In vivo detection of single cells by MRI

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Introduction: Non-invasive MR imaging of single cells in live animals would have an enormous impact in all fields involved in cell transplantation, early detection of cell homing, and monitoring cell migration. There has been increasing emphasis on improving the relaxivities and delivery of MRI contrast agents, with one goal being *in vivo* single cell detection. Cell labeling contrast agents are primarily composed of iron oxide or gadolinium, either as coated particles, dendrimer based structures, or simple chelates. Some strategies for cellular uptake of contrast agents in culture include the use of very large particles[1,2], transfection agents[3], receptor-mediated delivery[4], and conjugated cellular translocation signal peptides[5]. To date, single cells have been imaged in culture using MRI[6] with a recent extension to visualizing single cells and single particles in *in vitro* embryos[7].

Here it is demonstrated that single cell detection using MRI can be accomplished in a mouse, *in vivo*. Primary mouse hepatocytes were double labeled with 1.63 micron diameter, iron oxide particles (MPIOs) and fluorescent cell tracker agents and transplanted into the spleens of recipient mice. This is a well developed experimental model for hepatocyte transplantation[8], where cells migrate out of the spleen to the liver and engraft as single cells[9]. One month later, animals underwent MRI investigations, followed by harvesting of the tissue for histological analysis. MRI detected isolated, punctuated contrast in the livers of animals that received live, labeled hepatocytes. Histological analysis of the tissue revealed the presence of isolated, transplanted cells in the livers of hosts. Control experiments with dead cells and free particles did not produce this contrast or histology pattern. Measured sizes of the contrast regions in the livers, both *in vivo* and *in vitro*, and contrast regions in a single cell hepatocyte phantom were identical. Correlation of the histology to the MRI demonstrated that the contrast in the MRI was due to transplanted single cells.

Materials and Methods: Primary mouse hepatocytes were isolated and cultured using standard methods. Hepatocytes were labeled with 1.63 micron diameter MPIO's by overnight incubation of confluent cell culture dishes with $3x10^8$ particles. These MPIO's are polystyrene/divinylbenzene polymer coated iron oxide cores with a fluorescein derivative impregnated into the shell. Free particles were removed by rigorous washing followed by Ficoll-Paque density centrifugation. Host mice were injected in the spleen with one of the following: 10^6 labeled cells (n=12), 10^6 dead labeled cells (n=3), $3x10^7$ free particles (n=3), 10^6 labeled mouse embryonic fibroblasts (n=3) or without injection at all (n=3). One mouse received $3x10^7$ particles intravenously. At one month post transplant, mice underwent MRI examination. T_2^* weighted multi-slice gradient echo imaging was performed using a transmit volume coil in conjunction with a 35 mm surface receive only coil. Images were acquired at 100x100x300 micron resolution with 100 ms TR, 4 ms TE, FOV = 4x4 cm, 4 - 6 slices, with respiratory gating. Mice were then perfused, fixed with 4% paraformaldehyde, and the livers were removed and placed in saline. 3D gradient echo MRI was then performed (TE = 4 ms, TR = 100 ms) at 100 microns isotropic resolution. Livers and spleens were then sectioned for histology. Sections were either stained for iron with Prussian Blue staining or left unstained. Dual channel fluorescence confocal microscopy, was used to identify transplanted cells by observing both the fluorescent green MPIOs and the red cell tracker dye on unstained sections. A labeled hepatocyte agarose phantom was also constructed and imaged identically to the in vivo and in vitro livers. The sizes of the dark contrast spots in the livers were measured and compared to the sizes of the contrast regions measured in the cell phantom.

Results: Fig. 1 is a confocal fluorescent image of a labeled cell pre-transplant, displaying both green fluorescence from the MPIOs and red fluorescence from the cell tracker. More than 50 MPIOs can be counted in most cells, surrounding the nuclei in nearly every case. As each MPIO contains 1.1 pg of iron, cells were labeled with >50 pg of iron, consistent with previous reports of cellular labeling of hepatocytes with 1.63 micron MPIOs[2]. Labeling efficiency was >90% with viability >95% as assessed with Trypan blue. Fig. 2 shows in vivo MRI evaluation of A) a mouse that received live labeled cells into the spleen, and B) a control, uninjected mouse one month post hepatocyte transplantation. The experimental livers showed scattered dark, punctuated contrast, while livers from uninjected mice showed no susceptibility induced dark contrast. Livers from the other control experiments all showed grainy contrast, suggestive of isolated, single MPIOs. 9 of 12 mice that underwent labeled hepatocyte injection displayed dark, punctuated contrast regions in the liver; all 3 uninjected mice were negative for contrast and all controls were negative for punctuated dark contrast regions. Fig. 3 shows microscopic analysis of histological sections for the liver in Fig. 2A. Fig. 3A shows Prussian Blue iron staining within a grafted cell, again surrounding the nucleus as in Figure 1. Fig. 3B is a dual channel fluorescence confocal image showing co-localized green fluorescent MPIOs and red fluorescent cell tracker in the cell in the same liver. This can only happen if a transplanted cell grafts to and survives in the liver the full one month duration of the experiment. Histological analysis of control livers showed only scattered, isolated MPIOs, with no co-localized red and green fluorescence. Lastly, comparisons of the sizes of the contrast regions in the liver samples with a labeled hepatocyte phantom were identical. These sizes were 7.50 +/- 1.18 and 7.34 +/- 0.78 pixels for the in vitro liver and the phantom, respectively, at 100x100x300 microns image resolution, and 6.30 +/- 0.95 and 6.40 +/- 1.10 pixels for the in vivo liver and the phantom, respectively, at 100 microns isotropic image resolution.

