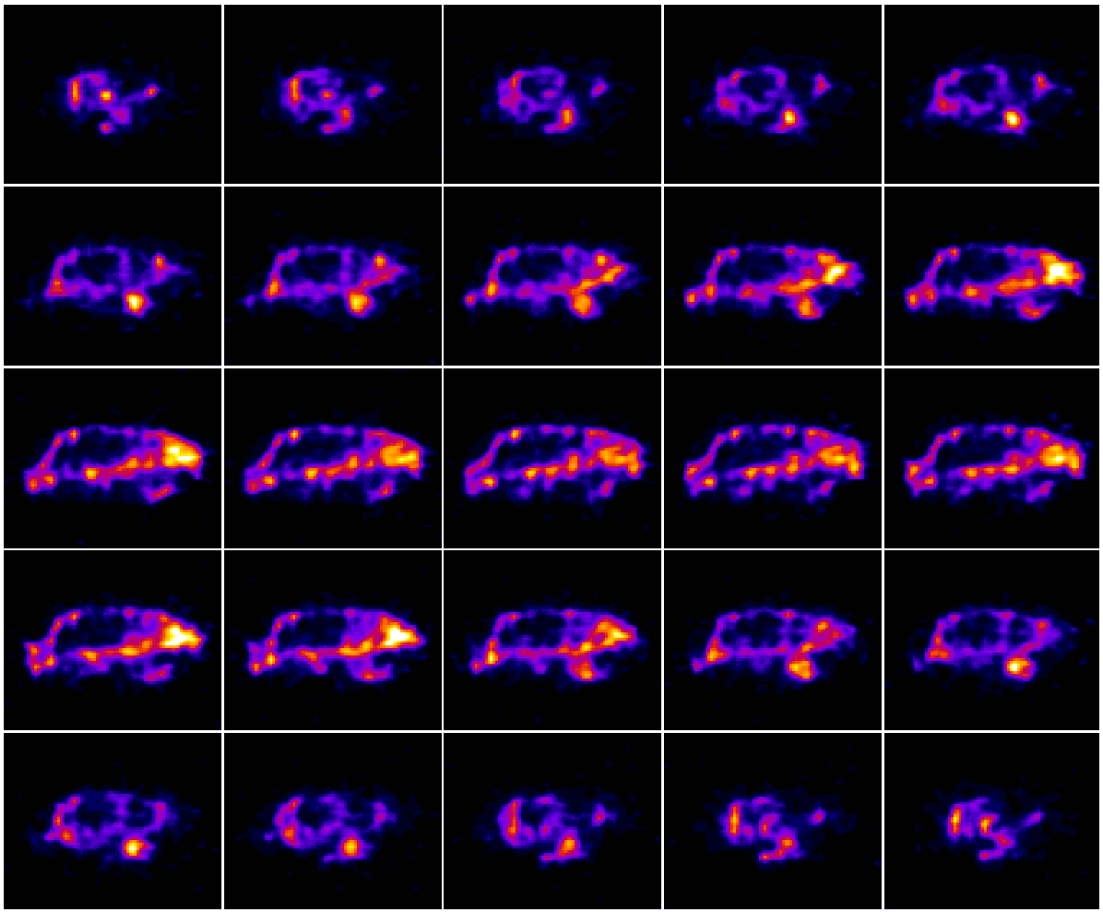

Figure 9: Consecutive sagittal section PET images of the mouse skull and facial bones after labelling the skeleton with F-18 fluoride. The animal's nose is toward the right in each panel and the back of the skull toward the left. The image at the upper left is most distant from the observer while the image at the bottom right is nearest. Images equally spaced away from the central sagittal slice (center image) appear similar due to left / right skeletal symmetry. These images, obtained with an experimental PET scanner designed solely to maximize spatial resolution, are among the highest resolution images ever made with PET (about 1 mm).

Future Uses and Enabling Technologies

Applications will include imaging animal models of human disease, establishing the mechanism and site of action of new drugs and radiopharmaceuticals, and identification and evaluation of phenotype changes resulting from genetic manipulations, particularly in the mouse. In addition, it is likely that several of the imaging methods described at this workshop will be used together in the same experiment (e.g., PET with CT or PET with MR, etc.). If so, development of combined instruments may be both useful and cost effective (e.g., a single device capable of simultaneous PET and MR imaging). As such studies become more common, whole-body digital data bases of all common laboratory animals will need to be developed, along with methods for cross-modality image registration and analysis.

Small Animal Support and Monitoring

Laurence W. Hedlund, Ph.D.
Center for *In Vivo* Microscopy
Duke University Medical Center

Biologic motion, physiologic monitoring, and life support are major biological issues in performing high-resolution imaging of the live, small animal. For animals as small as mice, these issues are quite challenging and they become even more challenging when magnetic resonance microscopy (MRM) is used. With small animal MRM, space and access are severely limited and strong magnetic and electrical fields pose unique problems. Solutions for many of these problems are summarized below.

