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## Essential Elements to Consider for MRI Cell Tracking Studies with Iron Oxide-based Labeling Agents

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### Abstract

Personalized diagnosis and treatment with allogenic or autologous cells have been intensively investigated over the past decade. Despite the promising findings in preclinical studies, the clinical results to date have been largely disappointing. Some critical issues remain to be solved, such as how to monitor the migration, homing, survival, and function of the transplanted cells *in vivo*. In the past years, imaging techniques have been introduced to solve these issues based on a concept that cells can be transformed to a cellular imaging agent following labeling of the cells with an imaging agent. For this purpose, magnetic resonance imaging (MRI) is so far the first choice imaging modality and iron oxide-based nanoparticles are the most frequently applied labeling agents. However, most MRI cell tracking studies are currently still limited in *in vivo* visualization of the labeled cells, some critical elements for cell tracking studies are often incompletely characterized, which makes it difficult to validate and meta-analyze the data generated from different studies. Incomplete information on preclinical studies also slows the transition of the findings to clinical practice. A robust protocol of MRI cell tracking studies is apparently critical to deal with these issues. In this review, we first briefly discuss the limitations of MRI cell tracking based on iron oxide nanoparticles and then recommend a minimum set of essential elements that should be considered in MRI cell tracking studies at preclinical stage.

### Keywords

Contrast agents; characterization; cell tracking; magnetic resonance imaging

### Introduction

How to monitor the migration and homing of transplanted cells as well as their engraftment efficiency and functional capability remains a critical issue to be solved in the field of cellular therapy (1, 2). Because magnetic resonance imaging (MRI) offers a good depth penetration and high spatial resolution, and exhibits a superior ability to extract molecular and anatomic information simultaneously, it has been actively investigated in past years and so far the first choice for tracking implanted cells (3, 4). Fundamentally, MRI cell tracking includes three components: labeling agents, labeling of cells of interest, and MRI tracking. The labeling agents are synthesized with procedures similar to those developed for organ imaging, with more attention in their cellular internalization, intracellular retention, and cytotoxicity. Cell labeling can be achieved through three ways: 1) *in vivo* labeling by systemic application of a contrast agent with subsequent phagocytosis of the agent by the cells of interest; 2) *in situ* labeling by injection of a contrast agent into the tissue area of

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interest to label the local cells; and 3) *ex vivo* labeling by incorporation of a contrast agent into a population of purified cells *in vitro* (4, 5). To date, *ex vivo* cell labeling is the approach that has been most frequently applied for cell tracking purpose. With *ex vivo* labeling, excess contrast agents and dead cells can be removed simply; the labeled cells can be thoroughly characterized before transplantation; and non-specific labeling of irrelevant cells can be well controlled by purifying the relevant cell population before labeling (2, 6). Regarding contrast agents, although diverse contrast agents (superparamagnetic, paramagnetic, ferrimagnetic, and ferromagnetic) have been developed, superparamagnetic iron oxide nanoparticles (SPION) are probably the agents that have been most extensively explored so far.

MRI cell tracking studies in animals first started in the early 1990s, however, the first study in humans was performed delayed to 2005 (7–10). In this study, autologous dendritic cells were labeled with SPION and  $^{111}\text{In}$ -oxiquinolone *ex vivo*. Migration of the cells after intranodal injection was tracked in patients with melanoma with 3T MRI and scintigraphy. With promising results from this study and others reported later, a huge challenge to turn MRI cell tracking into a robust technique for clinical application is the difficulty to study all the relevant features of the labeling contrast agents and the labeled cells *in vivo* (11, 12). None of the labeling agents to date has been approved by the U.S. Food and Drug Administration (FDA) for use specific to label cells in clinical practice. Most clinical investigations on cell tracking are based on the market-available SPION contrast agents, initially developed for enhancing the contrast of diseased lesions. There is a strong need to develop more sensitive and less toxic labeling agents as well as a robust protocol of cell tracking study. The critical elements in cell tracking studies should be characterized as completely as possible for allowing validate meta-analysis between studies and establishing a robust protocol (2). In this review, we will briefly discuss the limitations of MRI cell tracking with iron oxide-based agents and recommend a minimum set of essential elements that should be considered in MRI cell tracking studies (Table 1).

