

Video Article

Labeling Stem Cells with Ferumoxytol, an FDA-Approved Iron Oxide Nanoparticle

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Abstract

Stem cell based therapies offer significant potential for the field of regenerative medicine. However, much remains to be understood regarding the *in vivo* kinetics of transplanted cells. A non-invasive method to repetitively monitor transplanted stem cells *in vivo* would allow investigators to directly monitor stem cell transplants and identify successful or unsuccessful engraftment outcomes.

A wide range of stem cells continues to be investigated for countless applications. This protocol focuses on 3 different stem cell populations: human embryonic kidney 293 (HEK293) cells, human mesenchymal stem cells (hMSC) and induced pluripotent stem (iPS) cells. HEK 293 cells are derived from human embryonic kidney cells grown in culture with sheared adenovirus 5 DNA. These cells are widely used in research because they are easily cultured, grow quickly and are easily transfected. hMSCs are found in adult marrow. These cells can be replicated as undifferentiated cells while maintaining multipotency or the potential to differentiate into a limited number of cell fates. hMSCs can differentiate to lineages of mesenchymal tissues, including osteoblasts, adipocytes, chondrocytes, tendon, muscle, and marrow stroma. iPS cells are genetically reprogrammed adult cells that have been modified to express genes and factors similar to defining properties of embryonic stem cells. These cells are pluripotent meaning they have the capacity to differentiate into all cell lineages¹. Both hMSCs and iPS cells have demonstrated tissue regenerative capacity *in-vivo*.

Magnetic resonance (MR) imaging together with the use of superparamagnetic iron oxide (SPIO) nanoparticle cell labels have proven effective for *in vivo* tracking of stem cells due to the near microscopic anatomical resolution, a longer blood half-life that permits longitudinal imaging and the high sensitivity for cell detection provided by MR imaging of SPIO nanoparticles²⁻⁴. In addition, MR imaging with the use of SPIOs is clinically translatable. SPIOs are composed of an iron oxide core with a dextran, carboxydextran or starch surface coat that serves to contain the bioreactive iron core from plasma components. These agents create local magnetic field inhomogeneities that lead to a decreased signal on T2-weighted MR images⁵. Unfortunately, SPIOs are no longer being manufactured. Second generation, ultrasmall SPIOs (USPIO), however, offer a viable alternative. Ferumoxytol (Feraheme™) is one USPIO composed of a non-stoichiometric magnetite core surrounded by a polyglucose sorbitol carboxymethylether coat. The colloidal, particle size of ferumoxytol is 17-30 nm as determined by light scattering. The molecular weight is 750 kDa, and the relaxivity constant at 2T MRI field is 58.609 mM⁻¹ sec⁻¹ strength⁴. Ferumoxytol was recently FDA-approved as an iron supplement for treatment of iron deficiency in patients with renal failure⁶. Our group has applied this agent in an "off label" use for cell labeling applications. Our technique demonstrates efficient labeling of stem cells with ferumoxytol that leads to significant MR signal effects of labeled cells on MR images. This technique may be applied for non-invasive monitoring of stem cell therapies in pre-clinical and clinical settings.

Video Link

The video component of this article can be found at <http://www.jove.com/video/3482/>

Protocol

1. Day 1

1) Plate cells

1. Plate hMSC in a T75 flask at a confluency of 80% at least 18-24 hours prior to labeling. Refer to Table 1 for instruction for alternate vessels.

2. Day 2

2) Prepare labeling solution. This preparation will label one (1) T75 flask at 80% confluency with a concentration of 400 µg Fe/ml. Refer to Table 1 for instruction for alternate vessels.

1. Create a solution (solution 1) by mixing 1 ml of serum-free media and 93.1 µl of ferumoxytol (stock: 30 mg/ml) in a 15 ml conical tube.
2. Create a second solution (solution 2) by mixing 1 ml of serum-free media and 7 µl of protamine sulfate (stock: 10 mg/ml) in a second 15 ml conical tube.

3. Allow each solution to rest for 5 minutes.
4. Add solutions 1 and 2 together. Gently mix the new labeling solution. Allow the solution to rest for 5 minutes to permit USPIO-protamine sulfate complexes to form.
5. Add 5 ml of serum-free media to the mixed 2 ml of SPIO/protamine sulfate solution for a final volume of 7 ml.

3) Prepare cells for labeling

1. Aspirate the media from cells.
2. Gently wash cells once with 2-3 ml of pre-warmed Mg/Ca-free D-PBS or serum-free media to rinse away residual serum proteins and or other media components that could impair contrast agent uptake and labeling efficiency. Aspirate out the rinse fluid.

