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MR Imaging of Transplanted Stem Cells in Myocardial Infarction

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Abstract

Recently, several protocols for labeling of stem cells with superparamagnetic iron oxides (SPIOs) have been developed, leading to an active and growing field aimed at visualizing stem cells using MRI (magnetic resonance imaging), including image-guided stem cell injections. This development occurred simultaneously with a significant rise in the number of cell therapy clinical trials for cardiovascular applications and their preceding pre-clinical studies in animal models. In this chapter, we will describe several labeling strategies that can be used to label cells with SPIO nanoparticles. This is followed by a discussion of current strategies for using MRI to visualize these cells in myocardial infarct.

Keywords

Magnetic resonance imaging (MRI); stem cells; superparamagnetic iron oxide (SPIO); cellular labeling; cellular imaging; myocardial infarct

1. Introduction

Magnetic resonance imaging (MRI) is an ideal technique for precise MR-guided delivery of cells followed by monitoring of their trafficking within the body. On the one hand, MRI offers the interactivity of X-ray interventional techniques without exposing the patient or cells to ionizing radiation. On the other hand, the high spatial resolution and exquisite soft tissue detail of MRI are superior to X-ray cardiac interventional methods, which can only provide information about the lumen of the heart or vessels in combination with iodinated contrast agents. Moreover, MRI allows non-invasive, serial imaging for dynamic tracking of cell migration and engraftment (1–12).

There are many magnetic labeling methods to detect cells with MRI. Pre-labeling with superparamagnetic iron oxide (SPIO) contrast agents is currently the most widely used method (13–17). SPIO-labeling methods are relatively simple, fast, and inexpensive. Among the different MR contrast agents that are available, SPIO particles offer currently the highest sensitivity (18). Several clinically approved formulations of SPIO-based contrast agents are available that have been used for cell labeling in a variety of diseases. The toxicity of the magnetic nanoparticles is low, since they are composed of biocompatible iron which can be recycled using endogenous iron metabolic pathways. Compared to gadolinium-based contrast agents, SPIOs become more effective upon cell internalization due to particle clustering and, thereby, create large “blooming” hypointensities on standard clinical MRI scanners (18). While SPIOs are not internalized by non-phagocytic cells without further modification, simple methods to induce internalization and uptake have been developed and tested in a variety of stem cells. One of the most commonly used method is “magnetofection” – a method where transfection agents (TAs) are complexed with SPIOs to

provide the formation of SPIO oligomers with a highly positive surface charge (15), which induces macropinocytosis (endocytosis) of the SPIO-TA complexes (13,14). Concentrations of 2–20 pg iron/cell can be easily achieved after 24–48 h incubation in vitro (13). After magnetic labeling of stem cells, SPIOs are stably maintained in endosomes enabling the imaging of stem cells for several months after delivery to the heart (3,5,9,18).

Magnetoelectroporation (MEP) is another method of SPIO cellular labeling. MEP uses small pulsed voltages to induce intra-cellular uptake of SPIOs (16,17,19). No transfection agents are needed, which may aid in more rapid clinical translation. In addition, millions of cells can be labeled in seconds using MEP, which may be important in certain cell lines that are altered by culturing in vivo due to adhesion to tissue culture plastics leading to morphological changes. Furthermore, for cardiac cellular delivery, MEP may prove to be the method of choice where cellular delivery cannot be delayed by 24–48 h after an acute cardiac event, in particular when using off-the-shelf frozen stem cells within hours after a patient would be brought in.

Both magnetofection and MEP can be used to label cells with a variety of contrast agents, including manganese oxide nanoparticles (19). A recent study performing a head-to-head comparison of magnetofection and MEP demonstrated preserved cell viability and proliferation in embryonic stem cells by both techniques (20). However, cardiac differentiation of embryonic stem cells was most attenuated by MEP and iron uptake was greatest with magnetofection (20). This study has shown the importance of carefully selecting the magnitude and form of the applied electrical pulses. Detailed methods using these techniques to label stem cells for cardiovascular applications using SPIO contrast agents are given below.