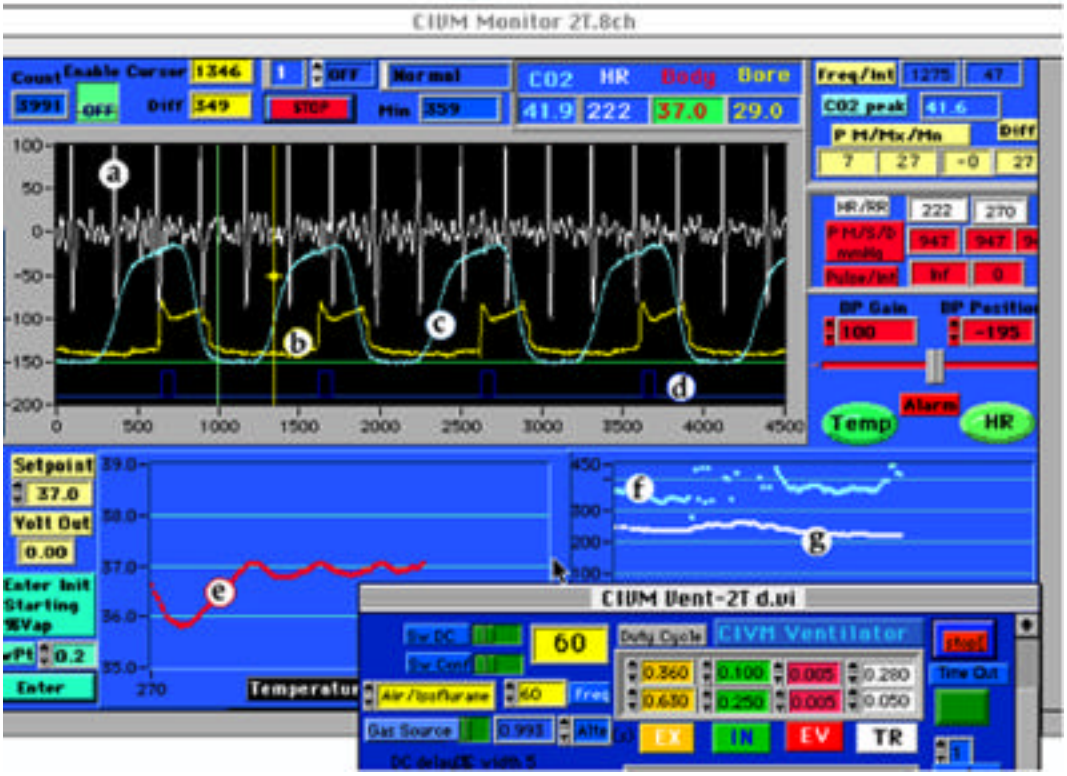

Figure 10

Shown in Fig. 10 is the display of a Power PC Macintosh computer running physiologic monitor and mechanical ventilator applications written in LabVIEW (National Instruments, Austin, TX). Label (a) shows the ECG QRS spike recorded from electrodes taped to the footpads of a small rat in an imaging coil in the bore of a 2 Tesla magnet. Heart rate is calculated (HR) and used for determining physiologic status and for adjusting the level of isoflurane anesthesia. Label (b) shows the airway pressure wave form recorded from a solid state transducer mounted on a complex, MRM-compatible breathing valve attached to the animal's endotracheal tube. The exact parameters of breathing

(duration of INspiration and EXpiration) are controlled by the ventilator application at the lower right of the screen. Mechanical ventilation maintains proper gas exchange and supports administration of easily controlled gas anesthesia. Label (c) is the waveform of exhaled CO_2 (out of phase with pressure waveform) used to determine metabolic status and proper ventilation settings. Label (d) shows a DC output trigger from the ventilator computer coincident with the beginning of inspiration, and this is used to synchronize imaging with the phase of breathing. This output trigger can positioned to occur at any point of the breathing cycle. Label (e) is the cumulative record (several hours) of body temperature from a rectal thermistor. This temperature is used by a feedback control loop in the monitor program to adjust the temperature of heated air flowing through the bore of the magnet. Body temperature control is critical for maintaining physiologic stability of small animals not only for survival but also for insuring maximal image quality and resolution, especially for cardiac-gated and diffusion-based imaging. Several hours of cumulative records of exhaled CO_2 (f) and heart rate (g) are also shown. When body temperature and anesthesia are controlled, heart rate can be maintained with minimal variation and this can significantly improve quality of images of thorax and upper abdomen.

When all the details of physiologic monitoring and biologic support are addressed, the full power of high resolution, in vivo imaging of small animals can be achieved-performing longitudinal studies on the same individuals.

Probes for *In Vivo* Imaging of Molecular Events

Ralph Weissleder, M.D., Ph.D.
Massachusetts General Hospital

Molecular imaging refers to the visualization of specific molecular events at tissue, cell, or subcellular level. With the continuing development of microscopic imaging techniques (MR microscopy, microPET, microSPECT, optical imaging), we are now approaching previously unprecedented resolution capabilities. Yet the image contrast of most currently available imaging techniques is based on physical parameters. The use of specific molecular probes, imaging reporter genes, and transgenic cell lines or animals will be a pre-requisite for expanding on current capabilities.

It is now possible to image gene expression by both MR (Fig. 11) and nuclear imaging at high resolutions and high sensitivities. A major advantage of optical imaging is the fact that “activatable” probes can be developed to visualize highly specific molecular events, for example, enzyme pathways and cascades in tumors (e.g., cathepsin, matrix metalloprotease, viral protease, or other molecules). Combining microscopic imaging techniques with highly specific molecular probes thus represents a unique opportunity to the imaging and molecular biology research community to visualize molecular events in intact micro- and macro-environments.

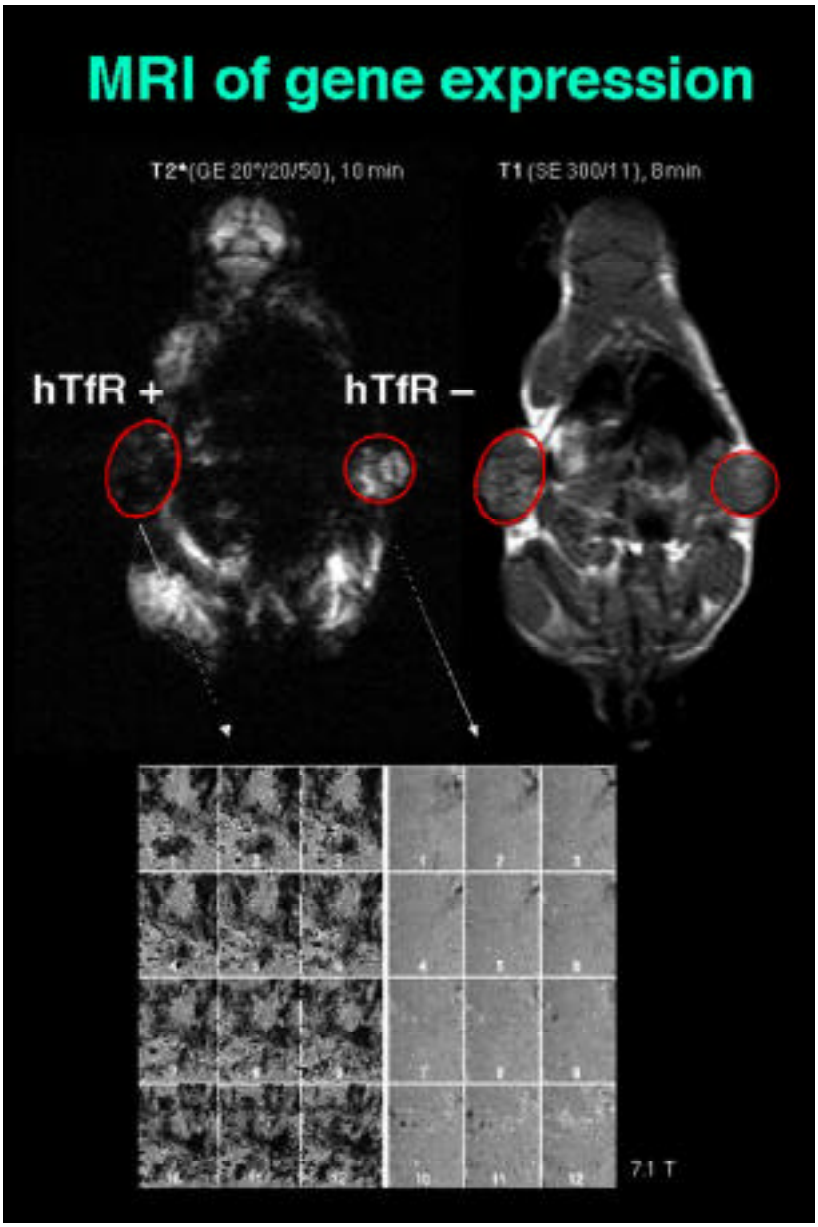

Figure 11: Transgene expression in tumors can be visualized directly by MR imaging either *in vivo* (top) or by MR microscopy (lower part). The transgene was the cDNA encoding for an engineered internalizing but not regulatable transferrin receptor (which was used to transfect tumors) and the probe was MION-Tf.