## I. Limitations of MRI cell tracking

There are several limitations for MRI cell tracking, especially when long-term tracking of the cells is necessary (6, 11). These limitations are either technical or physiology-pathology-related. These limitations can be summarized into four major aspects, which should be considered in designing studies of MRI cell tracking.

### 1. Live vs. dead cells

The signal intensity in MRI depends primarily on the local values of longitudinal and transverse relaxation rates of water protons (13, 14). SPION agents are not detectable themselves, but are detected by their effects on surrounding water protons. When the transplanted cells of interest die, the SPION agents may remain in or around dead cells until the agents are cleared away. These agents produce signal that is detected by MRI. Therefore, the MRI signal cannot indicate whether cells are dead or alive.

### 2. False positivity

Except for the cell death, several other situations can also lead to false-positivity of MRI cell tracking (3, 4). First, endogenous host cells such as macrophages can phagocytize dying or dead SPION-labeled cells and these cells may actively move away from the site of cell implantation. These host cells can be mistaken to be the transplanted cells. Second, certain physiological and pathological conditions can result in hypointense signal, which can be confused with the presence of SPION agents (11). For example, macrophages loaded with hemosiderin from hemorrhage are often present in infarcted myocardium and these cells are

indistinguishable from labeled cells of interest. Third, tissues with high iron content such as bone marrow and hemorrhage can lead to misinterpretation of MRI signal.

### 3. Dilution of the labeling agents among daughter cells

Immature cells such as stem cells continue to divide after transplantation. In such cases, the labeled contrast agents are diluted among daughter cells, which results in the loss of MR signal over time (12, 15). This issue is more prominent for rapidly dividing cells and stem cells. Stem cells may divide asymmetrically, leading to an unequal distribution of the labeled agents among daughter cells (16). The unequal distribution not only leaves some cells having less contrast agents and undetectable quickly, but also makes the quantification of cell number less precisely.

### 4. Quantification of cell numbers

Although MRI visualizes cells *in vivo*, cell number quantification is challenging. Cells may be quantified by counting areas of hypointensity against a homogeneous background (e.g., in phantoms) in *in vitro* experiments. However, quantification of absolute cell number *in vivo* can be extremely difficult because of the agent dilution during cell division, agent transfer to other cells or extracellular space, and irons of other sources (14, 17). In addition, MRI quantification of iron concentration is still not reliable, although various mathematical methods have been suggested. There is no clear correlation between the SPION signal and the absolute number of live cells.

## II. Essential elements that need to be considered in MRI cell tracking studies

### 1. Physicochemical and magnetic properties of the labeling agents

The fundamentals of labeling agent development are similar to those of contrast agents developed for organ imaging (18, 19). As mentioned above, most labeling agents that have been developed so far are iron oxide (IO, mainly  $\text{Fe}_3\text{O}_4$ )-based. Although most of these nanoparticles are readily taken up by cells when added to the culture medium, they are often further functionalized with target-specific ligands or internalization-enhancing agents to achieve optimal internalization. A detailed guideline regarding the characterization of MRI contrast agents has been previously proposed by Shan et al. and this guideline is also suitable for characterization of an agent developed for MRI cell labeling (20). All newly developed labeling agents should be thoroughly characterized for their physicochemical properties (chemical yield, chemical purity, structure/composition, size, and shape) and magnetic properties (relaxation time and relaxivity) before cell labeling.