2. Materials

2.1. Cell Culture and Preparation

1. Stem cell media, suitable for specific cell type, e.g., MEM alpha supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic containing penicillin, streptomycin, and amphotericin B for mesenchymal stem cells.
2. Phosphate buffered saline (PBS), 10 mM phosphate, 0.9% NaCl, pH = 7.4.
3. Trypsin (0.5 g/l) with ethylenediaminetetraacetic acid (EDTA 0.2 g/l), prewarmed to 37°C in a water bath before use. Long-term storage of trypsin should be at −80°C.

2.2. Labeling with Transfection Agents

Transfection agents (TAs) are highly charged molecules that will form complexes with iron oxide particles through electrostatic interactions. There are several classes of these agents, but the most convenient and most commonly utilized labeling methods are those based on commercially available TAs that are polycations, including dendrimers, such as Superfect®, poly-L-lysine (PLL), Lipofectamin®, and FUGENE®. SPIOs are used in conjunction with TAs to label cells and can be distinguished primarily based on the size of the nanoparticles. For brevity, we list several formulations that are approved or under development by major pharmaceutical companies.

1. Iron oxide contrast agents:
    a. Commercially available ferumoxides are Feridex® (Berlex Laboratories Inc., Wayne, NJ, USA) or Endorem™ (Guerbet SA, Paris, France). Ferumoxide stock solution contains 11.2 mg Fe/ml with particles

approximately 80–150 nm in diameter (21). Ferumoxide stock solution should be stored at 4°C, and should not be frozen. Feridex® is an FDA-approved liver contrast agent since 1996. In Europe, this compound is registered under name Endorem; both are identical. Both agents contain a dextran coating as a stabilizer.

b. Ferucarbotran (Resovist, Bayer Schering Pharma AG, Berlin, Germany) is an SPIO composed of nanoparticles coated with carboxydextran. It is currently used for the detection and characterization of focal liver tumor lesions and approved for clinical use in the European, Australian, and Japanese markets (*see* Note 1), as well as labeling cells.

c. Ferumoxtran (Sinerem™, Guerbet SA, Paris, France, or Combidex®, AMAG Pharmaceuticals Inc., Cambridge, MA, USA) is a member of the ultrasmall superparamagnetic iron oxide (USPIO) class of contrast agents with a median diameter <50 nm. Due to the smaller diameter, these particles will not be taken up by the reticuloendothelial system as quickly as SPIOs when injected intravenously. Thus, they tend to accumulate in lymph nodes and are used to distinguish normal from metastatic nodes (*see* Note 2). The particles are much less magnetic than SPIOs and also complex less well with TAs and have therefore not been widely used for magnetic labeling.

2. Transfection agents:

a. Poly-L-lysine (PLL, Sigma, St Louis, MO, USA) as a hydrobromide with a molecular weight of 388,000 Daltons is the most commonly used TA. A stock solution of PLL in sterile water at a concentration of 1.5 mg/ml should be stored in −20°C.

b. Protamine sulfate (American Pharmaceuticals Partner, Schaumburg, IL, USA), which is a drug used clinically to reverse the effects of heparin therapy, is another commonly used TA. It is available in bottles at a concentration of 10 mg/ml, and stored at 4°C.

Both transfection agents may be used (*see* Note 3).

2.3. Magnetoelectro - poration

1. Ferumoxide stock solution. (*See* **Section 2.2**.)

2. Electroporation cuvettes, 0.4 mm gap (Gene Pulser BioRad, Hercules, CA). It is important to deliver electrical pulses with the proper field strength and duration. The exact pulse delivery will be dependent on the type of cells that will be labeled. Mammalian cells typically require field strengths up to 6.15 kV/cm, which can be obtained using a 0.4-cm cuvette.