Applications

The applications of small animal imaging are extraordinarily broad. Recent interest from the molecular biology community has been particularly strong. But the application of small animal imaging has already been firmly established in drug discovery, toxicology, and studies in fundamental

physiology and disease mechanisms. The recurrent theme, particularly in longitudinal studies is the power of being able to use an animal as its own control by studying pre- and post-treatment. This biostatistical power is enormous resulting in tremendous cost saving by reducing the number of animals required in any given study. This section of the conference was dedicated to exemplary applications of small animal imaging.

MR Microscopy of Mouse Brain

Helene Benveniste, M.D., Ph.D.
Center for *In Vivo* Microscopy
Duke University Medical Center

Transgenic murine models have the potential to make significant contributions to our understanding of neurodegenerative disorders. Magnetic resonance microscopy (MRM) is uniquely suited to study these small animal models because it allows for sequential, non-invasive, three-dimensional analysis. MRM can be used to characterize both morphology and physiology in the transgenic mice. The latter comprise functional (perfusion and metabolic) information whereas morphological analysis can be used for phenotyping of "gross anatomy" (shape and volume) and/or "microanatomy" (tissue structure). An example of gross anatomical phenotyping by MRM in a transgenic mouse model is shown in Fig. 12.

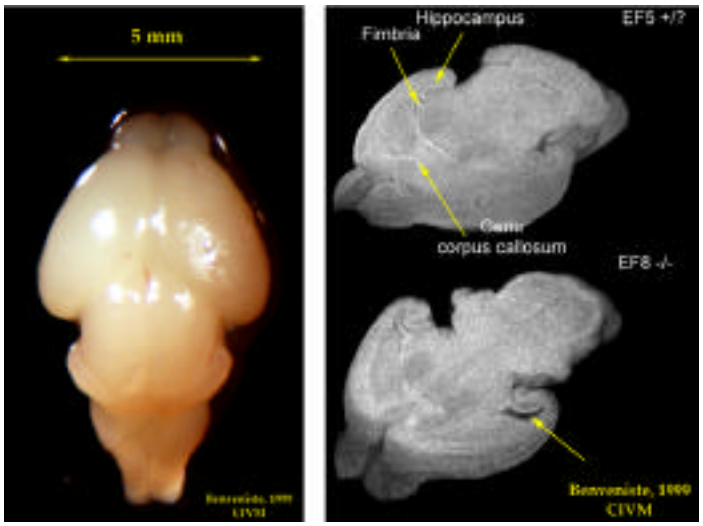

Figure 12: Left: dimension of neonatal mouse brain specimen used in the MRM study. Right: 3D diffusion-weighted MRMs of normal (EF5 +/?) and ankyrin B (-/-) mouse brain (EF9 (-/-). The 3D MRMs reveal enlargement of the lateral ventricle in the ankyrin B (1/1) brain.

As shown above, gross anatomical phenotyping by MRM is relatively straightforward, provided that sufficient image resolution can be obtained for the analysis. Microanatomical phenotyping by MRM can be more challenging because in addition to adequate image resolution, it also requires precise knowledge of the MR appearance of the various microstructures. For example,

most hippocampal subregions of the mouse brain can be visualized by MRM but not by the same MR proton stain. The granular cell layer can be visualized by T2* proton staining but visualization of the mossy fiber pathway and the pyramidal cell layers require diffusion proton staining (Fig. 13).

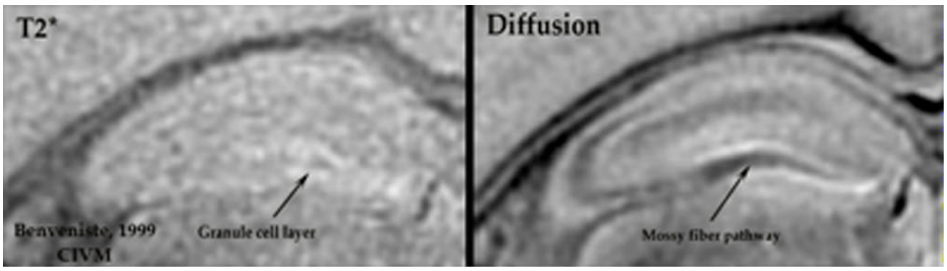

Figure 13: T2* proton stain (left) and Diffusion proton stain (right) of mouse hippocampus *in vitro*. The voxel resolution of the MRMs is 39 x 39 x 156 mm^3.

In vivo MRM studies of the mouse brain are extremely challenging due to the small size of the mouse (20-40 g). Motion from breathing and cardiac activity can superimpose artifacts in the images and decrease overall signal-to-noise ratios (SNR). Further, the small mouse needs to remain anesthetized during the imaging period. For longitudinal imaging studies, it is important also to be able to conduct MRM studies with minimal mortality and morbidity. We have recently performed a longitudinal *in vivo* MRM study on C-57 Wild Type and Apolipoprotein E deficient mice. The animals were imaged 3 times over a 40-day period. Mortality was found to be ≈ 15%. Our current efforts are directed towards reducing this mortality rate by improving our anesthetic techniques for MR imaging and by reducing our MR microscopy scanning time.

MR Methods in Pharmaceutical Research: Application to Drug Discovery and Development

Markus Rudin, Ph.D.
Novartis Pharma Inc.

In the last decade, in vivo MR methods have become established tools in the drug discovery and development process. Several potential and successful applications of magnetic resonance imaging (MRI) and spectroscopy (MRS) in stroke, rheumatoid and osteo-arthritis, oncology, and cardiovascular disorders have been reported by various research groups.[9]

Versatility is a major strength of MRI and MRS (i.e., a manifold of complementary data may be collected using the same experimental set-up). A typical example is an MRI application in stroke research where, in the same patient or animal, information on local brain perfusion, local cerebral blood volume, oxygen deficit, cytotoxic and vasogenic oedema, and functional responsiveness can be obtained with high spatial and temporal resolution in one imaging session. In addition, MRS can provide data on energy metabolism and

[9] Rudin M et al.: NMR Biomed. 1999; 12:1-29.

tissue acidosis. Similar comprehensive characterizations have been obtained in the study of tumors and for cardiovascular disorders.

A second important advantage of MRI is that it is non-invasive—a prerequisite for studying chronic diseases both in humans and animals. In preclinical research, apart from the obvious statistical advantages when monitoring disease progression and therapy response in an individual, there are also economical and animal welfare aspects to be considered. Non-invasive methods are also highly desirable for the phenotyping of transgenic and knockout animals. Statistics is a critical factor in drug testing, and throughput is a quantity that cannot be neglected when evaluating an analytical method. Other advantages of MRI's non-invasive nature include the ability to provide functional information (i.e., physiological readouts, such as the analysis of heart wall motion), perfusion MRI, tracer uptake and clearance studies, and neuronal activation studies. Functional information may also be derived from experiments using target-specific contrast agents, which will become important tools in future MRI applications.