### 2. Cell information and labeling condition

Efficient cell labeling is generally based on receptor-mediated endocytosis, cell phagocytosis, or permeability change of the cell membrane. Besides the characteristics of labeling agents, the labeling efficiency is also dependent on the cell types, cell state, and labeling condition. Some cell types allow for efficient uptake of the nanoparticles (NPs) by mere incubation with the NPs over a 24–48-h period. However, some cell types require additional enhancing methods to take up the labeling agents (6). Even for same type of cells, their growth status is an important determinant for labeling efficiency. Cells at different growth state can exhibit extremely different phagocytotic activities and express different amount of receptors and membrane transport proteins (21, 22). Transient changes of cell membrane permeability with the use of electroporation or ultrasound pulses allow for NP agents to pass through the membrane and into the cytosol, which may be less influenced by the cell types and cell growth status, compared to other labeling mechanisms. Therefore, the

cell information includes the cell source, cell type, activation status, culture condition, antigen loading, etc. The cell labeling condition should include the medium, cell density or number, labeling agent concentration, and incubation time.

### **3. Cell labeling evaluation: labeling efficiency, intracellular localization, label retention, detection limit, and cytotoxicity**

**Labeling efficiency**—Stable labeling of cells with a contrast agent is usually achieved through endocytosis, phagocytosis, lipofection, electroporation, or combined (3). Efficient labeling is necessary to maximize the signal that is generated from the label, while long retention within cells is critical to ensure that the label agent is not rapidly lost with time nor transferred to other cells. High stable labeling agents prevent their degradation within cells with time, allowing long-term visualization of the cells. However, agent synthesis is complicated and the materials used for synthesis are quietly diverse, which result in the development of diverse labeling agents that possess physicochemical and biological properties significantly different from the parent compounds and from each other. Furthermore, many other factors such as the cell type, cell growth status, agent concentration, and exposure time also influence agent internalization. All these make the labeling efficiency difficult to predict and the labeling efficiency should be determined individually (19).

Cell labeling efficiency is usually expressed as the percentage of labeled cells in total culture cells and the amount of iron per cell. We have noticed that only the percentage of labeled cells has been reported in many publications. For *in vivo* cell tracking, the amount of Fe per cell appears more important than the percentage of labeled cells because of the close relationship between Fe concentration and signal intensity in MRI. We strongly recommend investigators to report the labeling efficiency with both the percentage of labeled cells and the amount of Fe per cell (e.g., 10 pg Fe/cell) for IO-based agents. A particular concern for IO-based agents is their aggregation and sedimentation, which often take place in medium when long incubation times are necessary. The aggregates can be on the culture dish surface or on the cell surface, which may be difficult to wash away. In such cases, quantification with techniques such as inductively coupled plasma atomic emission spectroscopy, R2 relaxometry, and colorimetric assays can be misleading (23). It is, therefore, important to eliminate any unbound NPs for accurate quantification. Density gradient centrifugation and flow cytometry are potentially useful to exclude extracellular aggregates.

**Intracellular localization**—Intracellular location of the labeling agent is associated with both the strength of local contrast enhancement and cytotoxicity. Studies with Prussian blue staining and light and/or electron microscopy have shown that inorganic NPs often accumulate in well-defined endosomal compartments resembling lysosomes within the cytoplasm. On the one hand, accumulation in the lysosomes may limit the exposure of sensitive cell organelles to the NPs and prevent protein absorption to particle surface. Adsorption of proteins on surfaces can be irreversible and may lead to protein conformation changes, altering the biological stability and activity of the proteins. On the other hand, localization into lysosomes poses a potential drawback for long-term tracking, because lysosomes may degrade the NPs quickly. Importantly, observation of either the intact agent or any parts of the agent in the cell nuclei should be documented seriously because of potential damage of the labeling agent to cell genome.

### **4. In vitro characterization: Label retention, detection threshold, and cytotoxicity**

**Label retention**—This is an important issue that must be considered, especially in longer-lived, rapidly dividing and migratory cells (12). In general, label retention time in cells is not a problem soon after transplant, but the issue arises when labeled cells begin to divide,

migrate, or die. The labeling agents can be taken up by and/or integrated into the host cells. The labeling agents can also remain in the extracellular matrix for a relatively long time or are cleared through unknown pathways. It may be difficult to clearly answer this issue through *in vitro* studies alone. However, it is possible to define the pathways and timelines of agent clearance within cells. The retention information provides an important reference for predicting the time frame available for detection and the fate of labeling agents *in vivo*.