3. BTX electroporation system (Harvard Apparatus, Holliston, MA).

4. Culture media and 10 mM PBS as in **Section 2.1**.

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[1]Instead of using complete medium, PLL-Feridex® complex formation may also be carried out in serum-free medium.
[2]The amount of TA should be carefully titrated and optimized for each cell type. The suggested concentration of 375 ng/ml is only a guideline; this amount has been found to provide sufficient SPIO endocytosis without affecting cell proliferation or differentiation for most cell types.
[3]Either PLL or protamine sulfate works well for cell labeling. For future clinical applications, protamine sulfate may be preferred as it is already clinically used as an anti-heparin product; however, in our experience, PLL labels most cells more effectively than protamine sulfate.

2.4. Magnetic Resonance Imaging

1. Clinical MRI scanner equipped with surface coils to image the heart.
2. MRI-compatible ECG leads and monitoring equipment.

3. Methods

3.1. Cell Culture and Preparation

1. Prior to labeling, the cells must be prepared in a clean, sterile environment. The cells can be frozen and thawed immediately prior to labeling. To obtain a high fraction of viable cells, they can be cultured overnight following the removal of dead cells.
2. When stem cells approach confluency in a culture dish/flask, remove the old media and wash the monolayer once or twice with PBS.
3. Remove PBS with a pipette and add a minimal volume (~1 ml for a T-75 flask) of prewarmed trypsin.
4. Incubate the cells in trypsin for at 37°C in humidified, enriched in 5% CO_2 air, then check microscopically to determine whether the monolayer of cells is lifting off the culture dish.
5. When single cell suspension has been obtained, add ~10 ml of complete media and transfer the cell suspension to a sterile 15-ml conical tube and spin the cells on tabletop centrifuge (~ 600×*g* for 10 min).
6. Discard the supernatant carefully, so as not to disturb the cell pellet and resuspend the cells in complete media for counting.
7. Once the number and concentration of cells has been determined, reseed the cells. For example, MSCs typically will be reseeded into a fresh T-75 flask at a concentration of ~2×10^5 cells/ml.

3.2. Complexing of SPIO and Labeling Using Transfection Agents

Each combination of TA and (U)SPIOs should be carefully titrated and optimized, since too low concentrations may not lead to sufficient cellular uptake, whereas too high concentrations may induce precipitates or may be cytotoxic. The examples below are given for PLL and protamine sulfate (21). Figure 10.1 shows a representative example of intracellular labeling with Feridex® -PLL for a variety of cell types.

3.2.1. Poly-L-lysine

1. Use sterile culture medium, specific for the cell type being used, and add 2.2 μl Feridex® stock (11.2 mg Fe/ml, Berlex Laboratories) per ml of medium in order to prepare a medium solution containing 25 μg Fe/ml. Mix well (*see* Notes 1–4).
2. Add transfection agent to the Feridex® -medium at the appropriate concentration, i.e. 375 ng/ml (250 nl/ml medium using the 1.5 mg/ml stock). Mix well (*see* Notes 2 and 4).
3. Incubate the PLL-Feridex® medium for 60 min at room temperature using a rotating shaker. This allows the formation of PLL-Feridex® complexes through electrostatic interactions.

[4]It is mandatory to first add the Feridex® to the medium and mix very well before adding the PLL or other TA. If this is not done, formation of large TA-SPIO aggregates and precipitation will occur.

4. For adherent cells, remove the old medium, and add the PLL-Feridex® containing medium. For floating cells, spin the cells down at 400×*g* and resuspend the pellet in the PLL-Feridex® medium. For cells that are very sensitive to (autocrine) growth factors and supplements in the medium, spin the cells down at 400×*g*, resuspend the cells in 50% old medium, and add 50% PLL-Feridex® medium containing 50 μg Fe/ml Feridex® and 750 ng/ml PLL.
5. Incubate cells for 24–48 h. Shorter incubation times will induce less uptake.

