AD_____

Award Number: DAMD17-02-1-0034

TITLE: Image Guidance and Assessment of Radiation Induced Gene Therapy

PRINCIPAL INVESTIGATOR: Charles A. Pelizzari, Ph.D.
Howard J. Halpern, M.D., Ph.D.
Chad Haney, Ph.D.

CONTRACTING ORGANIZATION: The University of Chicago
Chicago, Illinois 60637

REPORT DATE: February 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 2003	Annual (7 Jan 02 - 6 Jan 03)

4. TITLE AND SUBTITLE
Image Guidance and Assessment of Radiation Induced Gene Therapy

5. FUNDING NUMBERS
DAMD17-02-1-0034

6. AUTHOR(S) :
Charles A. Pelizzari, Ph.D.
Howard J. Halpern, M.D., Ph.D.
Chad Haney, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Chicago
Chicago, Illinois 60637

E-Mail: c-pelizzari@uchicago.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Image guidance and assessment techniques are being developed for combined radiation / gene therapy, which utilizes a radiation-inducible gene promoter to cause expression of tumor necrosis factor alpha in irradiated tissues. TNF attacks vasculature, increasing the tumor killing effect of radiation. The radiation confines TNF toxicity to the irradiated region. This therapy has proven effective in several animal tumor models and phase I clinical trials. This project is developing imaging to visualize the effects of the combined modality therapy, by combining electron paramagnetic resonance imaging (EPRI), mapping oxygen concentration in 3D in vivo, and nuclear magnetic resonance imaging (MRI), measuring quantities such as vascular permeability and perfusion rate that reflect the status of vasculature. EPRI and MRI of prostate tumors in mice and rats will be conducted. Fusing the two sets of images from the same tumor before, during and after therapy, an image-derived signature will be developed which identifies regions responding well to therapy. In the final stage, this information will be used as the basis of an adaptive treatment regimen where regions that have responded less well will be given a boost, via image guided injection of additional gene vector prior to the next fraction of radiation.

14. SUBJECT TERMS:
radiation therapy, gene therapy, angiogenesis inhibitors, magnetic resonance imaging, electron paramagnetic resonance imaging, adaptive therapy planning, image guided therapy

15. NUMBER OF PAGES
40

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Combined radiation and gene therapy is a promising modality for cancer treatment. At the University of Chicago, a combined therapy utilizing a radiation-inducible tumor necrosis factor alpha (TNFα) gene vector has been studied extensively. In this treatment modality, vascular destructive effects of TNFα amplify the tumoricidal effect of radiation therapy, while the gene expression is confined to the irradiated region, limiting TNF-induced complications. This research project is intended to test the hypothesis that a combination of magnetic resonance imaging (MRI) and electron paramagnetic resonance imaging (EPRI) can detect, characterize and quantify the distribution of tissue changes resulting from combined radiation and TNFα gene therapy, as validated by comparison with histology. EPRI using trityl spin probes and existing imagers can provide 3D image maps of oxygen concentration *in vivo*, with spatial resolution approximately 1mm and oxygen resolution approximately 2 torr in small animals. Dynamic MRI using suitable contrast agents can provide high spatial resolution (down to approximately 0.05 mm in our laboratory) images which can yield local measures of blood volume, capillary permeability, and blood flow. The characteristic size and density distribution of these image-defined changes is to be used as a "damage kernel" in a superposition based treatment planning system. Finally, an image-guided injection scheme utilizing such a treatment planning paradigm will be developed which can be used in an adaptive fractionated treatment course to achieve spatially uniform tumor response, in large prostate tumors grown in rats.

BODY

Progress has been made on each task which was programmed to be addressed in the first year of the project, as follows.

Task 1. Develop imaging techniques to assess early response of tumor to radiation induced gene therapy.

The following subtasks were programmed to be undertaken in year 1:

a. design and construct immobilization jig for mice (months 1-6)
b. adapt pathology sample preparation apparatus (months 1-6)
c. develop combined EPRI / MRI imaging protocols (months 1-3)
d. investigate vasculature-sensitive MRI of TNF-injected mouse tumors (months 1-24)
e. develop EPRI oxymetry of TNF-injected mouse tumors (months 1-24)
f. develop and test correlation of EPRI and MRI imaging (months 1-18)

The subtasks required to allow combined EPRI/MRI mouse imaging (a, c) have been accomplished, although important development work in subtask c continues, aimed at improved maintenance of good animal condition through the long imaging sessions. The animal care aspects of subtask c involving recovery from prolonged anesthesia, regulation of animal body temperature, animal hydration and removal of urine containing a high concentration spin probe from the mouse bladder, have proven to be a significant challenge. To accomplish the aims of the project, animals must be imaged three times over a period of two weeks: on the day of treatment, known as day 0; day 3 post treatment; day 16 post treatment. On each of these days two imaging sessions are conducted, one for EPRI and one for MRI, each of which lasts two or more hours. Ensuring the survival of the mice with tumors for this extended time, with three days of these multi-hour imaging sessions, the animal maintained under anesthesia during the entire session, has required significantly more effort than anticipated to be devoted to development of animal care protocols.

The unique requirement for this project that mice must be repeatedly imaged, and the potential for spin probe concentration in the bladder to spoil images, has necessitated the development of advanced animal handling procedures. Specific developments in animal handling protocols which have been made are detailed in the report Animal Procedure and Imaging Methodology Improvements for Combined EPRI/MRI Imaging and Radiation Mediated Gene Therapy, included as an Appendix. The developments described represent a previously unreported level of sophistication in control of mouse bladder emptying. We have found little or no literature describing the type of urinary catheterization of mice we now do on a routine basis. A manuscript will be prepared for publication describing our methods.

Those subtasks of Task 1 involving analysis of images (d, e, f) are underway and significant progress has been made. An image display and quantitative analysis program

useful for navigation of 3D and 4D EPRI datasets has been developed for use in this project. This Matlab program, "ortho3slice", is illustrated in Figure 1 below.

The basic functionality of the ortho3slice program is to navigate through a 3D (MRI, EPRI intensity or EPRI linewidth) or 4D (EPR spectroscopic image) matrix of image data, displaying three orthogonal slices through a user-defined "current voxel" of interest. The current voxel is chosen either with scrollbars that page through sections along each principal direction, or by clicking in one of the image windows, in which case the current voxel is set to the cursor location. A region statistics capability is provided which displays either a histogram and rudimentary descriptive statistics (mean, rms, quartiles) for all voxels within a user-defined distance of the current voxel for 3D images, or the spectrum at the current voxel for 4D images. More complete documentation in the form of a brief user's guide is included in the Appendices. This program has come to be used routinely in the EPRI laboratory since its introduction.

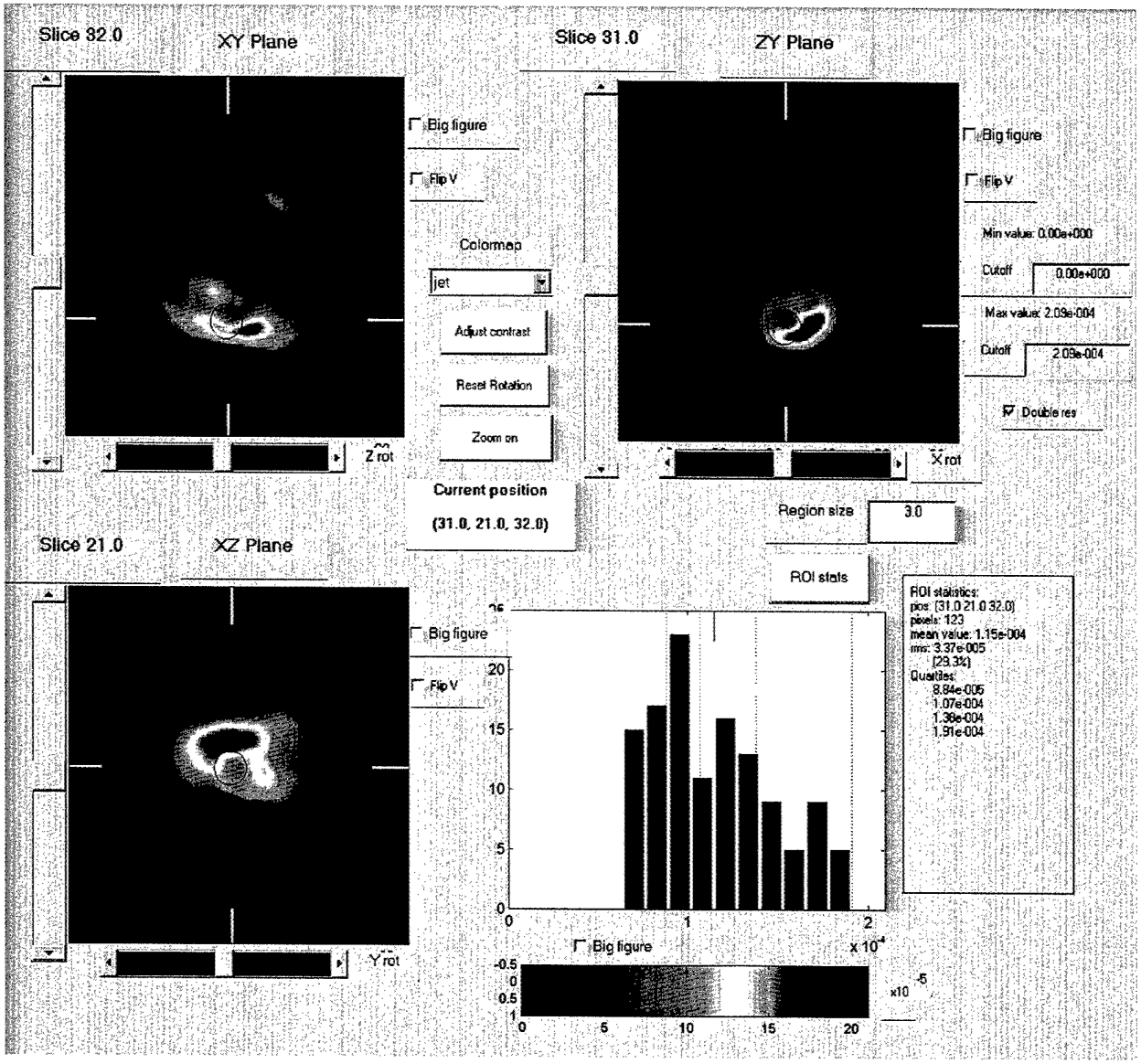


Figure 1. Example screen of the ortho3slice image navigation program.

Image Registration

Subtask 1f involves combining EPRI and MRI images from the same animal taken at each time point (days 0, 3 and 16) in order to identify multimodality image features demonstrative of the effects of the radiation and TNF treatment of the tumors. This task requires the development of image registration methodology for use with these images. Unfortunately due to differences in scanner geometry, it is not possible simply to acquire MRI image planes which are exactly aligned with the EPRI image planes, or vice versa. Image registration is the process of identifying the coordinate transformation which relates the position of the anatomy in the first set of images, to the position of the

anatomy in the second set of images. There are multiple approaches to this problem. One approach, which has proven useful in clinical practice for radiation therapy treatment planning, is to use an interactive method where the user "drags" and rotates one dataset in 3D relative to the other until they are visually overlaid. A second and more complex visualization program, expanded from the ortho3slice code, has been developed to facilitate interactive registration of 3D EPRI and MRI datasets. This Matlab program, "register_manual", is illustrated in Figure 2 below.

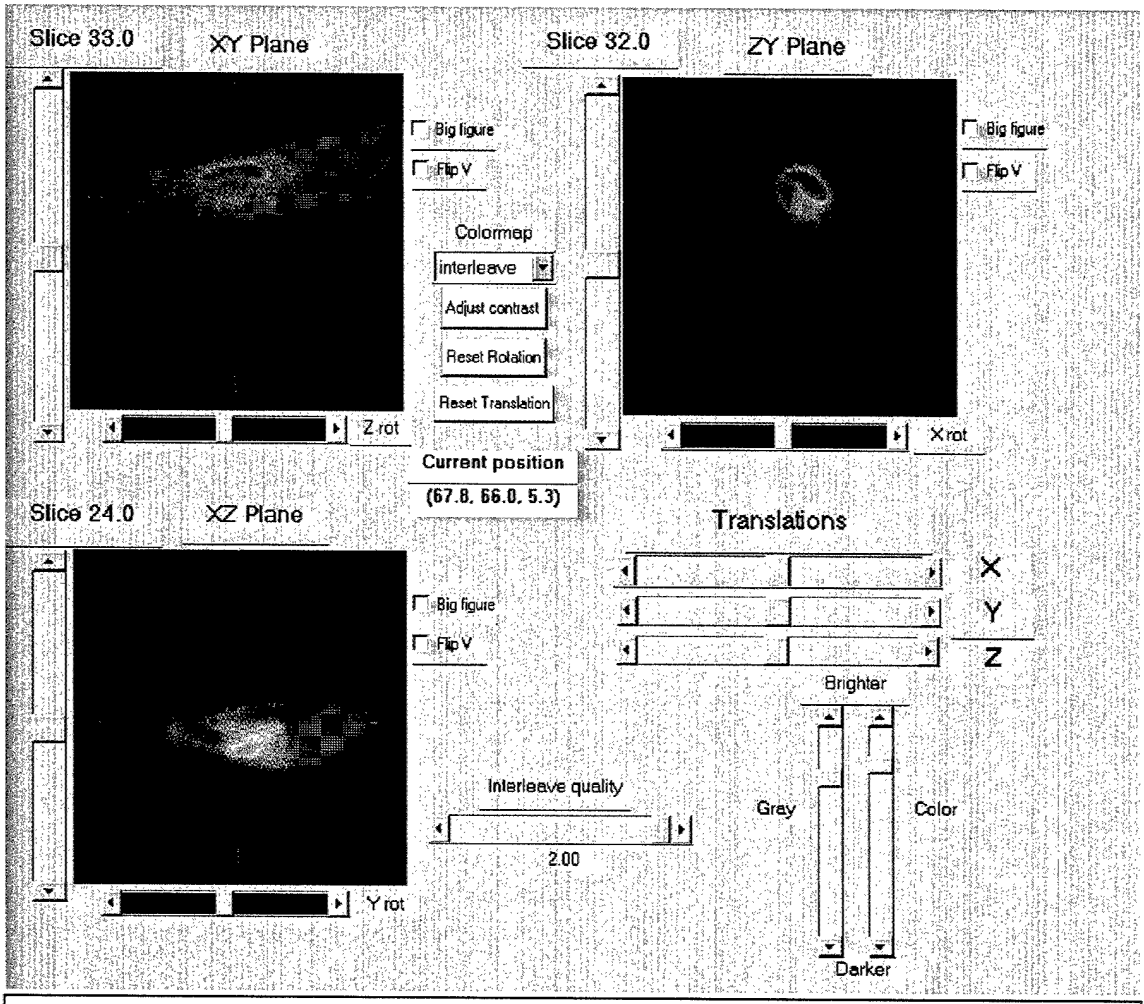


Figure 2. User interface of the manual image registration program showing EPRI and MRI datasets overlaid.