For routine drug testing, MRI procedures can be highly standardized, allowing in some cases the analysis of more than 50 animals per day. Successful applications in drug discovery exploit one or several of these aspects. In addition, the link between preclinical and clinical studies makes *in vivo* MR a highly attractive method for pharmaceutical research.

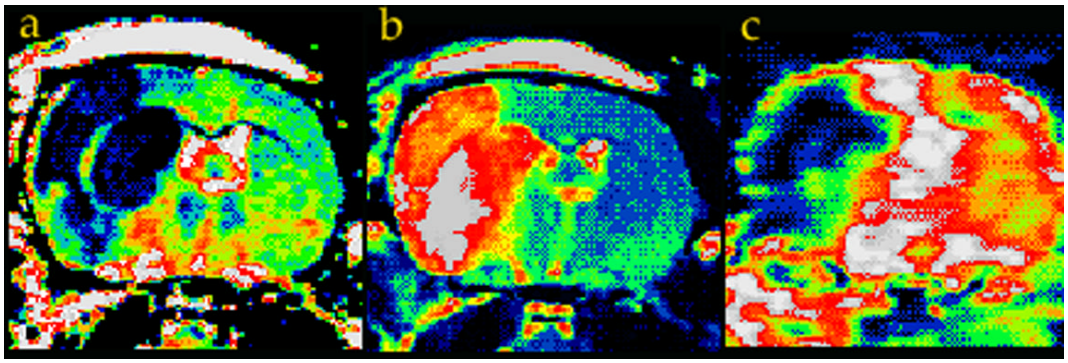

Figure 14: MRI of focal ischemia in rat brain following occlusion of the middle cerebral artery (MCA). The images were recorded 24 hours after MCA occlusion and represent maps of (a) the apparent diffusion coefficient (ADC), (b) T2 relaxation time (T2), and (c) relative cerebral blood flow (CBF). The ability to map different MRI parameters to reflect different pathophysiological processes offers great potential for tissue staging and therefore impact clinical drug studies (e.g., for patient selection). The quantitative assessment of tissue parameter and lesion volume is straightforward, allowing the evaluation of drug efficacy.

Imaging Reporter Gene Expression in Living Animals with PET

Harvey Herschman, Ph.D.
Jonsson Comprehensive Cancer Center
UCLA School of Medicine

Because of our ability to manipulate its genome, the laboratory mouse has become the workbench for most studies on models of human disease. We can now change, at will, any of the three billion nucleotides of the murine genome and create new strains of mice with directed point mutations, deletions, rearrangements, and transgenes. However, the breeding and care of these animals requires fastidious care and is expensive and time consuming. In order to create models of human disease in the mouse and carry out developmental, diagnostic, and therapeutic studies using conventional techniques, animals must be sacrificed at individual time-points for conventional biological analyses of gene expression.

The cell biology community has recently been galvanized around the use of green fluorescent protein, because this “reporter protein” can be used to repetitively and non-invasively monitor reporter gene expression in transfected living cells in culture and in transgenic organisms transparent to light. We have now developed two "PET reporter gene / PET reporter probe" systems in which reporter gene expression can quantitatively, repetitively, and non-invasively be monitored in mice using positron emission tomography (PET). In the first method, an [18F]-positron labeled derivative of spiperone (a dopamine receptor antagonist), fluoroethylspiperone (FESP), is used as a PET reporter probe to detect the expression of the dopamine D2 receptor used as the PET reporter gene. We have created a replication deficient adenovirus expressing the dopamine D2 receptor (D2R) PET reporter gene and demonstrated that we can quantitatively, repetitively, and non-invasively image the expression of the D2R gene in the livers of mice with [18F]-FESP after administration of the D2R adenovirus. We have also created tumor cells that express ectopically the D2R and demonstrated that we can repetively image expression of this PET reporter gene in the tumors following repetitive injection of the positron-labeled PET reporter probe and scanning in the tomograph (Fig. 15).

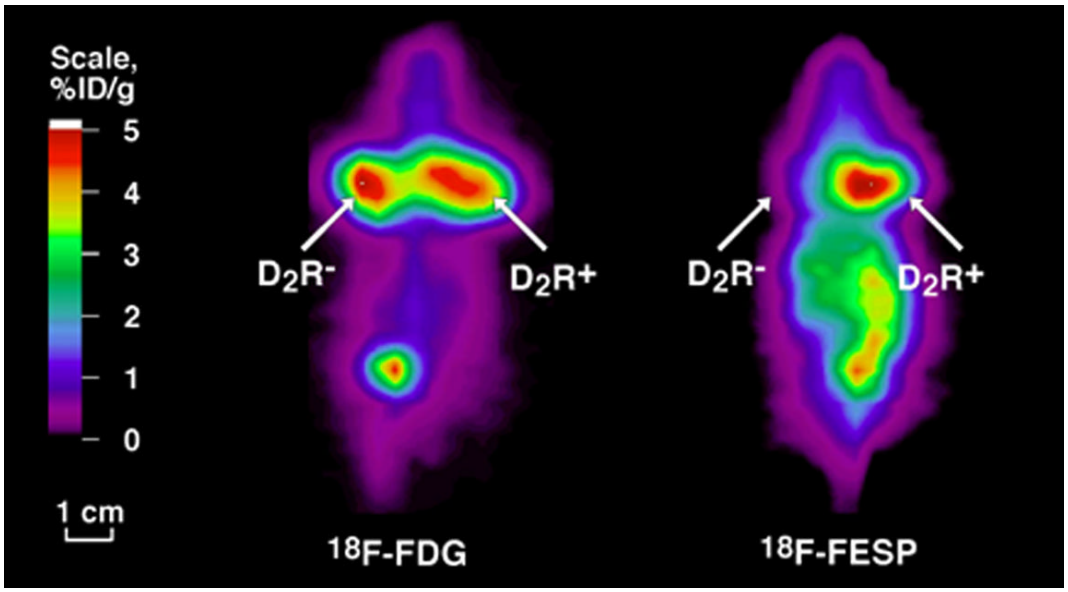

Figure 15: Imaging reporter gene expression in living mice with the FESP / D2R PET reporter probe / pet reporter gene system. A nude mouse carrying a control tumor on its left shoulder and a tumor that expresses a transfected dopamine D2 receptor (D2R) PET reporter gene on it right shoulder was first injected with fluorodeoxyglucose (FDG) and subjected to a PET scan (left image). Both tumors metabolize FDG at a high rate. The mouse was returned to the animal room. Several days later the mouse was injected with 18F-fluoroethyspiperone (FESP), a positron-labeled PET reporter ligand for the D2R PET reporter gene, and subjected to a second PET scan. Only the tumor that expresses the D2R PET reporter gene retains the FESP probe and is visualized by the second PET scan (right image).