4) Label cells

1. Add the complete 7 ml of labeling solution to cells.
2. Place cells into an incubator (37°C/ 5% CO₂) and allow the cells to incubate in the labeling solution for 4 hours.
3. Add 700 µl of FCS to the labeling solution of the cells to achieve a final serum concentration of 10%. Serum is added to re-establish the enriched environment of which the cells are accustomed, and to assist in minimizing cell death that could result from an abrupt shift to 24 hour exposure to a serum-free environment.
4. Allow the cells to incubate with the labeling solution for an additional 20 hours.

3. Day 3

5) Prepare labeled cells

1. Aspirate the labeling solution from cells.
2. Gently rinse cells with 2-3 ml of pre-warmed Mg/Ca-free D-PBS. Aspirate out the PBS. It is important to use Mg/Ca-free PBS as the Mg and Ca will impair the efficiency of Trypsin in the next step.
3. Add 2 ml of pre-warmed 0.05% Trypsin to the cells and tilt the flask back and forth to ensure the entire surface of the flask is covered with a thin layer of Trypsin. Place the cells in an incubator (37°C/ 5% CO₂) and allow the cells to rest for 5-7 minutes or until cells begin to detach from the plate. It may be necessary to confirm detachment under a microscope. Gently tap the sides of the flask if necessary to facilitate cell-surface detachment.
4. Add 4 ml of pre-warmed complete media to the flask to neutralize the Trypsin. Gently rinse the flask by pipetting the Trypsin/media/cell solution up and down several times. Collect the entire solution in a 15 ml conical tube. Centrifuge the cell solution at 400 RCF for 5 minutes.
5. Carefully aspirate the supernatant without disturbing the cell pellet. Resuspend the cells in 5 ml of media (serum containing or serum-free). Centrifuge the cell solution at 400 RCF for 5 minutes.
6. Repeat the cell wash described in step 5.5. In total, cells will be rinsed three times to remove residual, free contrast agent in media and on the surface of cells.
7. Count cells at this point to attain the most accurate cell count, as cells will be lost during previous washing. Perform a viability test of cells. Cells are now ready for subsequent analysis and or experimental application. MR imaging can be performed with T1-w and T2-w parameters, because although ferumoxytol is a T2-w contrast agent, ferumoxytol also exhibits T1 effects. Representative T2-w MRI parameters that can be used to image ferumoxytol labeled cells include: FSE or SE_Multislice sequences with a TR of 2000-2500 ms and a TE of 60-80 ms. To acquire a T1-w image, decrease the TE value on a FSE or SE_Multislice sequence or use a GRE sequence with a high TR and low TE. Figure 4 demonstrates a potential *in vivo* application for labeled stem cell imaging.

4. Representative Results:

Labeled cells demonstrate a significant darkening or negative contrast effect on T2-weighted MR images and a brightening or positive contrast effect on T1-weighted MR images (Figure 2). Longitudinal MR imaging and viability tests performed 5 days post labeling demonstrated no significant MR signal and no significant impact on viability compared to unlabeled controls (data not shown).

Labeled stem cells can subsequently be differentiated into various cell types or intravenously injected for *in vivo* investigation.

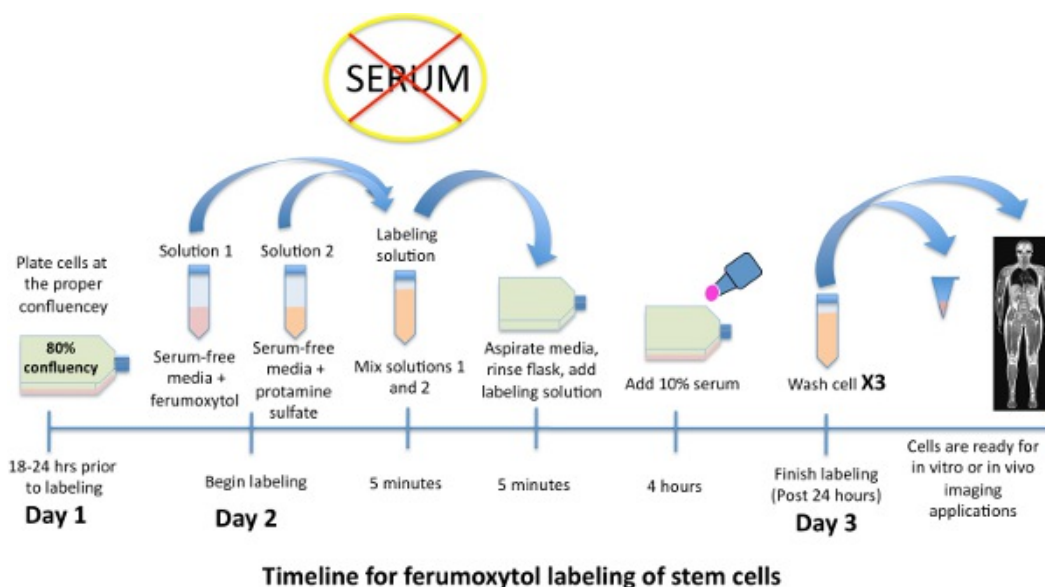


Figure 1. Schematic time line for labeling stem cells with iron oxide nanoparticle, ferumoxytol.