In this program, two 3D image datasets are input, and are displayed simultaneously with one (usually EPRI) in color and the other (usually MRI) in grayscale. The same navigation capability as in ortho3slice is used to display three orthogonal sections through the first dataset. Controls are provided to rotate and translate the second dataset

with respect to the first, and to display corresponding sections of the two datasets in an overlay presentation. Thus the first dataset (the EPRI displayed in color in this example) is seen to remain stationary, while the second dataset is interactively moved to subjectively match the first in the overlay display. This type of registration tool has proven useful in our clinical experience in radiation therapy treatment planning, where image datasets from X-ray computed tomograpy (CT), MRI and various functional modalities are routinely registered. The display quality on the computer is much better than the printed figure reproduced here.

A critical feature of this program is its ability to save the coordinate transformation which registers one dataset to another, and then to recall this transformation from a file. This allows both resuming a session at the point where one left off, and more importantly to apply a transformation relating one set of scans, to a second set of scans. For example, it is generally easier to register a 3D EPRI intensity image with MRI, than to register a 3D EPRI linewidth image with the same MRI. This is because the intensity image reflects the overall anatomy in a more intuitive manner, so its matching to the MRI is simpler. The EPRI linewidth, though, contains our essential functional information (oxygen concentration) and is thus what we really want to register with the MRI. Since the EPRI inensity and linewidth images are parametric images derived from the same underlying 4D dataset, they are inherently spatially registered, i.e., defined at exactly the same set of 3D points. Thus by registering the intensity image to the MRI, and then applying the same transformation to the linewidth image, the physiologic information in the linewidth image is registered with the MRI. Another important capability of this program is to save a set of slices from the second study, resampled along the planes of the first study. For example, a set of resampled MRI slices along the planes of the EPRI dataset can be saved for later multispectral analysis.

A more detailed description of features of this program is provided in a brief user's guide attached as an appendix.

Fiducial Development

Manual registration as described above was for a considerable time frustrated by inadequate EPRI image quality. This was due in large part to contamination of the images by a large signal from the bladder, where the spin probe accumulates as it is cleared from the mouse. This leads to image artifacts which severely distort the overall shape of the EPRI intensity distribution, as seen in the Animal Procedure and Imaging Modality Improvements report included in the Appendix. Anatomically based interactive matching was unreliable using these images. Recent improvements which have significantly reduced the bladder signal offer the potential to allow interactive matching to succeed. An alternative method based on matching of point landmarks has also been developed. In general, the position of a rigid object in 3D space can be fully characterized by specifying the positions of three or more points on the object. If the position of the same three or more points on the object are known when the object is in two different positions and/or orientations, the coordinate transformation (3 translations and 3 rotations) relating the two poses can be calculated using the "Procrustes" method,

which involves only simple linear algebra. The difference in pose may include reorientation of the object, a redefinition of coordinate systems, or both. In our case, the tumor bearing mouse leg is oriented differently in the two imaging instruments, and each instrument has its own coordinate system. In this situation, the Procrustes solution takes into account both effects, and yields a coordinate transformation which allows mapping any arbitrary point or plane in one image volume to the anatomically homologous point in the other. In order to use this method, however, it is necessary to locate the same anatomical points in each of the two image volumes. In human applications where multiple anatomical modalities are to be registered, it is frequently possible to identify three or more homologous anatomical features in the two sets of images. When this is not possible, externally applied fiducial marks can be used instead, and this is the approach we have taken. We have developed small (0.7mm diameter x 3mm length) fiducial markers which can be visualized in both EPRI and MRI. These can be used to register the two sets of images using the Procrustes method. Considerable effort has been expended in developing fiducials which are bright enough to see in both modalities, yet not producing signals sufficiently intense to cause image artifacts. Several stages of fiducial development, using different compounds and methods of attachment to the animal, are described in the appendix Animal Procedure and Imaging Methodology Improvements.

Experimental Information Management

In order to organize the large amount of image data produced, a database of experimental information associated with each imaging run was developed by Chad Haney, Ph.D., the postdoctoral research associate supported by this project. The database was built using Microsoft Access and is populated with information abstracted from hardcopy lab notebooks maintained in the EPRI laboratory. An example report from the database, illustrating the information fields which are available, is included as an appendix. As another tool to keep track of imaging experiments, a spreadsheet has been created which also contains a number of essential items of information about each imaging experiment. A printout of experiments in 2002 from this spreadsheet is included as an appendix. All runs for dates after 2002/04/02 are associated with this project. As can be seen, mouse survival has been dramatically improved following animal handling procedure optimization.

EPRI and MRI images

Examples are provided in the Appendices of EPR and MR images of our mice.

Task 2. Develop image guided injection system.

The following subtasks were programmed for work in year 1:

a. specify and acquire 3D translation stage (months 1-6)

b. design and construct stereotactic localizer frame (months 3-9)
c. integrate positioning and localizer components; interface with imaging (months 6-12)

The 3D translation stage has been produced, by adapting a device designed and built by the machinist associated with Dr. Halpern's EPRI Research Resource. The original application of this device was for making tissue oxygen tension measurements with an oxygen sensing probe, in a way that facilitates quantitative correlation of these physical

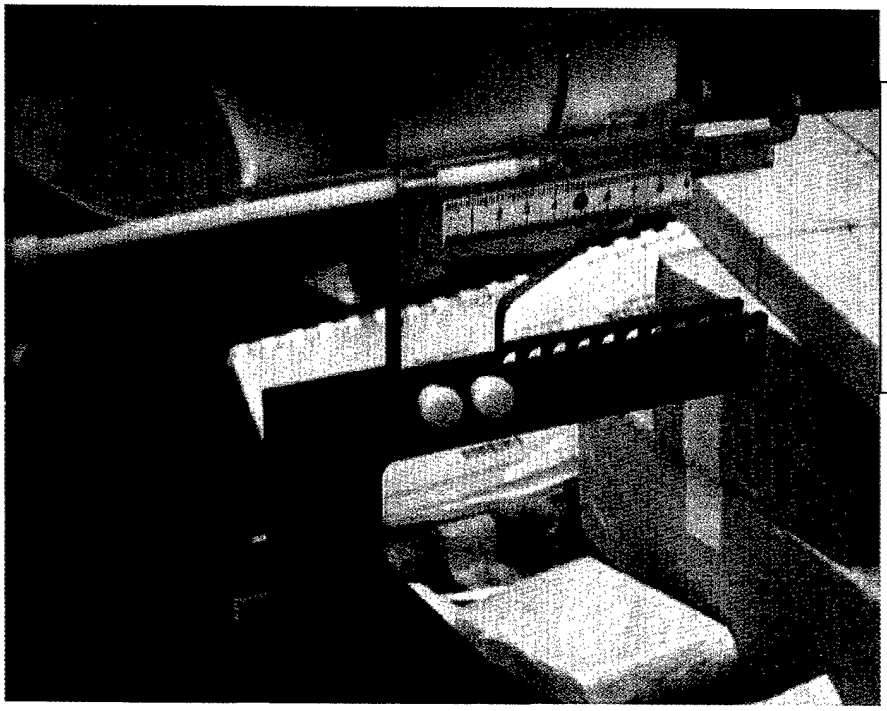


Figure 3. The translation stage developed for placement of needles and oxymetry probes. A simple adaptation of this stage may be used for image guided injection of adenoviral gene therapy vector.

measurements with EPR oxygen images at corresponding points. A photo of the device is shown in figure 3. Due to the unexpectedly large amount of time spent in developing the imaging techniques and animal handling procedures, work on the stereotactic localizer frame required for subtasks b and c has not begun. It may indeed be the case that the fiducial-based registration method that has been developed will obviate the need for the stereotactic frame; this remains to be seen as we gain more experience with the fiducial system.

Task 3. Develop injection planning software.

Activity in Task 3 has been substantially delayed by the diversion of effort to development of animal handling and imaging procedures. However, preliminary work has begun on the subtask of adapting an existing interstitial brachytherapy planning program (which was originally written by Dr. Pelizzari) to the planning of injections for the gene vector in the currently studied radiation mediated gene therapy. This program will require only straightforward modification to satisfy the needs of our planning task. The difference between brachytherapy dose calculations and prediction of the tumor damage distribution in radiation mediated gene therapy planning is mainly in the

distribution of effect (dose or vascular damage) around each source point (radioactive seed or injection point). The existing software is designed to allow the use of numerous radioactive seed types, each with its unique spatial distribution of response. Thus the addition of another response kernel, such as one to characterize the distribution of damage around a single injection site, requires minimal programming. Interface routines to allow import and export of EPRI/MRI images, contours produced in Matlab, and calculated "dose" distributions from the planning system, between Matlab, used in the EPRI lab, and AVS, used in the clinical brachytherapy planning system, are under development by Dr. Pelizzari.

Task 4. Test adaptive radiation induced gene therapy in rats

No activity was programmed for this task until the third year of the project.

KEY RESEARCH ACCOMPLISHMENTS

- First combined EPR and NMR imaging of tumors treated with combined radiation and TNFα gene therapy.
- Development of animal handling procedures required to allow multimodality imaging sessions of 4-6 hours' duration, and on several occasions over two weeks, to be tolerated by nude mice
- Animal immobilization which allows imaging in both EPRI and MRI instruments with minimal disturbance of animal position.
- Development of fiducial marker system to facilitate registration of EPRI and MRI of mouse tumors for multimodality analysis
- Development of unique dual lumen mouse bladder catheterization to allow flushing of accumulated contrast agent which would otherwise spoil images

REPORTABLE OUTCOMES

Presentations at national and international scientific conferences with published abstracts:

Pelizzari, CA. "Treatment Planning for Radiation Mediated Gene Therapy", Med. Phys. vol 29, p. 1297, 2002 (abstract) (Invited presentation at AAPM 2002 Annual Meeting, Montreal, CA)

Halpern, HJ. Quantitative, Calibrated Oxygen Images of Tumors in Living Micewith In Vivo EPR Imaging. 25th International EPR Symposium, Rocky Mountain Conference on Analytical Chemistry, Denver CO, July 31, 2002.

Presentations at academic institutions:

Halpern, HJ. Quantitative Oxygen Images in Tumors with EPR Imaging. Invited Colloquim Speaker, Department of Chemical Physics, Weizmann Institute, Rehovot, Israel. April 26, 2002.

Halpern HJ. What can oncologist learn from EPR Imaging? Presentation to Sigma Xi Research Society, University of Denver Chapter, and the Department Seminar, Department of Chemistry and Biochemistry and the Department of Biology, University of Denver, Denver CO Sept 19,2002.

Halpern HJ. Detecting and Imaging Oxygen and Other Free Radicals with Electron Paramagnetic Resonance. Free Radicals in Chemistry and Biology Seminar Course: Sponsored by National Jewish Medical and Research Center, Webb-Waring Institute for Antioxidant Research, University of Colorado School of Pharmacy, and the University of Denver. Denver CO Sept. 20, 2002.

Halpern HJ Quantitative Physiologic Imaging Deep in Living Animal Tissues with EPR Imaging at 250 MHz Colloquium, Department of Chemistry, University of Chicago. Nov.11, 2002.

CONCLUSIONS

Multimodality EPRI and MRI imaging of mouse tumors is feasible, though technically very demanding when mouse survival is required for serial imaging as needed in therapy evaluation. Important advances have been made in animal handling which now permit repeated imaging of mouse tumors in two modalities and on multiple days. Contrast enhanced MRI and oxygen sensitive EPRI both demonstrate changes following radiation plus antivascular therapy. Registration of these images is very difficult when image quality is not optimal, and further work must be done to improve image quality. In particular, contamination of EPR images by accumulation of spin probe in the bladder as it is eliminated from the mouse can cause artifacts which overwhelm the true signal. A dual lumen bladder catheterization method unlike any previously reported for use in mice has been developed which largely eliminates this problem. An innovative registration method has been developed which utilizes custom made fiducial markers containing two contrast agents - one visible in EPRI, one visible in MRI.

- **APPENDICES**

- Users' guide for ortho3slice program

- Users' guide for manual image registration program

- Animal Procedure and Imaging Methodology Improvements for Combined EPRI/MRI Imaging and Radiation Mediated Gene Therapy

- MR Imaging of Therapy Effect

APPENDIX: Using "ortho3slice" to navigate through 3D image datasets - May 2002, C. Pelizzari

The program "ortho3slice" provides interactive viewing of slices along the three principal planes of a 3D image array. A limited capability is also available for descriptive statistics (mean, standard error, histogram, quartiles) of the distribution of image values in a spherical region with user specified radius centered at the currently selected voxel.

ortho3slice depends on several other functions, each of which has its own .m file:

>> help ortho3slice

ORTHO3SLICE: 3D image navigator
ORTHO3SLICE(imagearray, options)
imagearray is a 3D or 4D array of data
    if a single 4D dataset is passed to ortho3slice, it will create a 3D dataset by summing over the fourth dimension, and this is what you will see displayed. Clicking on the "spectrum" button will plot the spectrum at the currently selected point (see below)
options are:
a second dataset - if the first two arguments to ortho3slice are a 3D and a 4D dataset (either order), then the 3D dataset will be displayed, and you can plot the spectrum from the 4D dataset at any point. So the 3D dataset (e.g. linewidth)is used to navigate through the 4D spectral image.
'xscale',number
'yscale', number
'zscale', number
to set the mag factors along each direction. If omitted they all default to 1.0

calls additional functions to do 4x4 homogeneous transformations:
htransform_vectors
hmatrix_translate_{x,y,z}
hmatrix_rotate_{x,y,z}
augment

also calls:
generate_image_coordinates
generate_slice_coordinates
windowlevel

So, you need to make sure that ortho3slice.m and all of these other functions are in your path in Matlab.