3.2.2. Protamine Sulfate

1. Dilute protamine sulfate in distilled water to a concentration of 1 mg/ml just before initiating labeling.
2. Combine (U)SPIOs with appropriate serum-free media based on the cell type to obtain a concentration of 100 μg Fe/ml. For example, add 9 μl of ferumoxide formulation per every 1 ml of media for MSCs.
3. Add protamine sulfate to the (U)SPIO solution to obtain a concentration of 4.5–6 μg/ml (22–24) and mix the solution for 5–10 min.
4. After 5–10 min, add an equal volume of standard cell culture media with a double concentration of serum to create a final ferumoxide concentration of 50 μg/ml.
5. Replace old media in cell culture with newly created media with (U)SPIO–protamine sulfate complexes and incubate with cells overnight.
6. After overnight incubation, remove the media containing (U)SPIO-protamine sulfate complexes, rinse the cells with warm PBS, trypsinize, and collect for counting and administration.

3.3. Magnetoelectro- poration

1. Remove media and wash cells with PBS.
2. Trypsinize and count cells. After counting, spin the cells using a tabletop centrifuge (~600×*g* for 10 min for MSCs) and wash with PBS.
3. Resuspend the cells in 10 mM sterile PBS at a density of 1–5 × 10^6 cells/ml (*see* Note 5) and transfer to sterile electroporation cuvette(s). While cell suspensions <1 ml/cuvette may be used, care must be taken to ensure that the cuvettes are entirely filled with the cell suspension. For example, using the BTX apparatus and 0.4-mm gap electroporation cuvettes, the total volume of cell suspension mixed with (U)SPIOs cannot be smaller than 700 μl.
4. Add (U)SPIOs to obtain a final concentration (after mixing with cell suspension) of 2 mg Fe/ml. For example, mix 130 μl of ferumoxides with 600 μl of cell suspension to obtain a final cuvette volume equal to 730 μl (*see* Note 6).
5. Using the BTX electroporation system (*see*Fig. 10.2), electroporate cells using the following conditions: 50 V pulse strength; 5 ms pulse duration; and 20 pulses in intervals of 100 ms.

[5]If cell density per cuvette exceeds 5 × 10 (6), cell clumping can occur during MEP. Thus, using less than 2 × 10 (6) cells per cuvette is recommended to avoid cell clumping (16).

[6]Iron uptake will be determined in part by of the type of cells. Cells with more cytoplasmic volume can be labeled with a larger amount of SPIOs. In addition, increasing the SPIO concentration can enhance intracellular iron uptake during MEP. Walczak et al. have demonstrated a correlation between concentrations ranging from 0.25 to 2 mg Fe/mL and cellular iron uptake (16).

6. Leave the cuvettes in the holder for 1 min, transfer to ice, and let them to rest for 5 min to allow for membrane recovery.
7. Remove the small top layer of foam and transfer cells to 50-ml conical tube containing culture media. Leave the tube with cells on ice for at least 15–20 min.
8. Wash the cells twice with PBS. Spin the cells in media using a tabletop centrifuge (~600×*g* for 10 min).
9. Remove the supernatant, resuspend MSCs in fresh, sterile 10 mM PBS, and spin down again. Repeat Steps 8 and 9 and then proceed to Step 10.
10. Discard the supernatant and resuspend cell pellet in 1 ml (or other desired amount) of PBS. Count the cells and dilute to final concentration for administration.

3.4. Cardiac Magnetic Resonance Imaging

SPIO-labeled stem cells can be delivered several ways including direct visualization during open-chest procedures, intracoro nary administration using conventional angiographic catheters, and transmyocardially using specialized catheters for delivery of therapeutics to the myocardium. High spatial resolution T2*-weighted images will depict the labeled cells as hypointensities (*see*Fig. 10.3). Because these hypointensities can be hard to distinguish from other hypointensities, such as calcified plaque or metallic objects like stents, off-resonance imaging techniques have been developed to portray the magnetic susceptibilities from iron-labeled cells as hyperintense signal (25–29) (*see*Fig. 10.4).