MR depictions of unlabeled and labeled cells

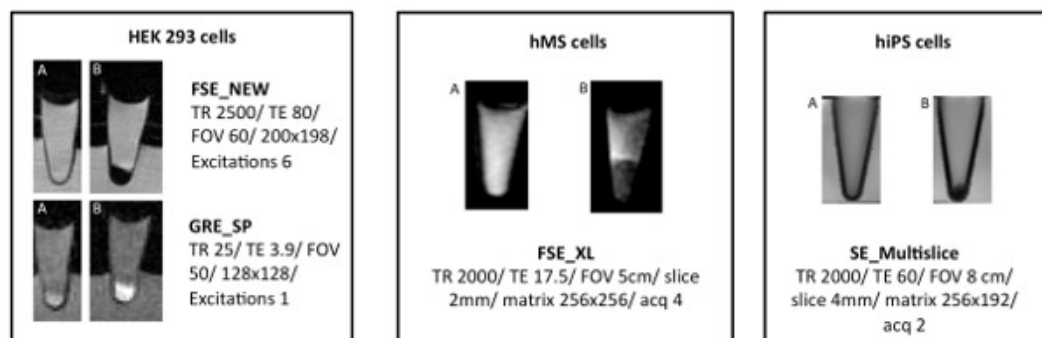


Figure 2. MR images of sagittal cross sections through eppendorf tubes with cells in a cell pellet. A) Unlabeled controls. B) Labeled cells demonstrating a significant T2 or negative contrast agent effect on T2-weighted FSE or SE_Multislice imaging and a T1 or positive contrast agent effect on the T1-weighted GRE sequence.

Detection limit of USPIO labeled cells

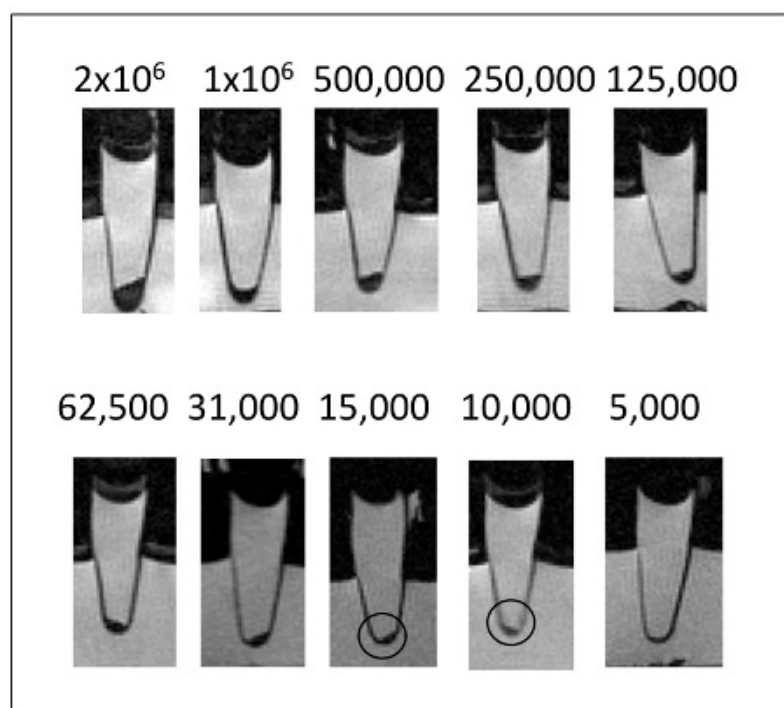


Figure 3. Sagittal cross sections of eppendorf tubes with cells in cell pellets. As little as 10,000 ferumoxytol labeled cells can be detected via T2-w MR imaging.