**Detection threshold**—*In vitro* detection threshold refers to the minimum number of cells detectable with MRI following labeling. This value forms the baseline for *in vivo* transplantation and cell number quantification (15). In the literature, detection threshold has been mostly determined by embedding the labeled cells in a phantom such as agarose. The data are usually expressed as cells/voxel at a given field strength. Theoretically, the *in vitro* detection threshold is much lower than the *in vivo* detection limit because of the cell distribution, division, migration, and death after transplantation (24). Several fold more cells than the detection threshold should be transplanted for *in vivo* tracking. Evidence has shown that the *in vitro* detection threshold for SPION-labeled human dendritic cells can be lower to ~125 cells/voxel at 7 T (~2000 cells/voxel for <sup>19</sup>F- or Gd-labeled cells) (25).

**Cytotoxicity**—Theoretically, the labeling agents and the labeling procedure should be non-toxic to labeled cells. Indeed, SPION seems no effect on the capability of cell proliferation and differentiation, although a few studies have reported that the stem cells labeled with SPION lose part of their differentiation capacity in a SPION concentration-dependent manner (26). However, NP imaging agents have been synthesized with various strategies and diverse nanomaterials. Each component of an NP agent can pose individual toxicity risks, and an intact agent might have toxic risks that differ from the toxicities of each component (27–29). The potential cytotoxic effects need to be addressed individually for each NP agent and cell type used. Given the large number of possible interactions between NPs and cells, the cytotoxic evaluation is not straightforward (30). A particular concern may be the induction of reactive oxygen species mainly by a reaction of hydrogen peroxide with iron-oxide moieties in lysosomes (31). Reactive oxygen species could also be generated as a cell response to the presence of a high load of NPs. To assess cytotoxicity, it is necessary to perform a multitude of cytotoxicity assays and to test a range of cell types for each type of NPs with multiple doses (30, 32). Immediate toxic effects can be tested with studies of cell growth, viability, apoptosis, phenotype, activation, differentiation, etc.

## 5. *In vivo* cell tracking: cell implantation, monitorable period, and label clearance

**Cell implantation (number of cells, route of implantation, and control)**—The goal of cell tracking is to track the cells qualitatively and quantitatively and long enough for evaluating the cell outcome. To reach the goal, precise control of all variables is necessary. Except for the information on experimental animals, the total implanted cell number and implantation route are also critical for the evaluation of cell tracking results (33). The former includes the cell number of each implantation and the times of implantation carried out. For the administration route, cells can be implanted through intravenous, subcutaneous, intraperitoneal, or intranodal injection. In addition, non-labeled cells as the control should also be included to establish a baseline for the experiment.

**Monitorable period**—The detection threshold for labeled cells is affected by several factors including field strength, pulse sequence, type of particles, signal-to-noise ratio, and voxel size. In typical settings for SPION- and <sup>19</sup>F-labeled cells, the minimum detection limit in animals is in the order of 10<sup>4</sup>–10<sup>5</sup> cells/voxel for clinical MRI systems and 10<sup>3</sup>–10<sup>4</sup> cells/voxel for high-field animal scanners (15, 25). In the case of cell vaccination, a typical study utilizes an intranodal or intradermal injection of ~10 million dendritic cells, with 3 × 10<sup>4</sup> – 2

$\times 10^5$  cells migrating to secondary lymph nodes. Although these data indicate that MRI is sensitive enough for cell tracking in clinical practice, however, mature dendritic cells will not divide further. For actively dividing cells such as T cells, SPION may be quickly divided among daughter cells to undetectable levels within a short period. The situation worsens in the case of stem cells that exhibit asymmetric cell division. Studies have shown that the cell number can be quantified for up to 3 weeks in actively dividing T cells in mice and the underestimation of cell numbers due to cell division is within tolerable limits. This error may be reduced if the cell division rate is known. Regardless the cell number determination, most studies indicate that the labeled cells could be visualized for up to 2–3 months in animal models (34).