We have also developed a second PET reporter gene / PET reporter probe system in which we use the Herpes Simplex Virus Type I thymidine kinase gene (HSV1-tk) as the PET reporter gene and [18F] positron labeled acycloguanosine derivatives (fluroganciclovir, FGCV and fluoropenciclovir, FPCV) as PET reporter probes. We have created an adenovirus that expresses the HSV1-tk PET reporter gene and similarly demonstrated that we can quantitatively, repetitively, and non-invasively image the expression of the HSV1-tk gene in the livers of mice with [18F]-FGCV and [18F]-FPCV after administration of the HSV1-tk adenovirus (Fig. 16).

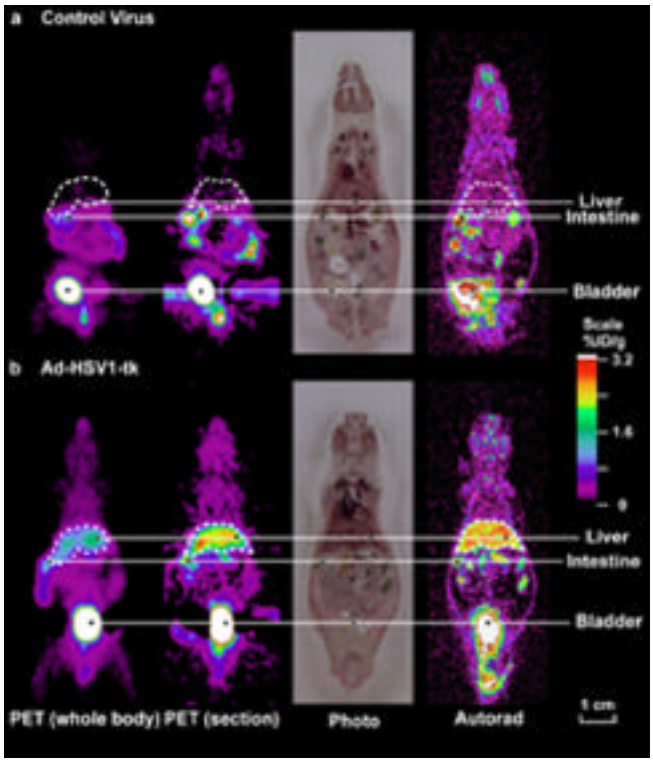

Figure 16: Imaging reporter gene expression in living mice with the FGCV / HSV1-TK PET reporter probe / pet reporter gene system. Mice were injected via the tail vein with either a control replication-deficient adenovirus or a replication-deficient adenovirus in which the Herpes Virus 1 thymidine kinase (HSV1-tk) gene, expressed from the CMV early promoter, is inserted into the viral E1A region. Two days later the mice were injected via the tail vein with F18-fluoroganciclovir (FGCV). The mice were then imaged in the microPET. For each mouse, a whole body mean coronal projection image of the fluorine-18 activity distribution is shown on the left. After the microPET scans, the mice were sacrificed, frozen, and sectioned. The third images show photos of tissue sections (45 microns) used for digital whole body autoradiography, shown in the fourth image. The second images from the left are microPET coronal sections, approximately two mm thick, that correspond to the autoradiographic sections. The color scale represents the FGCV percent injected dose per gram. Images are displayed on the quantitative color scale, to allow signal intensity comparisons among them.

The use of PET reporter gene / PET reporter probe technology can be used to "track" gene expression in transgenic mice, knock-in mice, and in models of gene therapy. We are currently creating models to demonstrate reporter gene expression in transgenic and knock-in mice as well as "bicistronic" viral vectors in which therapeutic genes and PET reporter genes are co-expressed. This new technology will permit us to non-invasively, quantitatively, and repetitively monitor the location, extent, and duration of reporter gene expression in living animals.

Ultrasound Micro-Imaging of Mouse Development

Daniel H. Turnbull, Ph.D.
Skirball Institute of Biomolecular Medicine
New York University School of Medicine

The extensive genetic information and rapidly expanding number of techniques to manipulate the genome of the mouse have led to its widespread and increasing use in studies of development and to model human diseases. In this rapid proliferation of methods to genetically engineer mice, technologies to investigate anatomical structure and function in the mouse have not kept pace. The results of transgenic and gene-targeting experiments, for the most part, are analyzed using histological methods that are static and two-dimensional, making it difficult to understand the underlying developmental and disease processes that are dynamic and three-dimensional.

We have developed ultrasound micro-imaging approaches for investigating embryonic development in the mouse. Ultrasound backscatter microscopy (UBM) is a high frequency (40–50 MHz) pulse-echo ultrasound imaging technique that provides high-resolution (20–40 µm axial; 50–100 µm lateral) images of live mouse embryos, in utero. In combination with high-frequency (40–50 MHz) ultrasound Doppler measurements, UBM allows real-time (5–10 images per second) imaging and image-guided blood velocity measurements over a wide range of early embryonic stages, providing a unique approach to studying normal and abnormal development of the brain, heart, and other organs, *in utero*, at critical early stages of development (Fig. 17).
The developing mouse cardiovascular system has been particularly difficult to study by traditional histological methods. The heart is the first organ to form, and the early establishment of a functional cardiovascular system is crucial for survival. Numerous genes have been shown in the mouse to be critical for normal cardiovascular development, but an understanding of the mechanisms regulating heart and vascular development are still incomplete, in part due to the lack of methods to measure hemodynamics in live mouse embryos. We have developed a noninvasive UBM-guided Doppler system for measuring blood velocity waveforms in the heart and large vessels (umbilical artery and vein, aorta, vena cava) in developing mouse embryos. The combination of these new ultrasound micro-imaging methods with the many available mouse mutants should yield important new insights into embryonic cardiovascular structure-function relationships.

Finally, we have developed a UBM-image guided micro-injection system for introducing cells, viruses, and other agents into targeted regions of early stage mouse embryos (Fig. 18). This technique has provided a powerful new tool to study cell lineage, fate, and developmental potential in the brain, limbs, and organ systems of early stage mouse embryos. Gene misexpression studies are being performed using UBM-guided injections of retroviruses and transfected cells into specific embryonic tissues at predetermined time points. The use of UBM-guided injections into mutant embryos lacking specific genes provides a unique system for testing in utero cell replacement and gene therapy approaches.