Potential application for USPIO-labeled stem cells: murine, cerebral injection

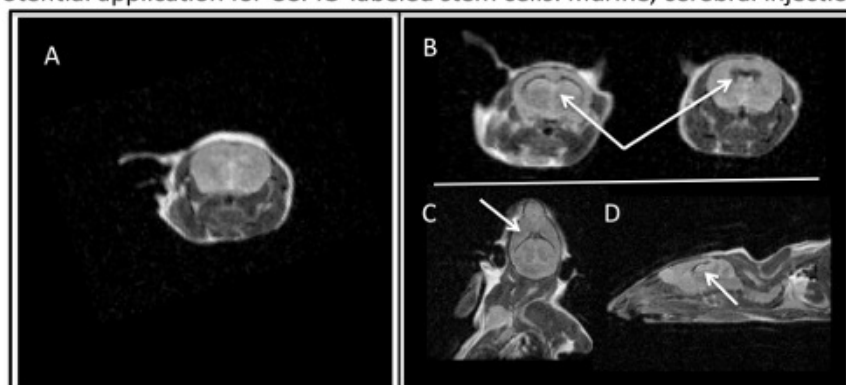


Figure 4. MR visualization of ferumoxytol-labeled stem cells injected into murine cerebral ventricles. A) Axial, MR image of murine brain with no injection of USPIO-labeled stem cells. B) Axial, C) coronal, and D) sagittal MR images depicting the T2, negative contrast effect of USPIO-labeled stem cells (white arrows) injected in a murine brain.

Labeling adaption for alternate vessels

Flask/ Dish Size	Volume to use	Estimate of cell count at confluency	Protamine Sulfate to use	Ferumoxytol to use
6 well plate	2 ml	1 million	2 μ l	26.6 μ l
T-25	2.5 ml	2.5 million	2.5 μ l	33.25 μ l
T-75	7 ml	8 million	7 μ l	93.1 μ l
T-175	14 ml	17 million ¹	14 μ l	186.2 μ l

Table 1. Quantity adaptations for labeling cells in alternate vessels.

Discussion

Improving the efficacy of stem cell engraftments is critical for the advancement of regenerative medicine. A non-invasive visualization technique for stem cells *in vivo* significantly enhances our ability to understand mechanisms that lead to successful engraftment outcomes. Magnetic labeling for MR visualization, such as the procedure we have demonstrated, allows *in vivo* tracking of stem cells with MR imaging. Magnetically labeled stem cells have previously been transplanted into target tissues and have been visualized up to weeks with MR imaging⁷. Long-term labeling and longitudinal imaging is possible due to the lysosomal storage of the iron oxide nanoparticles within cells⁸. Challenges, however, do correspond with long term monitoring of iron labeled cells. One challenge associated with longitudinal imaging of iron labeled cells is that as healthy, viable cells proliferate the contrast agent is distributed to daughter cells. Distribution of the contrast agent to daughter cells is uneven and results in a marked dilution effect of the contrast agent over time. A second challenge associated with long term monitoring of iron labeled cells is differentiating between originally labeled, viable cells from resident phagocytes, such as macrophages, that may have phagocytosed released contrast agent from originally labeled, dead cells⁴. Our group has identified differences in long-term signal kinetics of viable and apoptotic stem cell transplants, which had been labeled with iron oxide nanoparticles other than ferumoxytol⁹⁻¹¹. Future studies are required to determine whether these principles also apply to ferumoxytol-labeled cell transplants.

While in this study the potential effects ferumoxytol labeling could exhibit on the differentiation capacity of stem cells was not examined, previous studies have demonstrated an SPIO dose dependent effect on differentiation capacity and SPIO doses that do not impair differentiation potential, suggesting, a ferumoxytol dose that does not impair the differentiation capacity of stem cells can also be determined^{12, 13}.

Large SPIOs (hydrodynamic diameter > 50 nm) have been applied previously for cell tracking purposes following stem cell labeling via various labeling techniques including simple incubation and transfection with electroporation or transfection agents such as protamine, lipofectin and poly-L-lysine (PLL)^{3, 13-16}. Although not always necessary for cell labeling with SPIOs, transfection methods for cell labeling have proven beneficial for facilitating labeling and increasing labeling efficiency¹⁵. SPIOs which have been used for cell labeling, however, are no longer being produced for commercial reasons. Ultrasmall SPIO (USPIO, hydrodynamic diameter < 50 nm) such as ferumoxytol, on the other hand are still being produced but require the aid of a transfection agent to achieve efficient cellular uptake⁸. Protamine sulfate is a clinically applicable transfection agent that forms complexes with USPIOs to facilitate phagocytic uptake of the contrast agent by stem cells¹⁷. The combination of FDA-approved protamine sulfate with the FDA-approved USPIO, ferumoxytol, provides the potential for clinical translation of stem cell tracking techniques via off-label use of this agent. Of note, ferumoxytol has demonstrated an excellent safety profile in clinical trials¹⁸. Direct visualization of the transplanted cells via a technique such as the one demonstrated would allow for a better understanding of the factors that promote or impair successful stem cell transplantations and help to identify the most promising techniques for clinical applications. With the use of clinically applicable cell markers and imaging equipment, this imaging technique is in principle, readily accessible to patients who undergo stem cell transplants.

Disclosures

All contributors to this study offer no disclosures.

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