Discussion: This work demonstrates the first report of single cell detection in an animal, *in vivo*, by MRI, following cell labeling and transplantation. Due to the difficulty of comparing identical slices of *in vivo* MRI and *in vitro* MRI with histological sections, two lines of evidence are presented to demonstrate that single labeled cells were observed in the livers of animals that received live labeled hepatocytes. The first major line of evidence to prove that the dark contrast regions were due to single cells was the comparison of the sizes and hypointensity of the dark contrast spots observed *in vivo* and *in vitro* in the liver to labeled hepatocytes in an agarose phantom. Near identical sizes of contrast regions were observed in identical imaging conditions. The second major line of evidence was the fluorescence microscopy of the liver histology. The use of a double labeling scheme allowed the identification of grafted, transplanted cells in the liver, as opposed to other endogenous cells of the host that may have picked up the contrast agent following cell death. Only cells that had both green and red fluorescence could have been the originally transplanted cells. Additionally, only cells that had a large number of particles within them could have produced the large contrast regions visible in the livers of animals that received live, labeled cells. This is based on previously measured sizes of contrast regions of well labeled cells versus one or a few particles[2].

Finally, imaging conditions were crucial in detecting single cells *in vivo*. As the contrast mechanism is susceptibility based dephasing of water near the particles, gradient echo MRI is best suited. However, the liver has a short background T_2^*, so lengthy echo times would have resulted in undesirable signal loss from the liver. The echo time used in these experiments was 4 ms, which required high resolution MRI to detect single cells. The image resolution, 100 x 100 x 300 microns, was chosen to be able to detect well labeled cells, but free, single 1.63 micron MPIOs in the liver would contribute little to the contrast in the liver.

References: 1) Hinds KA, et al, *Blood*, **102**:3, 867-72, (2003); 2) Shapiro EM, et al, *MRM* (in press 2004); 3) Frank JA, et al., *Radiology* 228, 480-487 (2003); 4) Moore A, et al,, *Biochim.Biophys.Acta* 1402, 239-249 (1998); 5) Zhao M, et al, *Bioconjug.Chem.* 13, 840-844 (2002); 6) Dodd SJ, et al., *Biophys.J.* 76, 103-109 (1999); 7) Shapiro EM, et al., *PNAS* 101, 10901-6 (2004); 8) Fox IJ, et al, *Am.J.Transplant.* 4 Suppl 6, 7-13 (2004); 9) Ponder KP, et al., *PNAS* 88, 1217-1221 (1991)

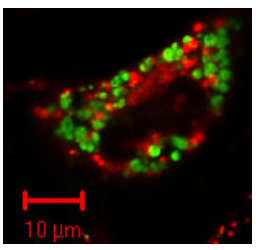

Figure 1

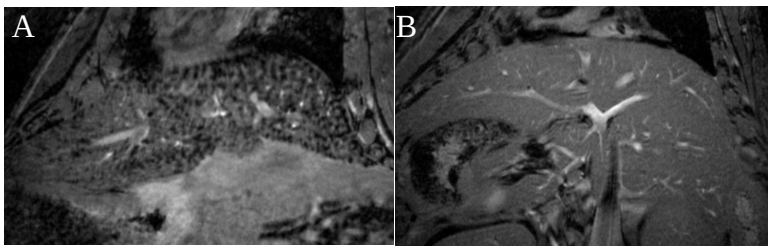

Figure 2

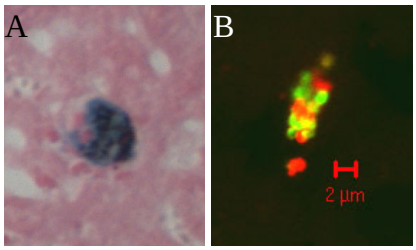

Figure 3