3.4.1. T2*-Weighted Imaging

1. For high-resolution imaging, images are acquired over multiple cardiac cycles using ECG-gating. Motion artifacts from breathing are suppressed using either navigator echo techniques or breath-holding.
2. While T2*-weighting can be obtained using several imaging techniques, gradient echo imaging with an extended echo time (TE) that does not degrade cardiac images appears to provide the best compromise in image quality on clinical scanners (2).
3. Typical gradient echo imaging parameters are 6 ms repetition time (TR); 1.6 ms TE; 20° flip angle; 512 × 512 image matrix; 5–8 mm slice thickness (ST): 32-kHz bandwidth (BW); and 2–4 number of signal averages (NSA). Images are acquired in the standard short or long axis planes to cover the extent of the left ventricle.

3.4.2. Off-Resonance Imaging—There are several types of off-resonance imaging techniques that have been used to image (U)SPIOs. One method uses a spectral excitation in combination with spin echo imaging, which is probably not well-suited for cardiac applications (26). Another method, GRadient echo Acquisition for Superparamagnetic particles/suscePtibility (GRASP), modifies the refocusing pulses to create positive contrast from iron-labeled cells (27,30). Background suppression using this technique is excellent. A third method, Inversion-Recovery with ON-resonant water suppression (IRON), uses frequency-selective prepulses to suppress the water signal leaving positive contrast from iron-labeled cells (28). While not providing as much background suppression as the GRASP method, IRON MRI provides the flexibility to be combined with either spin echo or gradient echo techniques as well as two-dimensional single plane or three-dimensional volume acquisitions (*see*Fig. 10.4). Recently, it has been demonstrated that these off-resonance imaging techniques will not benefit from field strengths > 4.7T (31). Thus, these techniques are ideal for use on currently available clinical scanners.

1. As with T2*-weighted imaging, ECG-gating and respiratory gating or breath holds are used to suspend cardiac and respiratory motion, respectively.
2. Typical imaging parameters for three dimensional fast spin echo IRON imaging at 3T are 2 ms TR; 11.6 ms TE; 24 echo train length (ETL): 11.6 ms interecho spacing; 170 Hz bandwidth water suppression; 95° iron saturation pulse. In the heart, fat suppression is recommended. Depending on the number of (U)SPIO-labeled cells per voxel and the image resolution, the off-resonant positive contrast will appear as hyperintense areas surrounding the cells and with a typical dipole appearance. The volume of the hyperintensities can be measured to determine a relative concentration of labeled cells.

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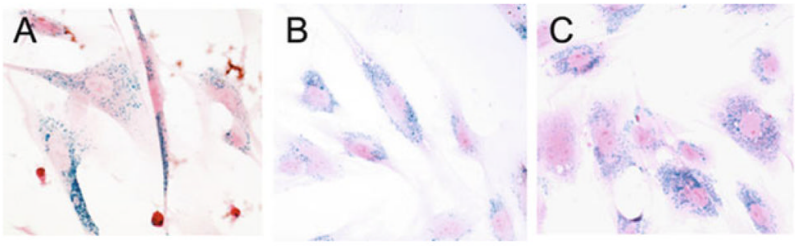

Fig. 10.1.

Labeling of cells using Feridex® -PLL complexes. Cells were labeled for 48 h with 25 μg Fe/ml Feridex and 375 ng/ml PLL. Prussian blue staining of labeled human embryonic germ-derived pluripotent stem cells (**a**), human mesenchymal stem cells (**b**), and swine mesenchymal stem cells (**c**) show an efficient intracellular uptake of particles into endosomes that is non-specific across species.

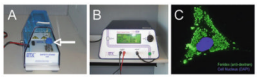

Fig. 10.2.