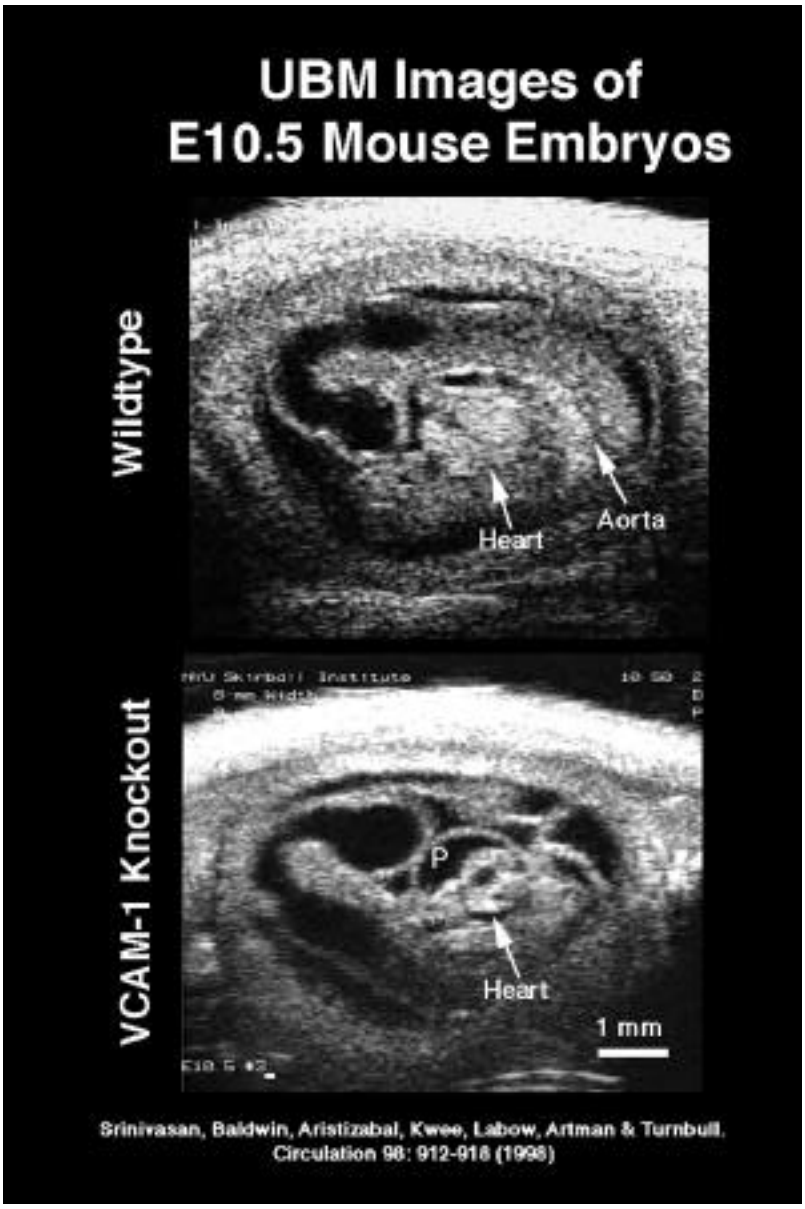

Figure 17: Ultrasound backscatter microscopy (UBM) has been used to image live mouse embryos, in utero, from early gestational stages. A comparison is shown between sagittal UBM images of a wildtype (normal) embryo and a VCAM-1 homozygous null mutant, both at 10.5 days of gestation (approximately equivalent to 4 weeks human). The reduction in cardiac dynamics and pronounced pericardial effusion in the VCAM-1 mutant embryos was obvious on real-time UBM images.

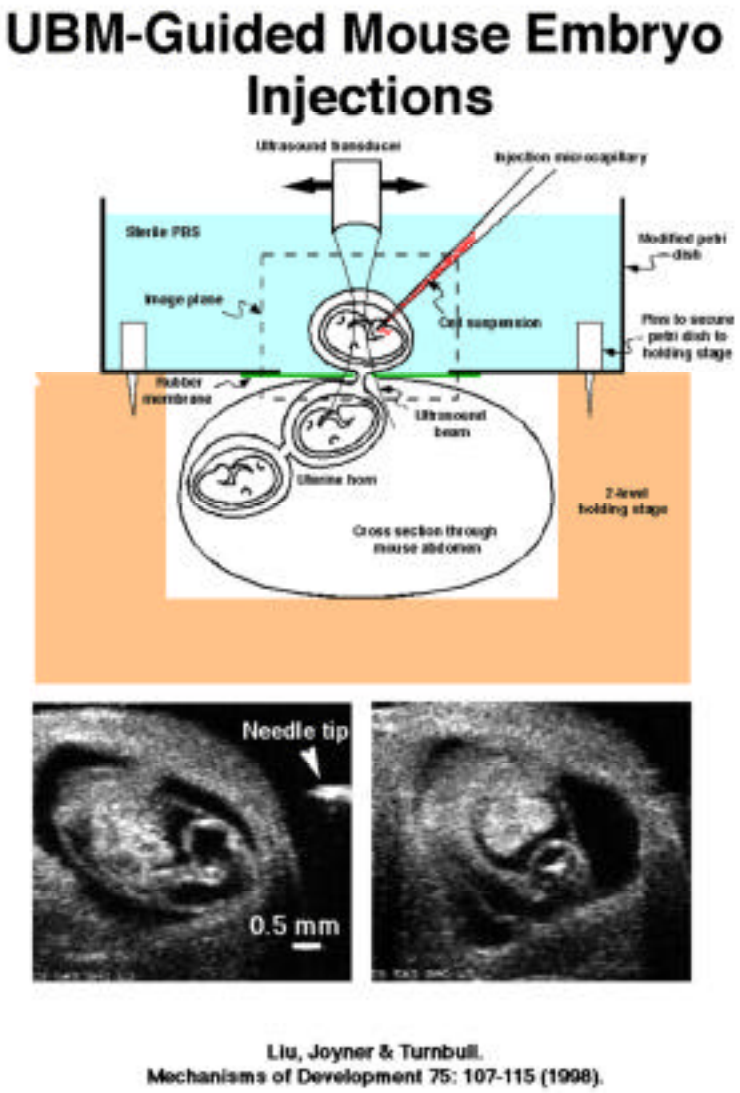

Figure 18: A UBM-guidance system has been developed, allowing cells, viruses, and other agents to be injected into specific mouse embryonic tissues at early developmental stages. A schematic of the system and surgical technique is shown, with an example of a cell being injected into the forebrain ventricle of a 9.5 day mouse embryo.

Imaging and the Mouse: Key to unraveling complex diseases?

Kevin L. Seburn, Ph.D.
The Jackson Laboratory
Bar Harbor, ME

It is now clear that many human disease conditions (e.g., cancer, cardiovascular disease, degenerative nervous system diseases) result from a combination of environmental and genetic factors that interact in a complex, variable manner with the physiology of an afflicted individual. It is equally clear that to understand these complexities requires a unified, integrated approach that examines the appearance and progression of a given pathology in the context of an individual's genetic background.

The mouse has emerged as a critical component in this effort because of: 1) its genetic similarity with humans, 2) its cost-effectiveness and experimental accessibility and, 3) the availability of increasingly sophisticated techniques for genetic manipulation. These advantages will increase with the expected completion of the maps for the entire genome of mice and humans. The availability of these maps will mean that as genes implicated in human disease processes are identified, we will be better able to study and understand their roles because we will be able to create better, more sophisticated, and accurate disease models in the mouse.