Scale Factors:

Arguments to ortho3slice are as listed above: a 3D or 4D image array, or both, and optional scale factors along x, y and z directions (x = across rows, i.e. second Matlab array index; y=down columns, i.e. first array index; z = third array index). Scaling will be most useful if the resolution of the image array is not isotropic, e.g. if you have an MRI volume with 0.3mm pixel size in plane, but 2mm slice separation, you could set zscale to 6.67, stretching the volume along the third dimension to make it isotropic.

A note about MRI "slice thickness" versus "slice spacing" - slice spacing is the thing we care about. If you have 10 slices, each 2mm thick, but separated by 1mm, then the extent along the z direction is not 10 times 2mm, but (about) 10 times 1mm. If you have 10 slices each 1mm thick, spaced at 2mm intervals, the z extent is 20mm, not 10mm. This is what we are interested in. The program actually interpolates in the data as if it were a function defined only at the grid points of the array, ignoring any effects due to finite pixel size or slice thickness.

So, if you have a nice isotropic dataset like a 64x64x64 EPR intensity or linewidth image, you can just say "ortho3slice(imlw)" or whatever, and off you go. If not, you will want to figure out the scale factors. Think of it like this: ortho3slice will stretch or shrink the data along each of the three axes (x,y,z) by the factor you give. ortho3slice(...,'zscale',3.0,...) is a factor of three stretch along z, appropriate if your MRI pixel size is 1/3mm and slice spacing is 1mm for example. If you were to reduce the number of pixels in your 64x64x64 EPRI dataset along the x direction for some reason, maybe by using "reducevolume(imlw, [1 2 1])", then without an xscale specification it would display as a tall narrow rectangle in the "XY plane". You could recover the square shape by using ortho3slice(...,'xscale',2.0,...). Remember that the (x,y,z) axes for these scale factors are not the EPRI (x,y,z) axes, but the (across row, down column, through page) directions of the 3D array. This would be correct for a transverse CT or MR scan.

Since there is no concept of distance in this program except in units of pixels, it might be less trouble to only use a zscale factor if the x and y pixel sizes are the same, as they usually are. In this case, the zscale factor should be the ratio of (slice separation) / (in-plane pixel size), like the factor 3 in the earlier example.

How to find the pixel size?

For EPRI, look in the ".0" file for your image study. There you will find an item called "Image length" which is the length in cm of the imaged region. This value divided by the number of pixels in the image width is the pixel size. For example, the ".0" file might say

E:\data\DD010606\m1.0
...

B mod frequency: 5.1200
B mod amplitude : 0.1500

Sensitivity : 500.0000
Length of the image : 3.5355
Current at the center:
Full Spectral acquisition (0/1): 1
...

If your image volume is 64x64x64, then the pixel size is 3.5355/64 = 0.055cm or 0.55mm.

For MRI, look in the header file for your image, which you may have in a directory called "Headers and Parameters" and will have the name of your slice with the file type".rh". In there you will find data items called "fov0", "fov1" and "fov". Hopefully these will all have the same value, something like 32.0 or 40.0, which is the field of view in millimeters, not centimeters as in the EPRI case. Again, dividing your field of view by the number of pixels (also in the header file under a data item called "dim0" and "dim1", if there is any doubt how big your images are) gives the pixel size, e.g. 32.0 / 128 = 0.3125mm/pixel.

Options and controls: scrolling / navigating

Once the program starts, you have a few options. There are three image windows. Upper left is a "transverse" slice of the image data, a cut at constant value of the third array index. Lower left is a "coronal" slice, a cut at constant value of the first array index. This corresponds to a single row of the image volume. Upper right is a "sagittal" slice, which is a cut at constant value of the second array index. This corresponds to a single column of the image volume. Each of the three image windows has three controls: a slider to scroll through a stack of parallel cuts, a checkbox to flip the image vertically, and a slider to rotate around the current center pixel. The "current center pixel" is the intersection of the three orthogonal planes currently displayed, and is initially the center of the image volume. As you either scroll through the images or click in any of the orthogonal views, the center pixel is updated to reflect the change. If you scroll through transverse slices (upper left view), you will see the position of the current transverse slice, indicated with tick marks on the other two views, change. If you click in any of the three views, the other two views will be updated to show the orthogonal slices through your chosen point. You can also rotate the image volume around the center pixel in any of the three views, but this may not be terribly useful just for exploring the image volume. These rotations are incremental, about the current center pixel and about the direction normal to the respective view; the sliders are also spring-loaded to return to the center. For convenience, pulling the slider under any view all the way to one end or the other and letting it go will perform a 90 degree rotation in that plane. This will have a dramatic effect on the other two views as well, of course. There is also a button to reset the rotations and return to the initial state, if you get frustrated with the rotation.

Inverting views:

The "flip V" checkboxes next to each view just invert the respective displayed slice, in case the image is upside down relative to how you'd like it to be. The three views can be flipped and unflipped independently.

Zooming views:

Activating the "Zoom on" button will allow clicking with the mouse buttons in any of the views to zoom in (left button) or out (right button). This cn be done independently in each of the three views. Deactivating the "Zoom on" button allows resumption of clicking on the views to choose slices. The current state of zooming in each of the three views is preserved. To reset to no zoom, activate "Zoom on" and right click several times in each zoomed view, then deactivate. It will never zoom out any farther than the original view sizes.

Color adjustment:

The "Colormap" dropdown list allows loading one of a number of predefined colormaps. The "Adjust contrast" button brings up the window/level widget to lighten or darken the images.

Regional analysis:

Pushing the "ROI Stats" button at any time will plot the histogram of image values within a user specified distance of the current center pixel. The distance is controlled by typing the desired value (in pixels) into the "region size" text box. Note that this is a distance criterion, so you are specifying the radius of a spherical region of interest. The intersection of the ROI with the three displayed slices is then drawn, and some rudimentary statistics are displayed in the "ROI Statistics" list box. At the moment these results are not saved to a file, though that can certainly be done.

Spectrum:

If ortho3slice has a 4D dataset to work with, there will be a button labeled "Spectrum" next to the "ROI Stats" button. Pushing this button will plot the spectrum from the 4D dataset at the current center pixel.

Printing:

There is a "File" menu at the top of the window, which has two options: Print and Quit. Print will bring up the normal print dialog to send the contents of the entire program window to the printer. Quit is hopefully self explanatory. Closing the window with the window manager ("X" in MS Windows) also quits of course. Note that you can also resize the window up and down and it will keep working, from full screen all the way down to a postage stamp-sized icon of itself. Of course the ROI statistics are kind of hard to read at that point, but it is very cute!

APPENDIX: Using "register_manual" to register 3D image datasets - May 2002, C. Pelizzari

The program "register_manual" allows interactive translation and rotation of one image dataset relative to another. Three orthogonal slices through both datasets are shown with the data from one dataset in color and the other in gray. The user can evaluate the quality of the fit from the fused images and adjust the transformation relating them as needed.

register_manual depends on several other functions, each of which has its own .m file:

---

htransform_vectors
hmatrix_translate_{x,y,z}
hmatrix_rotate_{x,y,z}
augment

interleave
twodmap
generate_image_coordinates
generate_slice_coordinates
windowlevel

So, you need to make sure that register_manual.m and all of these other functions are in your path in Matlab.

Scale Factors: (see discussion in the "ortho3slice" documentation)

Arguments to register_manual are as follows: a 3D image array, optional scale factors along x, y and z directions (x = across rows, i.e. second Matlab array index; y=down columns, i.e. first array index; z = third array index); a second 3D image array, optional scale factors for the second image. Scaling is used to correct for non-isotropic resolution, as in ortho3slice, and in addition to scale the second image volume to match the first. Assuming you have an isotropic image volume like a 3D EPRI dataset as the first array, then the (x,y,z) scales for the second dataset should be the ratio of (second image pixel size) / (first image pixel size) along the respective directions. So, if your EPRI pixel size is 0.55mm in all three directions, and the MRI pixels are 0.3125mm in plane with 1mm slice spacing, the (x,y,z) scales would be (0.3125/0.55, 0.3125/0.55, 1.0/0.55). Remember that the (x,y,z) axes for these scale factors are not the EPRI (x,y,z) axes, but the (across row, down column, through page) directions of the 3D array. This would be correct for a transverse CT or MR scan. The calling sequence in the case of this example would be

register_manual(imepr, imri, 'xscale',0.57, 'yscale',0.57, 'zscale', 1.82)

How to find the pixel size? (see discussion in the "ortho3slice" documentation)

Options and controls: scrolling / navigating

This program looks like ortho3slice, with a few more controls. There are the same three image windows. Each of the three image windows has the same three controls: a slider to scroll through a stack of parallel cuts, a checkbox to flip the image vertically, and a slider to rotate around the current center pixel. The "current center pixel" is the intersection of the three orthogonal planes currently displayed, and is initially the center of the image volume. As you either scroll through the images or click in any of the orthogonal views, the center pixel is updated to reflect the change. If you scroll through transverse slices (upper left view), you will see the position of the current transverse slice, indicated with tick marks on the other two views, change. If you click in any of the three views, the other two views will be updated to show the orthogonal slices through your chosen point.

You change the orientation of one image volume relative to the other by translating and rotating the second image volume around the center pixel in any of the three views. These rotations are incremental, about the current center pixel and about the direction normal to the respective view; the sliders are also spring-loaded to return to the center. For convenience, pulling the slider under any view all the way to one end or the other and letting it go will perform a 90 degree rotation in that plane. This will have a dramatic effect on the other two views as well, of course. There is also a button to reset the rotations and return to the initial state, if you get frustrated with the rotation. Translations are also incremental, and the sliders for translation are also spring-loaded. Translations are in the plane of the displayed view, not in the native coordinate system of the transformed volume, so the "X translation" and "Y translation" sliders move the transformed data left and right or up and down in the "XY plane", and so forth. This seems to be the least confusing way to define the translations. As you change the orientation of the second image volume by translating and rotating, the overlaid display is updated by reslicing the second image volume along the three orthogonal planes of the first volume, which are currently displayed. If you change the three views by scrolling or clicking on one of the views, the overlaid slice will be recalculated and updated on the screen. You can control the saturation of the color, and the brightness of the grayscale image, using the "Gray" and "Color" sliders at the lower right of the GUI.

Inverting views:

The "flip V" checkboxes next to each view just invert the respective displayed slice, in case the image is upside down relative to how you'd like it to be. The three views can be flipped and unflipped independently. Flipping a view does not change the relationship of the two image volumes - it just inverts the view. To introduce a reflection along one axis into the interscan coordinate transformation, for example if you had two CT scans and in one the slice position (Z) values increase from head to feet but the other from feet to head, you would have to assign a negative value to one of the pixel sizes.

Zoomed views:

Each of the three views also has a checkbox labeled "Big figure" which will pop up a new window with a copy of the view. This can be panned, zoomed, printed, etc. like any normal Matlab figure window. If you close this extra figure window with the window manager, it will be recreated again whenever the view gets updated, as long as the checkbox is checked. Checking the box on one of the other views will take over the big figure window for that other view - there is only one extra figure window shared by all the views.

Color adjustment:

The "Colormap" dropdown list allows loading one of a number of predefined colormaps. The "Adjust contrast" button brings up the window/level widget to lighten or darken the images. Neither of these is probably useful when two studies are overlaid, except to reload the overlay lookup table.

Overlay quality:

The image overlay is accomplished by combining the two images in a checkerboard fashion: the slice from volume one on the white squares of the board, from volume two on the black squares. Initially the size of the checkerboard is equal to the size of the larger of the two image volumes, or 128 pixels, whichever is greater. The "image quality" slider increases the density of the checkerboard, making the overlay finer as the image quality index increases. There is some penalty in speed for this, but the improvement in overlay image quality in using the highest setting is considerable.

Printing:

There is a "File" menu at the top of the window, which has two options: Print and Quit. Print will bring up the normal print dialog to send the contents of the entire program window to the printer. Quit is hopefully self explanatory. Closing the window with the window manager ("X" in MS Windows) also quits of course.

Saving and restoring results:

In the "Parameters" menu are several options for saving and retrieving images and transformations. "Save resliced volume" will save an array the same size as the first image volume, with image data from the second image volume sliced along the planes of the first. In our example, this would be a set of MRI slices, one for each EPRI slice. "Save matrix" prompts for a filename and saves the current interscan coordinate transformation. If you were then to quit, and restart the program with the same command line you used the first time, and reload this transformation with the "Read matrix file" menu item, the images would be registered exactly as they were when the matrix was saved. This will be useful for reading in and displaying an EPRI linewidth image, say,

after the EPRI intensity image has been registered to the MRI. The "parameters" menu item allows changing a number of things, among them the pixel sizes and also the names of the axes that are used to label the three views. This can be used to have the labels agree with the EPRI coordinate system, if desired.

APPENDIX: Animal Procedure and Imaging Methodology Improvements for Combined EPRI/MRI Imaging and Radiation Mediated Gene Therapy

by: Chad Haney, Ph.D. and Gene Barth

DAMD17-02-1-0034 (Pelizzari)

Maintenance of animal temperature

Working with nude mice and knowing that many anesthetics effect temperature, the need to maintain the mouse body temperature is intuitively clear. However, methods for controlling the body temperature of a 25 g animal are not trivial. Considering the range of environments, e.g. surgery table, EPRI and MRI magnets, cage, etc. that the mouse is in, many methods need to be combined to maintain a core temperature near 37 °C. Heating lamps are used while the mouse is in the EPRI magnet and warm air is blown into the MRI magnet.