Instant MR labeling of cells using magnetoelectroporation (MEP). **a** Cells are suspended in a sterile electroporation cuvette (*arrow*), mixed with Feridex®, and placed in a cuvette holder. **b** Using the connected electroporator, cells are mildly permeabilized for 10–20 ms. **c** Magnetic labeling is instantaneous, and Feridex clusters are trapped in the cytoplasm as demonstrated by anti-dextran immunofluorescent staining (*green clusters*).

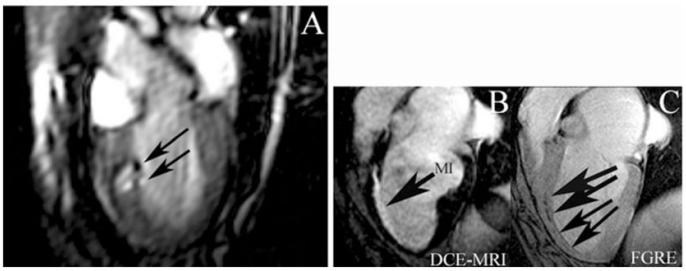

Fig. 10.3.

MR-guided real-time injection of Feridex® -labeled MSCs in a dog myocardial infarct model. **a** Still frame long-axis view from real-time MRI demonstrating Feridex® -labeled MSCs as hypo-enhancing artifacts (*arrows*) after initial two injections at 3 days post-infarction (MI). *Top* lesion is 7×10^6 Feridex® -labeled MSCs; *bottom* lesion is 3×10^6 labeled MSC with 4×10^6 unlabeled MSCs. At 8 weeks after injection, initial two injections (**c**, *upper arrows*) are still visible, as well as additional injections (**c**, *lower arrows*) with as low as 1×10^5 labeled MSCs at initial injection in fast gradient echo images (FGRE). Hypo-enhancing artifacts change from round lesions to linear lesions by 8 weeks and align along the edge of the infarct (hyper-enhancing artifact [MI]) on delayed contrast enhanced (DCE) MRI (**b**). (Adapted from Bulte et al. (5), with permission.)

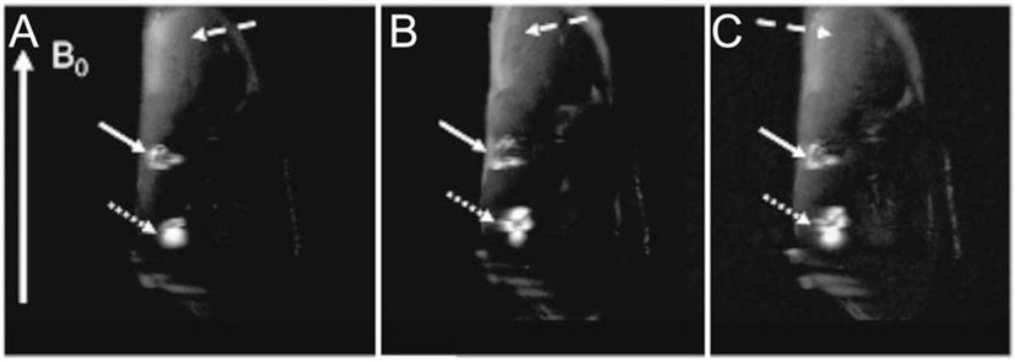

Fig. 10.4.

Positive contrast (IRON) imaging of Feridex® -labeled MSCs transplanted in the hindlimb muscle of an ischemic rabbit. Adjacent double-oblique slices (**a–c**) from an in vivo 3T FSE 3D IRON acquisition obtained in an ischemic rabbit hindlimb with two injection sites of SPIO-labeled MSCs (2.5×10^5 cells at *dotted white arrow* and 1.25×10^5 cells at *solid white arrow*). Note the excellent background suppression that leads to clear visualization of the stem cell injection sites with positive contrast and a larger volume of hyperintense signal for the 250,000-cell injection site. (Adapted from Stuber et al. (28), with permission.)