There continues to be steady progress in our ability to manipulate the mouse genome. Techniques are now available or are being refined that permit targeted, inducible, and reversible manipulations of gene expression. However, the usefulness of these and other manipulations for deciphering complex disease processes are significantly limited because interpretations are largely restricted to post-mortem observations made at single time points in the progression toward some pathological endpoint. This limitation makes interpretation difficult or impossible for individual within-strain variations in the response to a given manipulation, or even of systematic variation in the response of different strains. The limitation applies even, and perhaps especially, when manipulations are restricted to a single component of an identified pathway because of the likelihood of complex interactions or unrecognized redundancies and compensatory processes.

The ability to visualize disease progression in humans (e.g., tumor growth) through advanced imaging technology has significantly improved treatment options for cancer and cardiovascular disease as well as permitting better assessment of treatment effectiveness. However, the combined use of advanced imaging techniques with environmental or genetic manipulations for the study of human disease will always be severely constrained by ethical considerations. Therefore, our ability to study, understand, and develop better treatments for these and other complex diseases depends critically upon the development of in vivo imaging capabilities in the mouse that are comparable to or exceed those currently available for humans. The development of these capabilities will not only provide significant new insights from the study of the mouse, but could also help drive improvements in the imaging technology available for human applications.

Recommendations

The imaging technologies and applications discussed at this workshop were all at the cutting edge, but the crystal ball that allows us to see where these technologies will develop can be clouded by serendipity. Nevertheless, there was consensus on a number of general points:

• Additional NIH support should be provided for development of the core technologies (e.g., new detectors and novel encoding methods). Traditional "hypothesis-driven" proposals should be replaced by engineering proposals directed at specific goals (e.g., improve the sensitivity by x or resolution by y).

• NIH and their investigators must work to make the technologies more widely available. Availability might be enhanced by research to reduce the costs of the systems, by supporting industry via SBIR/STTR or other mechanisms to increase access, or through the Resource mechanism currently supported through NCRR.

• Computer technologies for automated image analysis, advanced reconstruction/correction, visualization, and databasing will be critical for dealing with the shear mass of data. Clearly the Web must become a part of all of our daily lives, and proposals to make that a reality should be encouraged.

• New biological approaches should be encouraged-the integration of molecular biology and imaging will be critical for the success of both fields.

• Support should be encouraged for bioengineering developments crucial to physiologic monitoring and support of small animal models and for development of new animal models appropriate for imaging studies.

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NIH AI 34567-06 (H.Herschman, PI)

Workshop Participants

Registrants:

Gail G. Weinmann, M.D.
Division of Lung Diseases, NHLBI
Two Rockledge Centre, MSC 7952
6701 Rockledge Drive
Bethesda, MD 20892-7952
(301) 435-0202

Barbara Y. Croft, Ph.D.
Diagnostic Imaging Program, NCI
6130 Executive Blvd., EPN 800
Rockville, MD 20892-7440
(301) 496-9531
bc129b@nih.gov, Barbara_Croft@nih.gov

Murali Cherukuri
National Cancer Institute, Radiation Biology Branch
NIH, 9000 Rockville Pike Bethesda, MD 20892
(301)496-7511

Subrmanian Sankaran
National Cancer Institute, Radiation Biology Branch
NIH, 9000 Rockville Pike
Bethesda, MD 20892
(301) 496-7511

James B. Mitchell
National Cancer Institute, Radiation Biology Branch
NIH, 9000 Rockville Pike
Bethesda, MD 20892
(301) 496-7511

John Cook
National Cancer Institute, Radiation Biology Branch
NIH, 9000 Rockville Pike
Bethesda, MD 20892
(301) 496-7511

Robbe Lyon
FDA Center of Drug Evaluation and Research
8301 Muirkirk Rd., Rm. 2009
Laurel, MD 20708
(301) 594-5855

Joe Hanig
FDA Center of Drug Evaluation and Research
8301 Muirkirk Rd., Rm. 2009
Laurel, MD 20708

Scott Pine
FDA Center of Drug Evaluation and Research
8301 Muirkirk Rd., Rm. 2009
Laurel, MD 20708
301-594-5855

Hirsch Davis
FDA Center of Drug Evaluation and Research
8301 Muirkirk Rd., Rm. 2009
Laurel, MD 20708
(301) 594-5855

David Lester
FDA Center of Drug Evaluation and Research
8301 Muirkirk Rd., Rm. 2009
Laurel, MD 20708
(301) 594-5855

Jan Johannessen
FDA Center of Drug Evaluation and Research
8301 Muirkirk Rd., Rm. 2009
Laurel, MD 20708
(301) 594-5855

Nathan M. Appel, Ph.D.
Medications Development Division
National Institute on Drug Abuse
6001 Executive Boulevard
Room 4123, MSC 9551
Bethesda, MD 20892-9551
(301) 443-8475
an69k@nih.gov

Jurgen Seidel
Nuclear Medicine, NIH
Rm 1C401, Bldg 10
10 Center Drive MSC 1180
Bethesda MD 20892-1180
(301) 402-1999
Jurgen_Seidel@nih.gov

Juan Jose Vaquero
NIH, Nuclear Medicine Department9000 Rockville Pike
Bldg. 10 Room 1C401. MSC 1180
Bethesda, MD 20892-1180
(301) 402.1999
e-mail: juanjo@nmdhst.cc.nih.gov

Calvin A. Johnson, Ph.D.
Center for Information Technology, NIH
Bldg. 12A, Room 2021, MS 5624
Bethesda, MD 20892-5624
(301)402-3045
johnson@mail.nih.gov

Delia McGarry
NIH / CIT / CBEL
301-435-8521
delia@helix.nih.gov

J. E. Fahner-Vihtelic
Office of Technology Transfer, NIH
6011 Executive Blvd., Suite 325
Rockville, MD 20852
301-496-7735, X270
jf36z@nih.gov

Neal B. West, Ph.D.
Comparative Medicine area, NCRR
6705 Rockledge Drive
Bethesda, MD 20892-7965
(301) 435-0749
nealw@ncrr.nih.gov

Dr. Abraham Levy
Biomedical Technology, NCRR
6705 Rockledge Drive
Bethesda, MD 20892-7965
(301) 435-0755

Dr. Jeffrey T. Mason
Department of Cellular Pathology
Room G137, Armed Forces Institute of Pathology
Washington, DC 20306-6000
(301) 319-0643

Col. William Inskeep
Armed Forces Institute of Pathology
Washington, DC 20306-6000
(202) 782-2601

Robert E. Cunningham
Department of Cellular Pathology, Room G137
Armed Forces Institute of Pathology
Washington, DC 20306-6000

Kevin J. Quinn, Ph.D.
Cognitive Neuroscience Program, NIH
Room: 7N-7172
6001 Executive Blvd. MSC 9637
Bethesda, MD 20892-9637
(301) 443-1576