An innovation in our preparation and transport procedure was to use the Deltaphase® Isothermal Pad (Braintree Scientific, Inc. Braintree, MA). It has two advantages over heating pads. It does not generate heat and therefore cannot burn the mouse. Secondly, it is portable. The pad can maintain a small animal at near normal and constant temperature for several hours. At room temperature the pad is in solid form. When heated to 39°C the solution in the pad becomes fluid. The pad remains isothermal until all the liquid phase has solidified. Only then does its temperature fall. The transition temperature has been preset by the manufacturer to 39°C so that a small temperature gradient always exists for heat flow from the pad to the mouse. This heat flow balances heat losses and maintains animal body temperature near 39°C for periods up to several hours. During recovery we combined the Deltaphase® pad with a Styrofoam box. This combination maintains the temperature, as measured by a rectal thermometer, for several hours.

Mouse weight

Following some of the early combined imaging experiments, several the mice appeared to be cachectic. It is not clear if this is a result of the tumor model or the length of the baseline (day 0) experiment. On day 0 the mouse is prepared for imaging, imaged with EPRI and MRI, injected with the adenoviral vector, and finally subjected to radiation. On average, that amounts to six hours of anesthesia. Mouse weight maintenance, and therefore mouse survival have been improved by better hydration. Changing the anesthesia regimen may also help, as discussed later.

Hydration

In an effort to ensure the survival of the mice to day 16, extra vigilance was required in hydration before and after the experiment. Maintenance of weight was facilitated by management of mouse hydration. When a mouse's weight dropped, 1 mL of either Ringer's lactate or 0.9% saline was given subcutaneously. This intervention resulted in a noticeable improvement in mice alertness and ambulation after overnight recovery.

Food

Access to food and water may be hindered during recovery from anesthesia or possibly due to the cachectic nature of mouse in this tumor model. Another improvement in our procedure was to place water and food in the cage directly as opposed to utilizing the suspended water and food built into the cage.

Bladder clearance

The triarylmethyl spin probe (OX063) used for EPR imaging is rapidly cleared by the kidneys. The accumulation of spin probe in the bladder creates a tremendous source of signal, often greater than that of the tumor. To reduce the artifacts created by the bladder

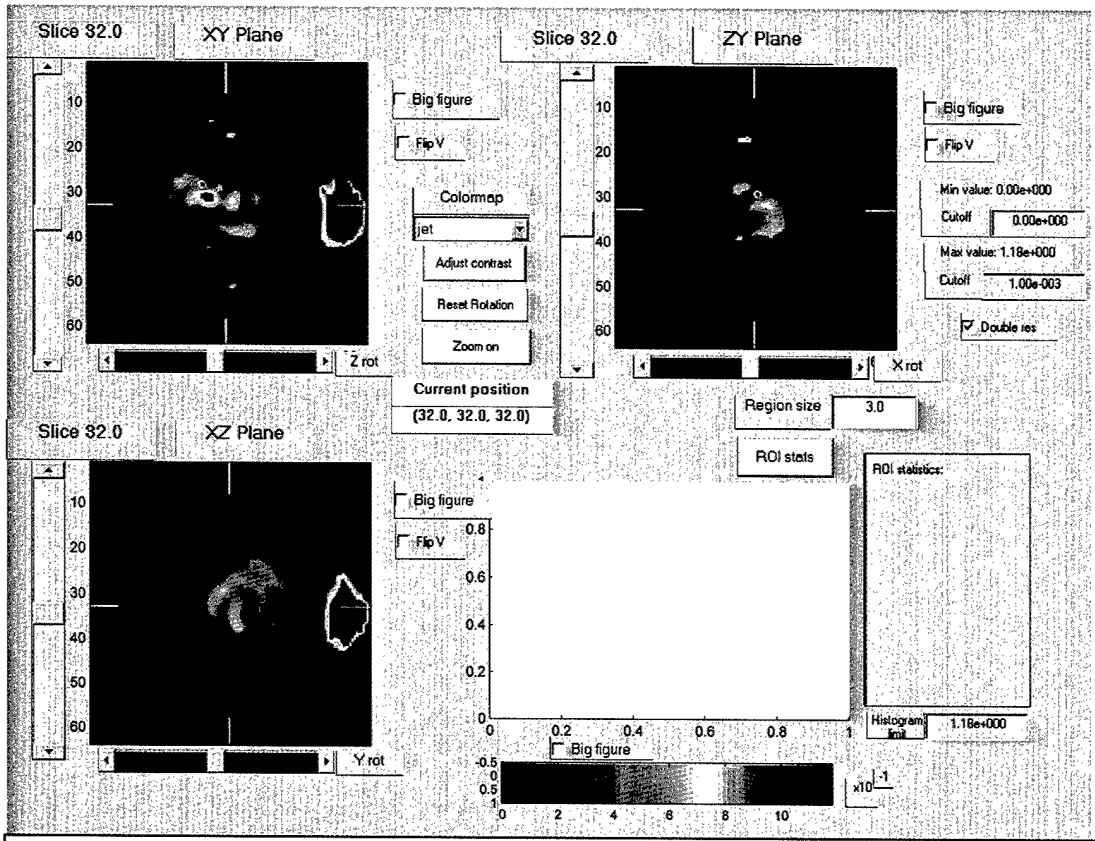


Figure A1. Slice through 3D EPRI intensity image showing intense bladder signal (right) which distorts signal from tumor bearing leg (left).

signal, bladder clearance became a priority. The high concentration of triarylmethyl in the bladder causes self broadening, not the broadening by oxygen (the desired measurement). In figure A1 the bladder (smaller structure to the right) is distorted and artifacts are present around the tumor, even after fitting and eroding one layer of pixels to remove edge effect artifacts.

PE10 tubing

Searching on PubMed for non-invasive techniques to flush the bladder led only to invasive techniques or techniques utilizing needles. For example, Eichel et al flush the bladder with a 24 G stainless steel ball tip needle (designed for oral lavage) [1]. This has two limitations: securing the needle to not leak and the requirement of non-magnetic materials. With the paucity of methods in the literature, our first iteration of bladder clearance was to simply insert PE10 tubing (0.28 mm ID, 0.61 mm OD, Clay Adams INTRAMEDIC® Polyethylene, BD Franklin Lakes, NJ). However, PE10 tubing is too flexible, requiring a trocar or stylet. Also the end was often sharp or abrasive regardless of attempts to "polish" the ends.

IV catheter

The next iteration was to use a standard 20 G IV catheter (1.1 mm ID, Introcan® Safety™ IV Catheter, B. Braun Medical Inc., Bethlehem, PA), with a rubber tube extension. This provided a more tapered, smooth end. It also has a trocar with it. With the use of visual and tactile indicators, cannulation of the bladder via the urethra became fairly easy. Because the IV catheter is more rigid than the PE10 tubing, it will change position inside the bladder rather than bend, when the tail is repositioned. This is likely the cause of occasional interrupted urine flow after positioning the mouse in the resonator. Lundbeck et al have used a similar setup for bladder filling [2, 3]. However, for flushing the bladder with a transurethral cannula, this is a new technique.

IV catheter with PE10 inside

Due to the anesthesia, it is likely that
micturition is interrupted. Another innovation in the procedure is to flush the bladder with saline via a double lumen tube. All of the catheter manufacturers contacted could not make a double lumen tube small enough to fit a mouse urethra, without significant investment. Fortunately the PE10 tubing fits inside the IV catheter providing a double lumen. De-ionized water is pumped into the PE10 tubing to flush the bladder with a Baxter Infusion Pump model AS40A (Baxter Healthcare Corporation, Deerfield, IL), while the bladder is emptied via the catheter. The pressure from the infused saline slightly inflates the bladder allowing positive pressure to clear the triarylmethyl. The flowrate has not been optimized yet. Currently 10 to 15 mL/hr works well. However, it is not clear yet, if this flow rate will influence the survival of the mice. Lundbeck et al, use 6 mL/hr saline [2, 3]. De-ionized water is used because the triarylmethyl is hypotonic. In figure A2, the improvement in tumor imaging is clear. The bladder signal is absent along with the artifacts it creates. Also the tumor closely resembles the shape of the leg (with the foot to the left).

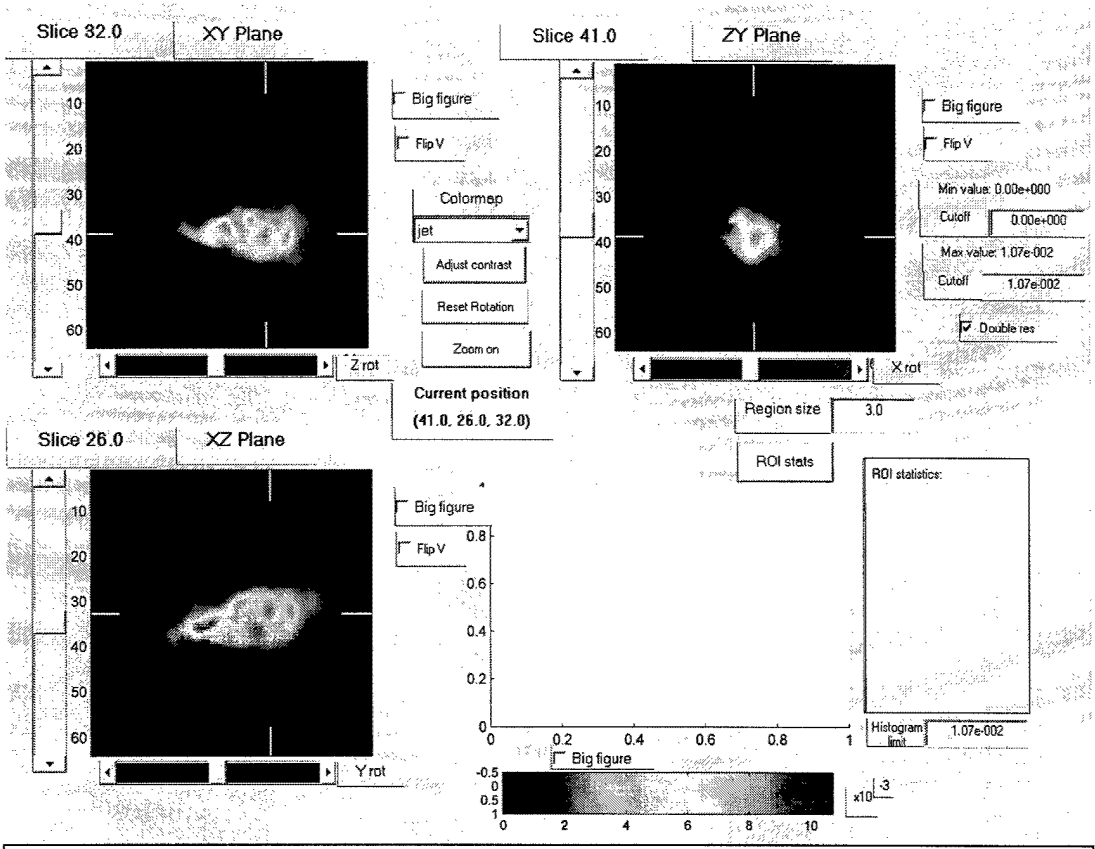


Figure A2. EPRI intensity image (parametric image from fitted 4D volume) showing nearly complete suppression of bladder signal.

Tail position

Securing the transurethral catheter to the tail is the easiest to manage but has its limitations. When the mouse is positioned in the resonator the tail is moved 90° relative to the leg. This changes the position of the catheter. Due to its rigidity, it would likely press against the bladder wall, occluding the opening, rather than simply bending. We have tried anchoring the transurethral catheter to the trunk of the body, but it often slips out. We are considering designing an adapter, like a right angle bend, to allow the tip of the catheter to be parallel to the trunk of the body while the part anchored to the tail is always parallel to the tail.

Perfusion holes

An important innovation on the transurethral catheter development is the use of perfusion holes. We have tried making holes in the side of the catheter to improve fluid exchange, even when the wall of the bladder occludes the tip. We were unable to make the holes such that the surface remained smooth enough as to not abrade the lining of the urethra. Access Technologies (Skokie, IL) has discussed making a custom catheter with perfusion holes, a rounded tip, and a 0.9 mm OD, using polyurethane. Access Technologies has been designing catheters and vascular access ports since 1981.

Anesthesia

After consulting the veterinary staff, it appears possible that the duration of anesthesia is too long for a species with a high metabolic rate, using the present agents. We currently use 50 μL of ketamine (100 mg/mL)/xylazine (5 mg/mL) mixture for induction of anesthesia and maintain with periodic injection of ketamine (14.7 mg/mL)/xylazine (0.7 mg/mL) mixture. It is possible that switching to a gas anesthetic like isoflurane will improve the survival rate, as isoflurane has a much faster recovery rate as it is cleared mainly through respiration and not via the kidneys and liver as in ketamine and xylazine. Equipment and training are required to implement gas anesthesia. Alternatively, dramatic speed improvements in the EPR image acquisition might obviate the need to changes anesthesia. Some of the improvements which are being pursued by the EPRI Research Resource could translate into a significant reduction in EPR image acquisition time.

MRI/EPRI fiducials

From the summer of 2001 through the summer of 2002, we experimented with the composition of EPRI/MRI visible fiducials, methods of attaching them to the tumor bearing legs of our experimental animals, and methods of efficiently imaging them without interfering with our EPR oxymetric imaging.

By the summer of 2002, we had succeeded in making fiducials with good visibility in both EPR images and MR images, reliably attaching them to our experimental animals, efficiently imaging the fiducials.

Chemical and physical development

As late as August of 2001, we were marking tumors with white stripes and then photographing the installed tumor with a digital camera. In anticipation of the need for accurate registration for the upcoming Army grant, in September 2001 spectra of a mouse tumor with lithium phthallocyanate (LiPc) fiducials attached to it were made. In the first mouse imaging runs with EPR fiducials, we kept the fiducials on the tumor during the oxymetric imaging. However, fiducial artifacts dominated the oxymetric image. Soon, we were pulling the LiPc fiducials off the tumor before starting the oxymetric image. Beginning in Winter 2001-2002, we were also acquiring "LiPc + tumor" one-spatial-dimension images for the X, Y, and Z axes ("2Dx", "2Dy", and "2Dz").