**Label clearance**—Some important issues remain to be solved concerning the clearance of contrast agents from cells (35). These issues include whether the agents remain in the cells or leak to extracellular space following cell death, whether the released agents are incorporated into macrophages or other host cells, and how tissues clear the agents. There is no consensus on how these issues should be solved with imaging techniques. Pathological examination and flow cytometry of surgical specimens may help answer some questions, but these procedures are invasive.

## 6. Imaging protocol

Imaging sequence selection and parameter setting can influence the image contrast or sensitivity of cell detection (4).  $T_2$  and  $T_2^*$  relaxation times are shorter at high field strength, increasing the ability to visualize cells labeled with IO particles. Protocols for the acquisition of MR data for cell tracking are similar to those for routine MRI/MRS (14). IO-based agents cause a hypointense contrast that can be confused with the susceptibility-induced field inhomogeneities *in vivo*, namely dark areas arising from airspaces, blood vessels, hemorrhages, and tissue interfaces. Modified acquisition protocols have been reported to convert IO-related hypointense to hyperintense spots ('bright contrast') through suppression of background tissue, which may help increase the robustness of cell detection with MRI (36, 37). Because the normal anatomic background of the image is lost with these bright contrast techniques, the bright contrast image needs to be overlaid with a standard MRI image.

## 7. Outcome evaluation

The outcome here refers to the outcome of animal models, functional outcome of implanted cells, and side effects of labeling agents on host. The function of implanted cells is dependent on the cell types and experimental purposes. Evaluation of the cell function is usually performed with a series of methods (38).

Different from the cytotoxicity of agents on cells *in vitro*, side effects are more referred to the negative effect on host and can be short-term and long-term. Because iron participates in cell metabolism, IO particles are well tolerated by living organisms. Iron presents in human body at a dose of ~4 g in the average adult, of which 80% is incorporated in cells of the haematopoietic system and another 10–15% is present in muscle fibers and other tissues. The total dose that would be introduced into the human body in MRI cell tracking would be ~1 mg, or 0.025% of total body iron, based on the calculation for a dose of  $1 \times 10^8$  cells and 10 pg Fe/cell (11). This amount of iron introduced by cell tracking purpose is far less than the iron amount causing toxicity to human body. However, the coating and functionalizing materials may cause problems and close observation is recommended.

In conclusion, the feasibility of MRI cell tracking with IO-based agents as labels has been well demonstrated in animal experiments. Clinical trials of MRI cell tracking are also

ongoing. Different to animal studies, some general requirements for clinical cell tracking should be met for the labeling agents. The labeling agents must be shown to be non-toxic to cells in culture and to animals. The labeled cells should be more extensively characterized to determine any effects of the labeling procedure on cell functionality. The labeling agents should be able to be synthesized in a reproducible manner in a GMP facility with compounds that are or can be approved for human use. Any design strategies for agent synthesis, cell labeling and *in vivo* MRI tracking would necessarily need to take the approval of regulatory agencies into consideration. A robust protocol for any MRI cell tracking studies is critical to obtain approval by the regulatory agencies to move to clinical trials or practice.

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**Table 1**

Essential elements to consider for MRI cell tracking studies

<b>Elements</b>	<b>Brief description</b>
Labeling agent	Physicochemical and magnetic properties
Cell information	Cell source, cell type, activation status, culture condition, antigen loading, etc.
Labeling condition	Medium, cell density/number, labeling agent concentration and incubation time
Cell labeling evaluation	
Labeling efficiency	% of labeled cells in total and Fe/cell
Intracellular localization	Lysosomes, cytoplasm, nuclei, etc.
<i>In vitro</i> characterization	
Label retention	Especially in dividing or long-lived cells
Detection limit	Cells/voxel at a given field strength
Cytotoxicity	Mainly the adverse effect on cell function,
<i>In vivo</i> analysis	
Cell implantation	Cell number, route, and control
Monitorable period	Period from implantation to the time undetectable
Label clearance	Clearance of label from both living and dead cells
Imaging protocol	Detail imaging parameters
Outcome evaluation	
Functional outcome	Influence on cell function <i>in vivo</i>
Side effects	Acute and long-term effect on host
Validation	e.g. histology, flow cytometry on biopsy samples