Dr. Ashraf Fouad
Dept. of Endodontology, School of Dentistry
University of Connecticut Health Center
263 Farmington Ave.
Farmington, CT 06030
(860) 679-2726

Dr. Linda Otis
Dept. of Oral Diagnosis, School of Dentistry
University of Connecticut Health Center
263 Farmington Ave.
Farmington, CT 06030
(860) 679-2773

David L. Wilson
Department of Biomedical Engineering
Case Western Reserve University
Cleveland, OH 44106
(216) 368-4099
dlw@po.cwru.edu

Dr. R. Mark Henkelman
Sunnybrook & Women's College Health Science Center
2075 Bayview Ave.
Toronto, Ontario CANADA M4N 3M5
(416) 480-4293
rmh@srcl.sunnybrook.utoronto.ca

Wayne Mitzner
Department of Environmental Health Sciences
The Johns Hopkins University
615 N. Wolfe St.,
Baltimore, MD 21205
(410) 614 5446

Brett A. Simon, M.D., Ph.D.
Johns Hopkins Dept. of Anesthesiology
(410) 614-1515

Alice M. Wyrwicz, Ph.D.
Center for MR Research
1033 University Place #150
Evanston Il 60201
(847) 492-0730

Youssef Zaim Wadghiri, Ph.D.
Skirball Institute - NYU Medical Center
540 First Avenue, 5th Floor, Lab #13
New York, NY 10016
(212) 263-7261
zaim@saturn.med.nyu.edu
Roger E. Price, D.V.M., Ph.D.

Dept. of Veterinary Medicine and Surgery - 063
U.T. M.D. Anderson Cancer Center
1515 Holcombe Blvd.
Houston, TX 77030-4009
(713) 792-2780

John Hazle, M.D.
U.T. M.D. Anderson Cancer Center
1515 Holcombe Blvd.
Houston, TX 77030-4009
Nancy Contel, D.V.M.
Dupont Pharmaceutical Co.
P.O. Box 804000
Wilmington, DE 19880-0400
(302) 695-1186
nancy.r.contel@dupontpharma.com

Giora Feuerstein
Dupont Pharmaceutical Co.
P.O. Box 804000
Wilmington, DE 19880-0400
(302) 695-1840

Dennis J. Kountz, Ph.D.
Product Manager, DuPont Superconductivity
DuPont Experimental Station
Wilmington, DE 19880-0304
(302) 695-4256

Robert J. Lontz, Ph.D.
R&D Analysis Inc.
3711-C University Drive, Suite C
Durham, NC 27707
(919) 493-9116

Eric Kaldjian, M.D.
Diagnostic Pathology
Parke-Davis Pharmaceutical Research
2800 Plymouth Rd.
Arbor, MI 48105
(734) 622-1455

William McNally
Parke-Davis Pharmaceutical Research
2800 Plymouth Rd
Ann Arbor, MI 48105
(734) 622-7329

Baldev Ahluwalia
SMIS USA Inc.
Division of Medical Physics
Nathan Kline Institute,
140 Old Orangeburg Rd
Orangeburg, NY 10962
(914) 398-5465

D. Geoffrey Vince, Ph.D.
The Cleveland Clinic Foundation
(216) 444-1211
http://www.ccf.org/ri/bme/staff/DGVince.html

Martin Tornai, Ph.D.
Dept. of Radiology
Duke University Medical Center
Durham, NC 27710
(919) 684-7791
mtournai@dec3.mc.duke.edu

Marielle Delnomdedieu, D.Sc.
Center for In Vivo Microscopy
Box 3302 Duke University Medical Center
Durham, NC 27710
(919) 684-7785

X. Josette Chen, Ph.D.
Center for In Vivo Microscopy
Box 3302 Duke University Medical Center
Durham, NC 27710
(919) 684-7781

Bradley R. Smith, Ph.D.
Department of Radiology
Box 3302 Duke University Medical Center
Durham, NC 27710
(919) 684-7852

Edward V. Staab, M.d.
NCI
6130 Executive Blvd., EPN 800
Rockville, MD 20892-7440
Staabe@mail.nih.gov

Stephen Haleen
Parke-Davis
1600 Plymouth Rd.
Ann Arbor, MI 48105

Larry Clarke
NCI Diagnostic Imaging
6130 Executive Blvd., EPN 800
Rockville, MD 20892-7440

Speakers:

Helene Benveniste, M.D., Ph.D.
Center for In Vivo Microscopy
Box 3302 DUMC
Durham, NC 27710
(919) 684-7789
hb@orion.mc.duke.edu

Simon Cherry, Ph.D.
Associate Director
Crump Institute for Biological Imaging
UCLA School of Medicine
(310) 825-4334
scherry@mednet.ucla.edu

Katherine W. Ferrara, Ph.D.
Professor of Biomedical Engineering
University of California, Davis
kwf8q@unix.mail.virginia.edu

Michael V. Green, M.S.
Chief, Imaging Physics Section
Nuclear Medicine Department, CC, NIH
(301) 496-5675
mike@nmdhst.cc.nih.gov

Laurence W. Hedlund, Ph.D.
Center for In Vivo Microscopy
Box 3302 DUMC
Durham, NC 27710
(919) 684-7767
lwh@orion.mc.duke.edu

Harvey Herschman, Ph.D.
Jonsson Comprehensive Cancer Center
Mail Code: 157005
UCLA School of Medicine
(310) 825-8735
HHerschman@mednet.ucla.edu

G. Allan Johnson, Ph.D.
Center for In Vivo Microscopy
Box 3302 DUMC
Durham, NC 27710
(919) 684-7754
gaj@orion.mc.duke.edu

Michael Paulus, Ph.D.
Instrumentation and Controls Division
Oak Ridge National Laboratory
P.O. Box 2008, M.S. 6006
Oak Ridge, TN 37831-6006
(423) 241-4802
paulusmj@ornl.gov

Markus Rudin, Ph.D.
Novartis Pharma Inc.
CTA / In vivo Models
S-386.2.02
CH-4002 Basel, Switzerland
markus-1.rudin@pharma.Novartis.com

Kevin L. Seburn, Ph.D.
Physiogenomics Program Supervisor
The Jackson Laboratory
600 Main St.
Bar Harbor, ME 04609
(207) 288-6447
http: / / www.jax.org

Daniel H. Turnbull, Ph.D.
Skirball Institute of Biomolecular Medicine
New York University School of Medicine
540 First Avenue, New York, NY 10016
(212) 263-7262
turnbull@saturn.med.nyu.edu

Ralph Weissleder, M.D., Ph.D.
Massachusetts General Hospital
149 13th St., Rm 5403
Charlestown, MA 02129
(617) 726-8226
weissleder@helix.mgh.harvard.edu

Meeting Coordinator:

Elaine Fitzsimons
Center for In Vivo Microscopy
Box 3302 DUMC
Durham, NC 27710
(919) 684-7758
egf@orion.mc.duke.edu