We continued using the LiPc fiducials through the beginning of 2002. We attached the LiPc fiducials to the mouse's leg by "double stick" tape. Each fiducial had a "collar" of heat shrink tubing. Using a needle, we passed a suture thread through the collar, and then secured the thread with a knot. We simply pulled on this thread to remove the fiducial. Beginning in 2002, Adrian Parasca and Gene Barth had attempted to manufacture the first OX031 filled fiducials. Following Dr. Halpern's suggestion, we also began attaching the fiducials to the mouse's leg by a loop of suture thread passing in part through the skin of the mouse.

By March of 2002, we were using Finland trityl (D) fiducials. In the spring of 2002, Adrian and Gene also made FNST (D) fiducials that contained either gadolinium or chromium oxalate (CrOx). The addition of the gadolinium or chromium oxalate doubled the MRI intensity of these fiducials under imaging conditions where faster proton relaxation returns a larger signal. Jonathan River (MRI) and Gene Barth studied the plain FNST(D) fiducials side-by-side with the FNST(D) + Relaxation Agents (CrOx or gadolinium) and observed the enhanced MRI visibility of the combined-agent fiducials.

On May 31, 2002, we made our first pair of two-spatial-dimension fiducial images, 3Dxy and 3Dyz, rather than a trio of one-spatial-dimension images. On July 01, 2002, we made our first three-spatial-dimension fiducial image, 3Dxyz. For this image, we sutured three "combo" fiducials to the mouse's leg by a single loop. To each fiducial, we had also permanently attached a suture thread. After finishing the fiducial 3D image, we simply pulled on each fiducial's permanent thread until the fiducial came out of its restraining loop and off the tumor. We then injected the OX031 or OX063 spin probe into the mouse and made our 4D oxymetric image. Before the MRI run, we reattached the fiducials and made our anatomic and gadolinium washout MR images with the fiducials on the tumor. This 07.01.2002 experiment is our present state of the art for working with fiducials.

Securing Fiducials

At first, tape was used to secure the fiducials to the tumor bearing leg. This was unreliable and sutures were the next evolution. Sutures work well to secure the fiducials in place but made it difficult to remove the fiducials prior to EPR imaging. However, we did not want to abandon the secure and reproducible placement of the fiducials that suturing affords. Therefore, silk thread was attached to the fiducials such that pulling on the string removes the fiducials without disturbing the position of the mouse leg, while in the resonator. With the sutures still in place, the EPR image is acquired. Prior to MRI imaging, the fiducials are slipped back into the sutures in practically the identical location. A photo of a mouse in the EPRI resonator and the MRI coil with sutured fiducials is shown in figures A3 and A4. A closeup of the sutured fiducials on a mouse leg is shown in figure A5. A photo of the shield used when irradiating the mouse tumors is also shown in figure A6.

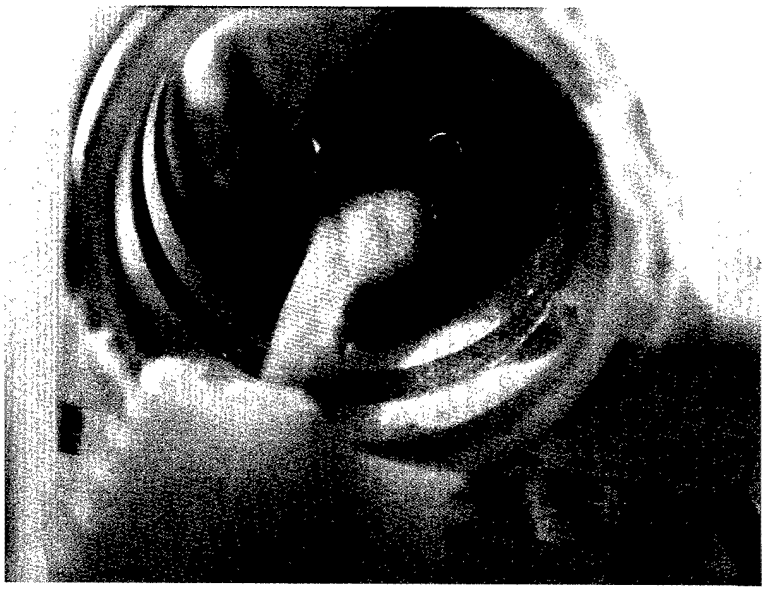


Figure A3.View of a mouse leg in the MRI coil with three fiducials attached.

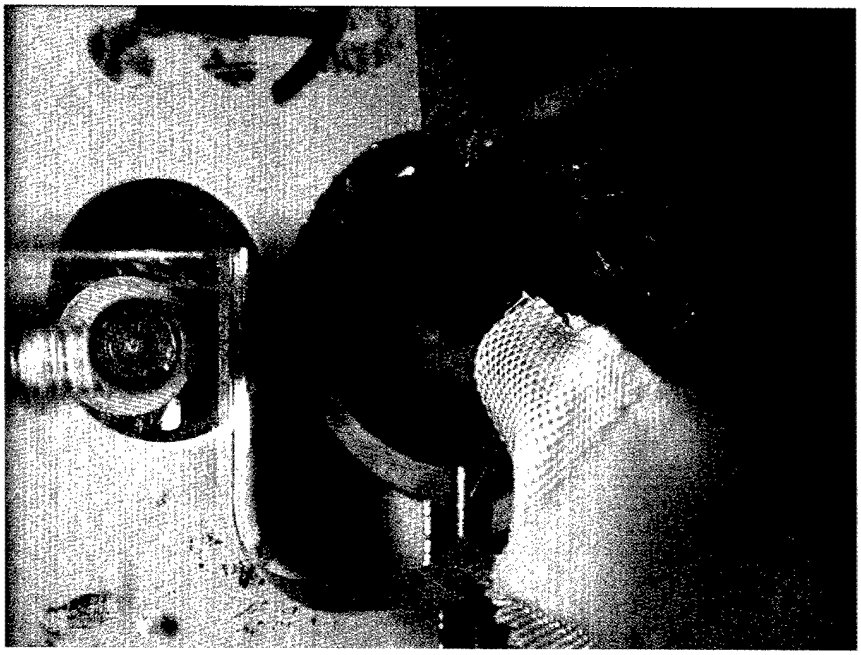


Figure A4. Mouse leg extending through EPRI resonator with fiducials attached.

Database

In order to find trends in the data and procedures, to improve survival, a Microsoft Access 2000 database was developed. Parameters and comments are recorded for both experiments and analysis. More importantly, the health of the mice are recorded, i.e., mouse initial weight, temperature, and tumor dimensions; total amount of anesthesia, injections of analgesia and Ringer's lactate; and daily weight measurements.

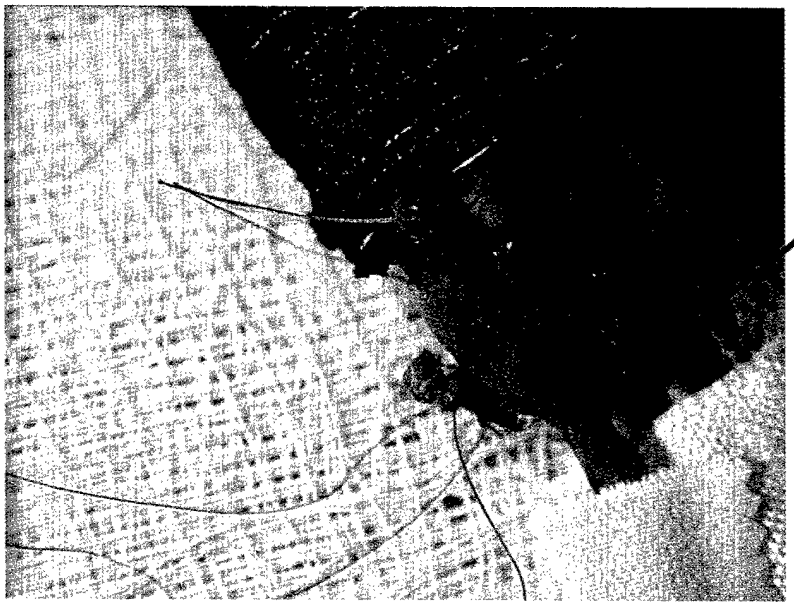


Figure A5.Closeup of tumor bearing mouse leg with suture attached fiducials . Note bladder catheter (arrow).

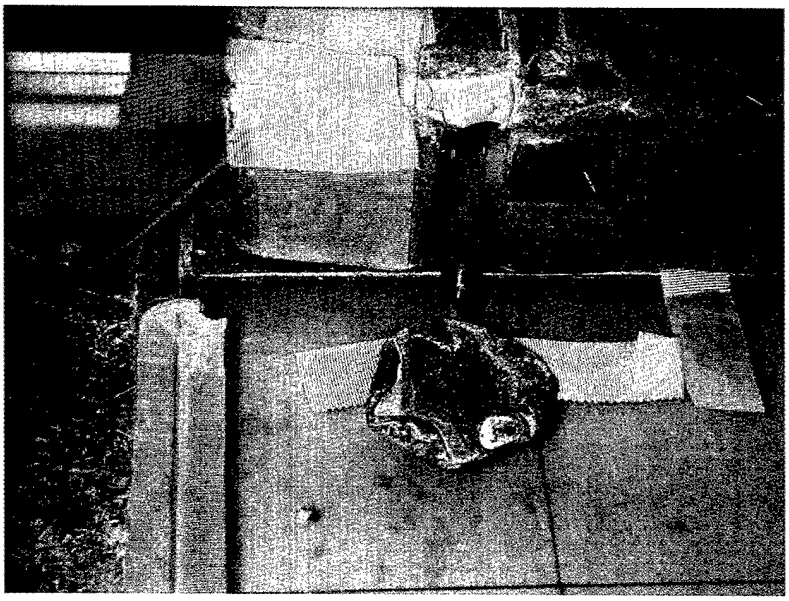


Figure A6.Mouse in whole body shield with leg tumor immobilized for radiation treatment at the 250 kV X-ray unit.

Summary

These improvements have led to 75% increase in the number of mice imaged on day 16. Prior to implementation of these techniques only one mouse survived to be imaged on day 16 of treatment. Similarly, there has been a 50% increase in the number of mice

surviving to day 3. With the change to gas anesthesia and refinements in the bladder flushing, it is likely that further increases in survival will be seen.

References

1. Eichel, L., et al., *Assessment of murine bladder permeability with fluorescein: validation with cyclophosphamide and protamine.* Urology, 2001. **58**(1): p. 113-8.
2. Dorr, W., *Cystometry in mice--influence of bladder filling rate and circadian variations in bladder compliance.* J Urol, 1992. **148**(1): p. 183-7.
3. Lundbeck, F., J.C. Djurhuus, and M. Vaeth, *Bladder filling in mice: an experimental in vivo model to evaluate the reservoir function of the urinary bladder in a long term study.* J Urol, 1989. **141**(5): p. 1245-9.

APPENDIX: MR Imaging of Radiation/TNF Therapy Effects

Following are summaries of two MR imaging runs on a leg-borne PC3 tumor in one of our mice. The first run, labeled 10-08-02, is a pretherapy or "day 0" session. The large set of images shows a dynamic series monitoring contrast uptake and washout on a single slice at longitudinal position z = 0 mm, and at the top of the first page are shown difference images (post contrast - pre contrast) for three slices, z = -2, 0, +2 mm. Contrast uptake shows a distinctive pattern of increased intensity consistent with a highly vascularized rim around the growing tumor.

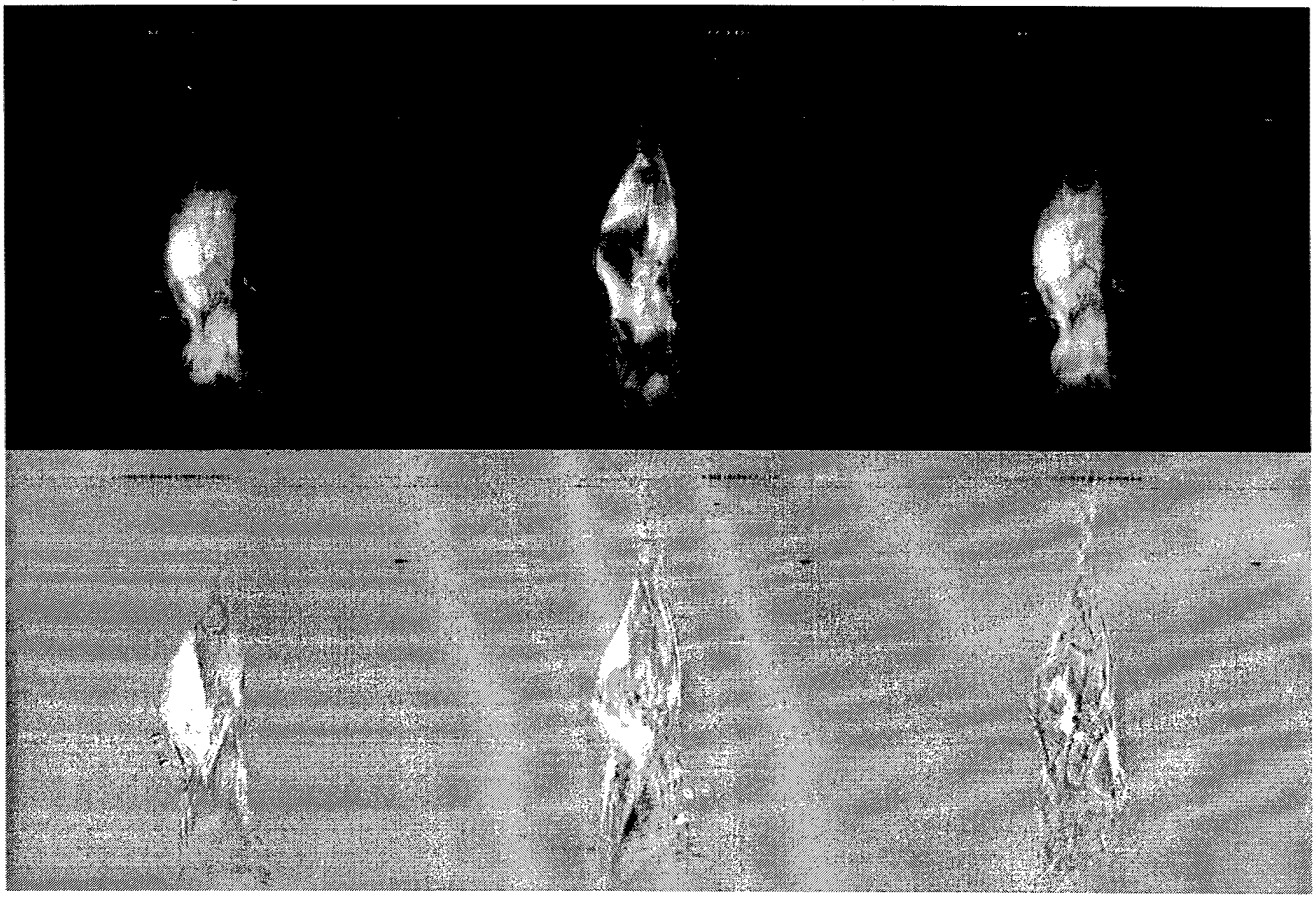
On day 0, following the EPRI and MRI imaging sessions, the tumor is injected by Dr. Helena Mauceri with the Ad.Egr-TNFα viral vector and irradiated with 250 kVp X-rays.

The second image set, labeled 10-11-02, is of the same tumor three days later, a "day 3" run. There is a marked difference in contrast uptake in the area of the tumor with no intense rim of uptake, and a general reduction in uptake, which is consistent with the expected disruption of vasculature by the TNFα and radiation.

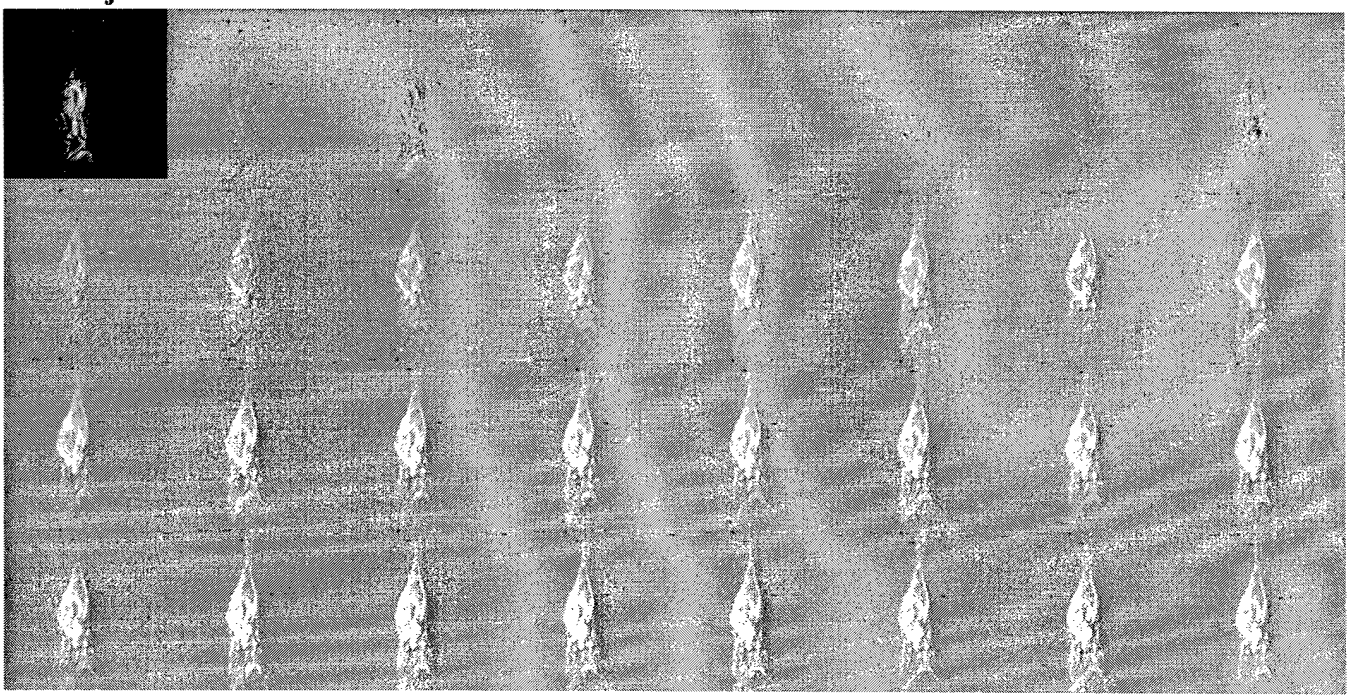
In the spin-echo images which are shown after the dynamic contrast studies, the fiducial markers which are used for registration of EPRI and MRI can be seen as hyperintense features - either short lines or points, depending on the relative orientation of the fiducial capsule with the imaging plane.

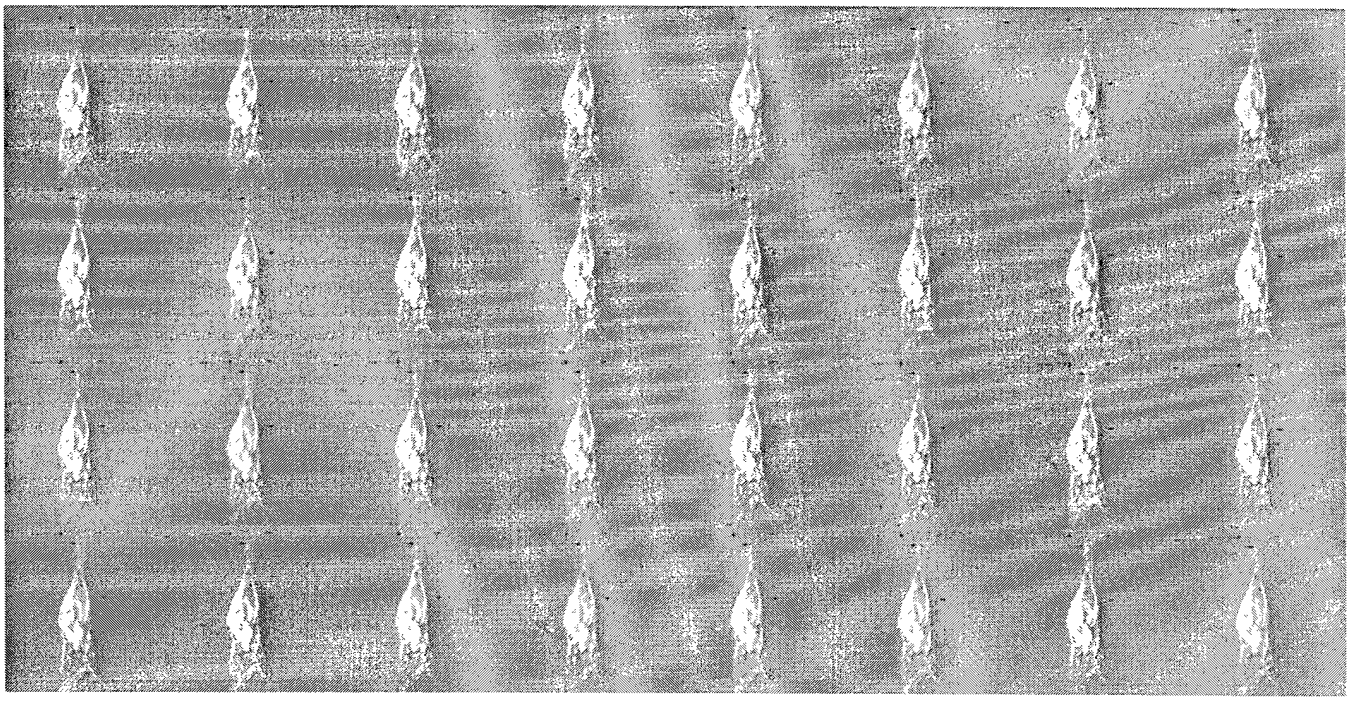
Pelizzari mouse 10-08-02

1st row: Before injection; 2nd row: after – before. Slice offset = -2, 0, 2

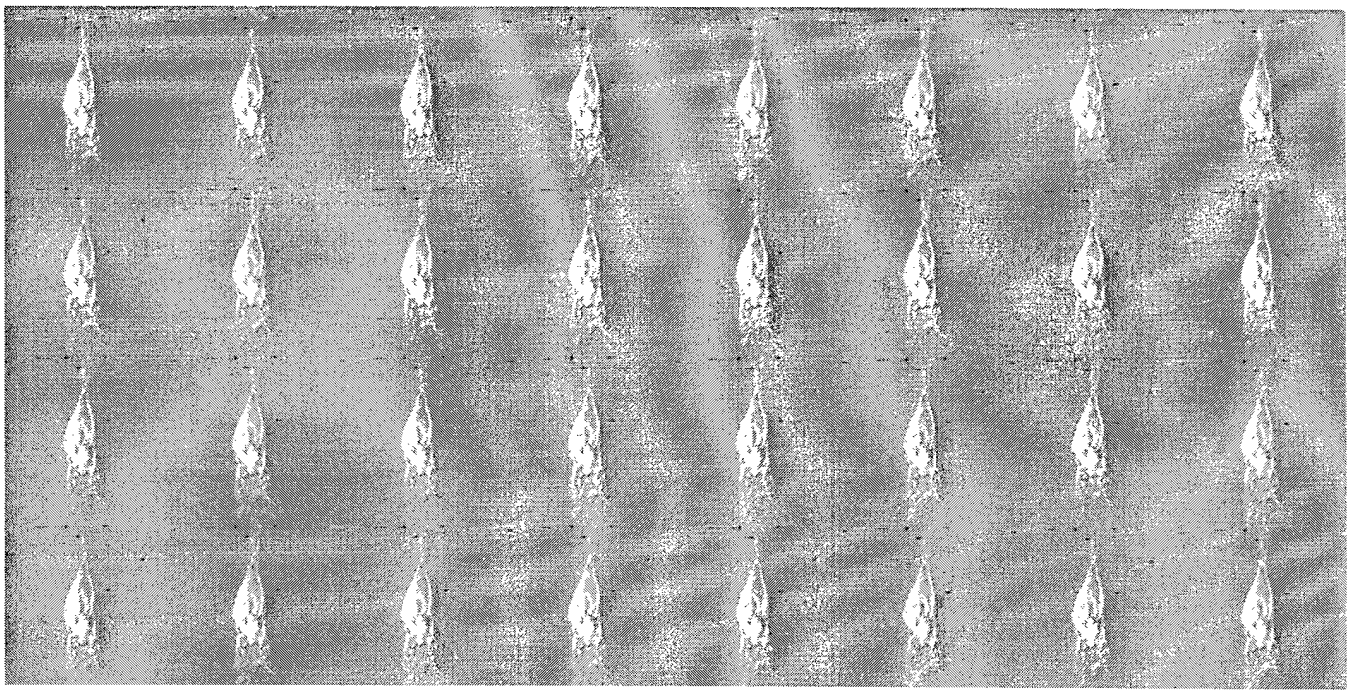


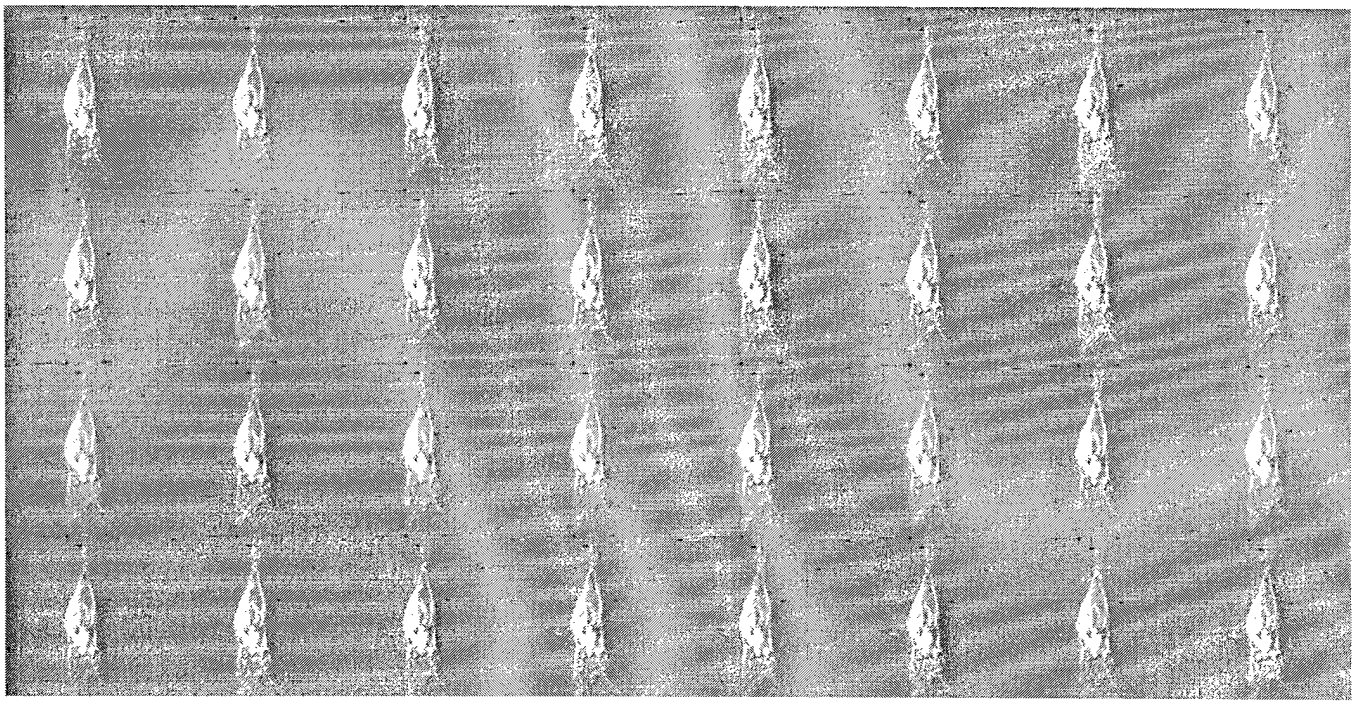
First Injection:



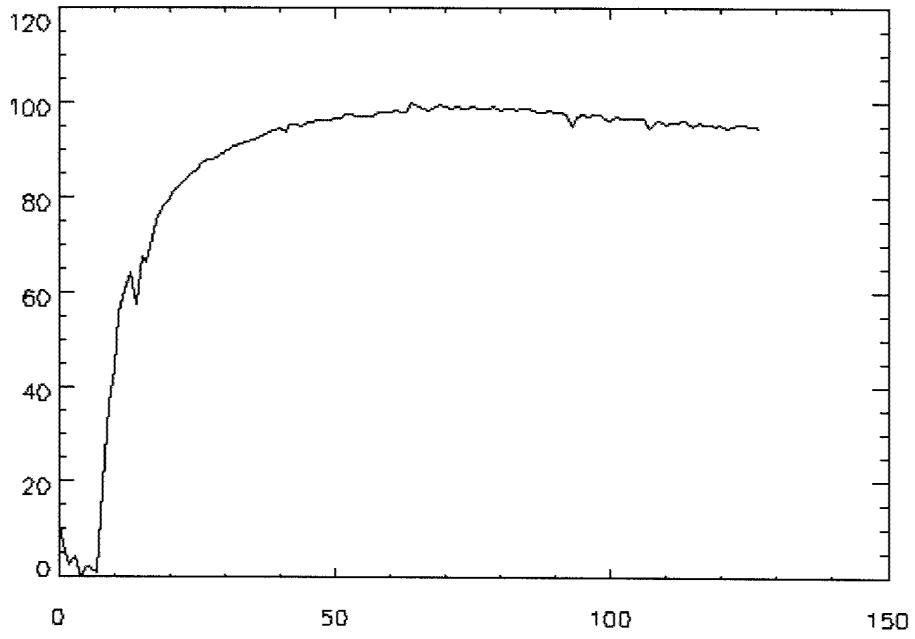


Second half of 1st contrast injection:

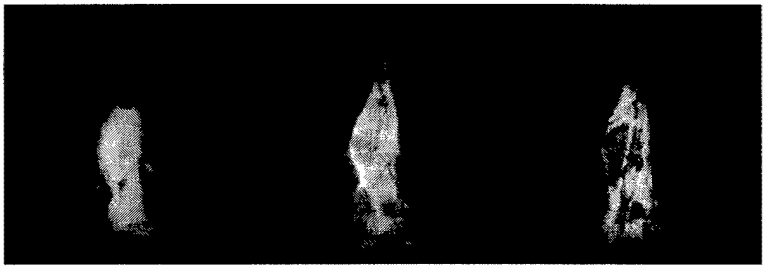




Contrast uptaken curve: 100.0*total(n^{th}_image-control)/control as a function of time

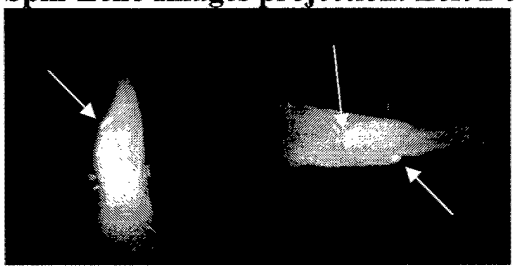


Gradient echo images: slice offset = -2, 0, 2



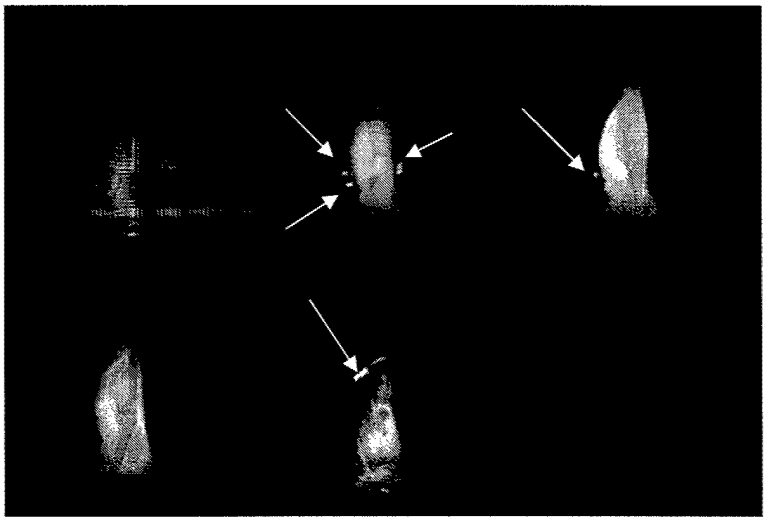
Note: bright spots and short lines (arrows) are fiducials for registration with EPRI.

Spin-Echo Images projection: Left z-direction; Right y-direction

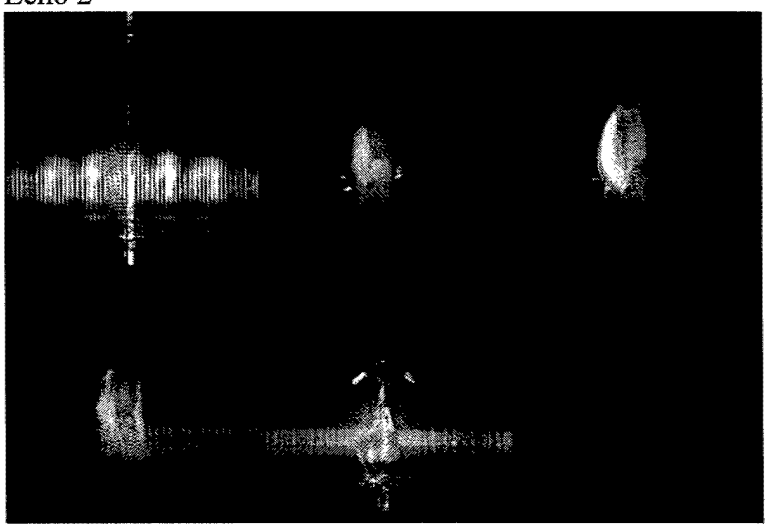


Spin-Echo Images: Slo=-4, -2, 0, 2, 4; te=15ms (For 3D reconstruction)

Echo 1

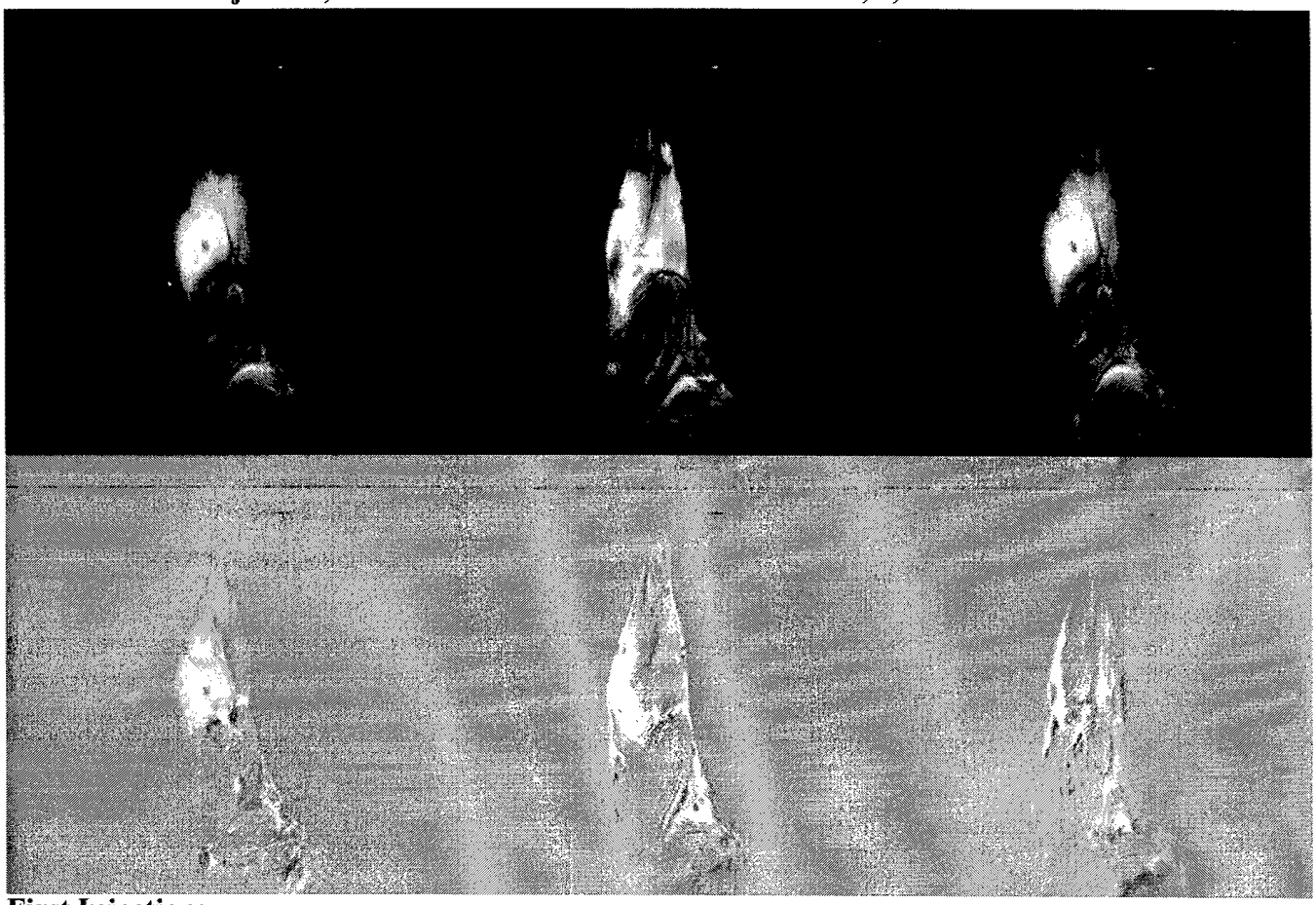


Echo 2

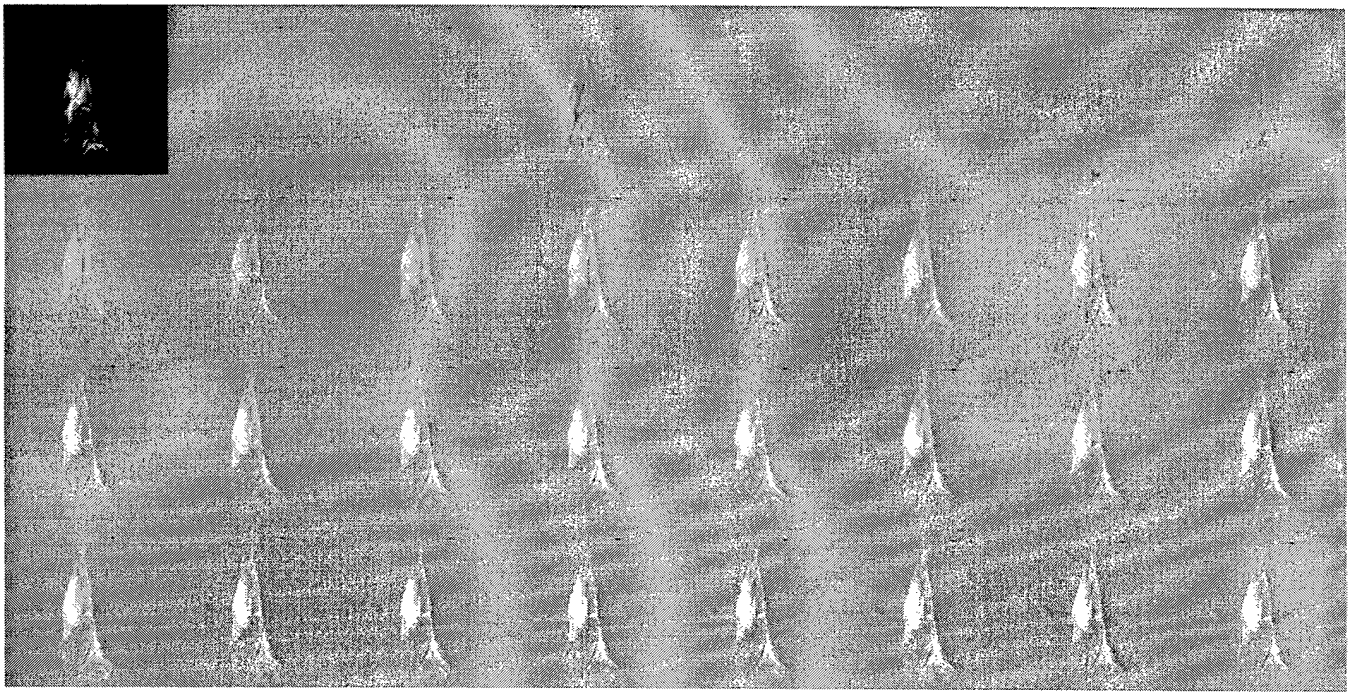


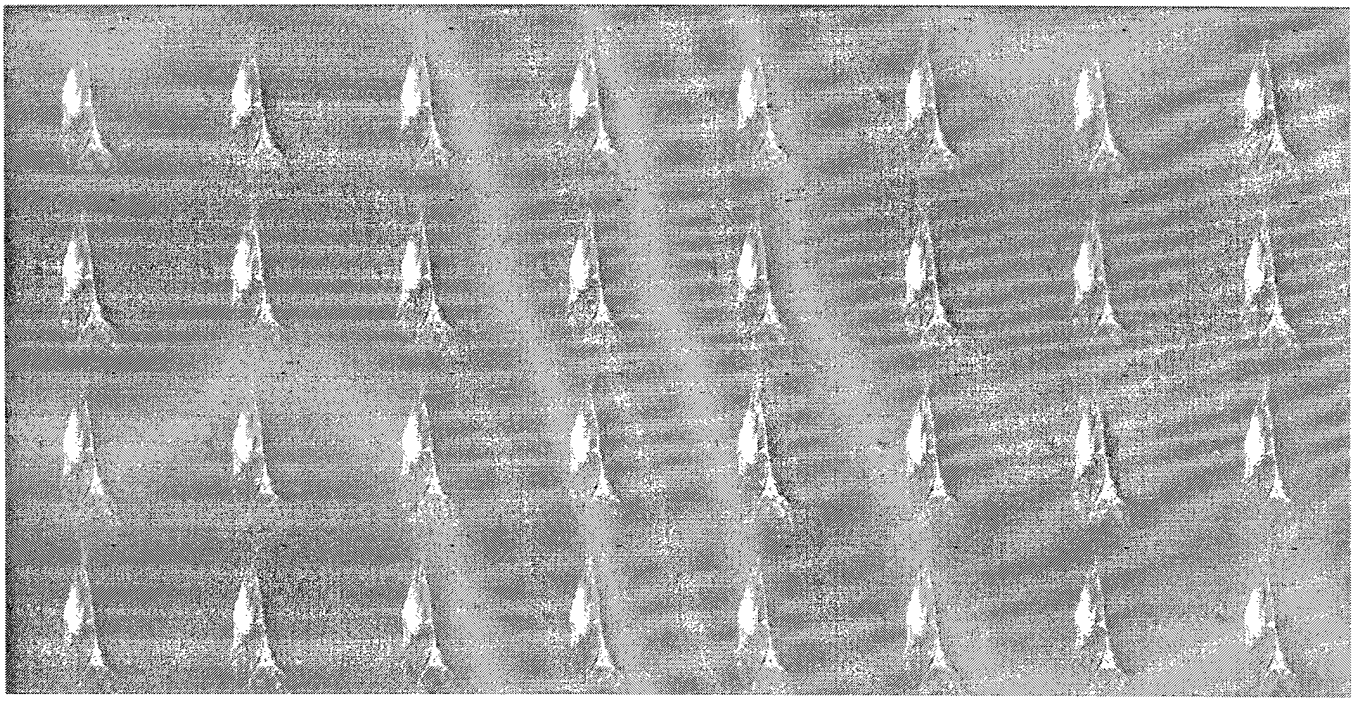
Pelizzari mouse 10-11-02; Day 3 for 10-08-02

1st row: Before injection; 2nd row: after – before. Slice offset = -2, 0, 2

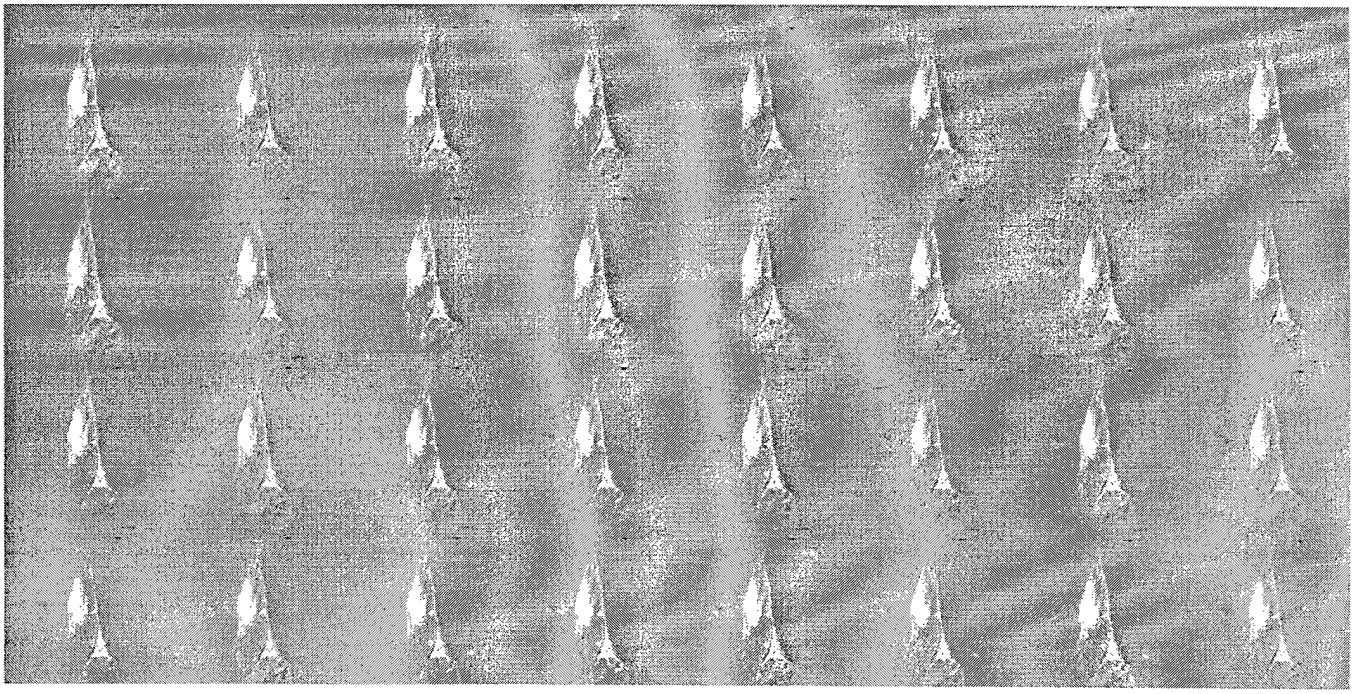


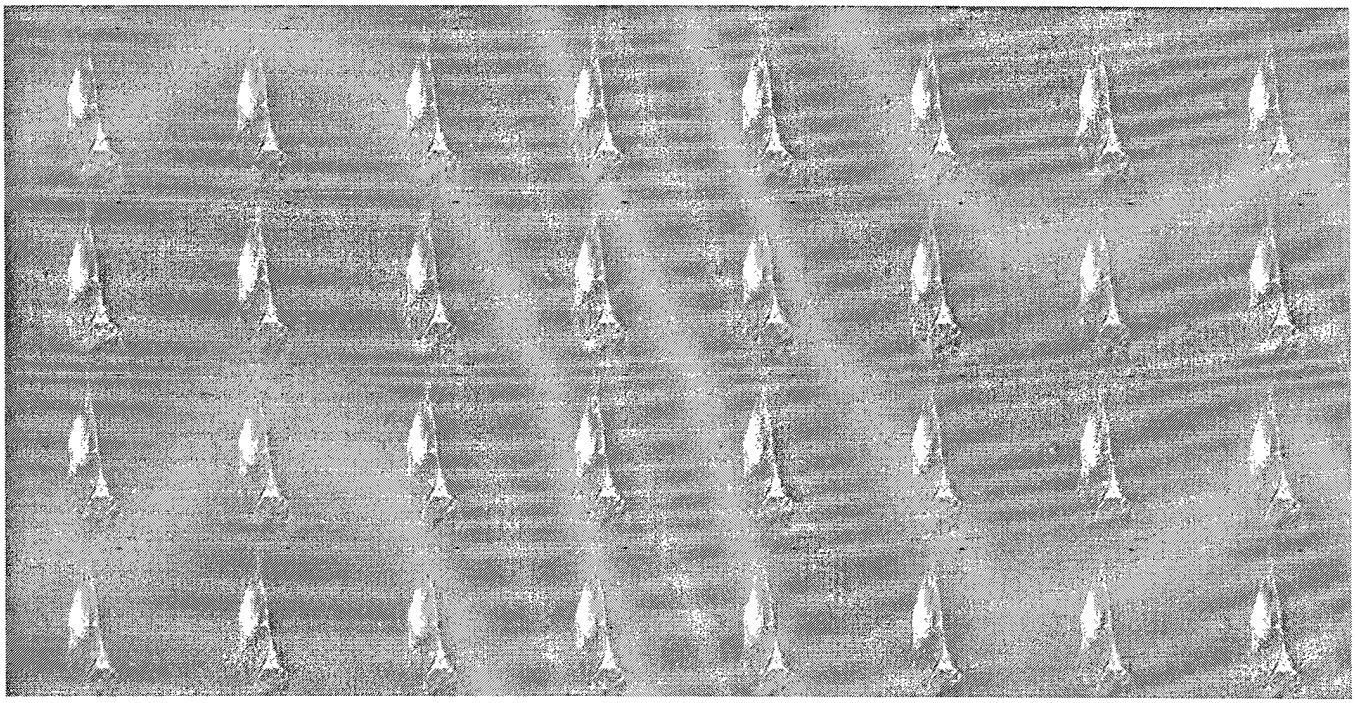
First Injection:



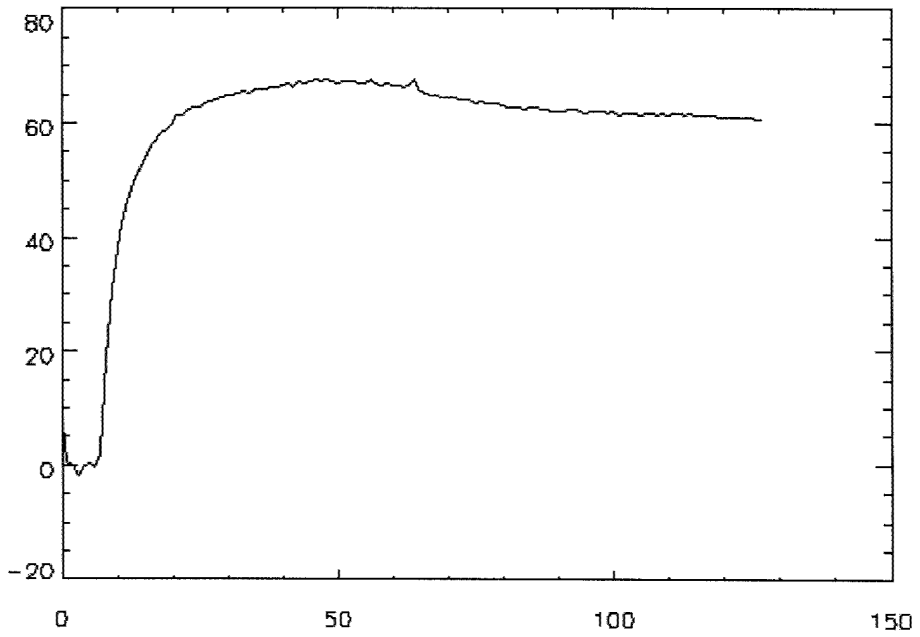


Second half of 1st contrast injection:

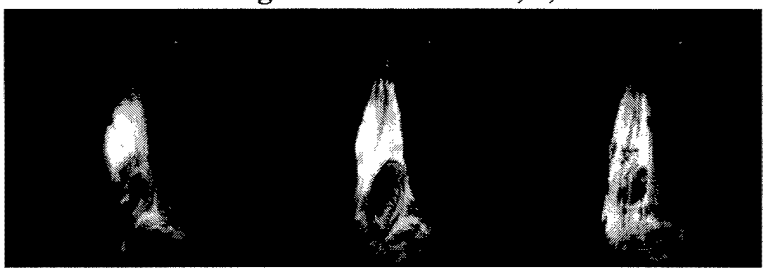




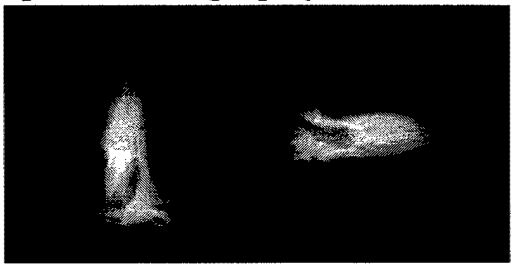
Contrast uptaken curve: 100.0*total(n^{th}_image-control)/control as a function of time



Gradient echo images: slice offset = -2, 0, 2

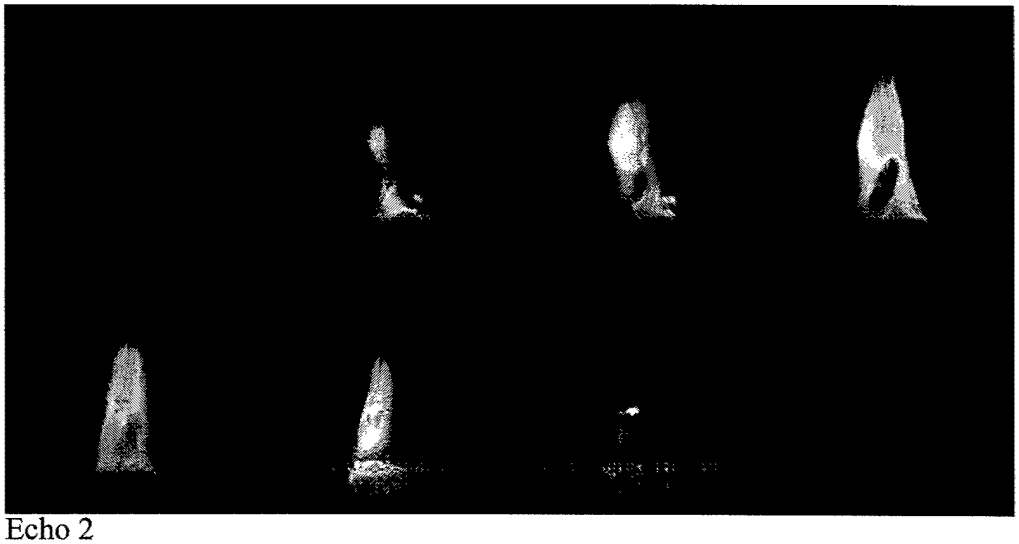


Spin-Echo Images projection: Left z-direction; Right y-direction



Spin-Echo Images: Slo=-6, -4, -2, 0, 2, 4, 6; te=15ms (For 3D reconstruction)

Echo 1



Echo 2