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Labelling of mammalian cells for visualisation by MRI

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Abstract Through labelling of cells with magnetic contrast agents it is possible to follow the fate of transplanted cells in vivo with magnetic resonance imaging (MRI) as has been demonstrated in animal studies as well as in a clinical setting. A large variety of labelling strategies are available that allow for prolonged and sensitive

detection of the labelled cells with MRI. The various protocols each harbour specific advantages and disadvantages. In choosing a particular labelling strategy it is also important to ascertain that the labelling procedure does not negatively influence cell functionality, for which a large variety of assays are available. In order to overcome the challenges still faced in fully exploiting the benefits of in vivo cell tracking by MRI a good understanding and standardisation of the procedures and assays used will be crucial.

Keywords Cell labelling · Contrast agents · Cell tracking · Apoptosis · MRI

Introduction

Labelling of cells with magnetic contrast agents has been the subject of research in a considerable number of studies. Because of the increased use of cells as therapeutic agents or vehicles, the need for noninvasive imaging of cells in vivo has developed. For studies regarding the development of cell-based therapeutic strategies, and for monitoring the efficacy and safety of such therapies, it is essential for the fate of transplanted cells to be monitored in vivo.

Various methods are available for in vivo cell imaging, including optical imaging techniques [1] and nuclear imaging techniques such as positron emission tomography (PET) [2], single-photon emission tomography (SPECT) [3] and MRI [4]. Each of these techniques has its own advantages and limitations. In vivo cell imaging by optical techniques has the advantage that it is a high-throughput method with high sensitivity and multispectral capabilities. Optical imaging techniques, however, are limited by their

poor spatial resolution and penetration depth making them mostly applicable in small laboratory animals or for superficial locations, and as yet they are not suitable for clinical translation. PET and SPECT offer high sensitivity for detection, and quantifiable results. However the spatial resolution of these techniques is low; the half-life of the tracers is short and there are safety issues involved with ionising radiation, limiting their use in longitudinal studies. MRI offers several advantages for in vivo cell tracking: it is an intrinsic noninvasive technique, it does not involve the use of ionising radiation, it has high spatial and temporal resolution capabilities, it provides information in an anatomical context and it offers the possibility to assess functional aspects of tissues. Because of these advantages MRI has been promoted as a promising technique for in vivo cell tracking. A prerequisite for imaging of cells by MRI is the association of contrast agents with the cells of interest. The contrast agent must alter the MR signal parameters to allow detection.

Various groups have demonstrated that cells can be labelled with paramagnetic particles allowing visualisation by MRI, even at the single cell level [5–11]. To label cells, contrast agents should be bound to the external cell membrane or internalised into the cytosol or intracellular vesicles. A variety of approaches, in terms of labelling method and type of contrast agent used, have been described for the effective labelling of cells with contrast agents [12–24]. For safe clinical application of labelled cells both short- and long-term toxicity studies are needed. However, before contrast agents can be administered to patients, potential adverse effects *in vivo* following their metabolism should be investigated as well. Imaging labelled cells with contrast agents can provide an exquisite approach to following the fate of the injected cells.

This article provides an overview of these different approaches used for cell labelling and their specific benefits and limitations. Furthermore, several methods to assess labelling efficiency will be compared and several assays to assess the toxicity of the contrast agents used for cell labelling and their effect on cell function will be discussed. Finally, a brief overview of image acquisition possibilities and limitations will be presented.

Applications of paramagnetic cell labelling

The fact that cells can be visualised by MRI, when labelled with paramagnetic nanoparticles, has created the possibility to assess the fate of transplanted cells *in vivo*. Various applications for *in vivo* cell imaging by MRI have been reported and proposed. The various applications include studies regarding the fate of transplanted stem cells in neurological disorders [25], cardiovascular disease [4] and joint disease [26], survival of transplanted pancreatic islets [27] and homing and function of immune effector cells in cell-mediated treatment of cancer [28]. For additional information on applications of cell imaging by MRI we refer the reader to some excellent recent reviews and a recently published book on cellular and molecular imaging by MRI [25, 29–35].

In vivo cell imaging by MRI has also already entered the clinical arena. The first report describing the use of MRI to monitor the fate of transplanted cells was by De Vries et al. [15]. In this paper, autologous dendritic cells loaded with tumour-derived antigenic peptides were labelled with superparamagnetic iron oxide particles (SPIO) before injection in melanoma patients. The study showed that MRI allowed for the assessment of the accuracy of dendritic cell delivery and of the inter- and intranodal cell migration patterns of the injected dendritic cells over the following days. Thereafter, Zhu et al. [36] reported on a feasibility study performed in two patients, of tracking neural stem cells labelled with SPIO in patients with brain trauma. They showed that the migration of the stem cells could be monitored by MRI. More recently, Toso et al. [37]

reported on MRI-based monitoring of pancreatic islet grafts labelled with SPIO in patients with type 1 diabetes. While some technical limitations in terms of visualisation of the grafts were encountered, the use of SPIO-labelled cells did not affect the *in vitro* or *in vivo* functionality of the grafts.

Contrast agents for cell labelling

To date, many different contrast agents have been used for cellular MR imaging [5, 20, 37–47]. MR contrast agents can broadly be divided into paramagnetic agents, the so-called T1 agents (gadolinium, manganese etc.), and superparamagnetic agents, the T2 agents (e.g. iron oxide nanoparticles) [48].

Iron oxide particles

The effect of iron particles on the contrast between labelled and unlabelled cells is stronger than that of other paramagnetic agents. Usually, iron oxide particles require stabilisation by a surface coating of dextran to prevent aggregation. Dextran-coated iron oxide nanoparticles are biodegradable and biocompatible. There are big differences between the sizes of the various magnetic nanoparticles [20]. For example, Feridex I.V.[®] (Advanced Magnetic Industries, Cambridge, Maryland, USA), which is a ferumoxide, has particles that range from 120 to 180 nm in size; Sinerem[®] (Guerbet, Villepinte, France), which consists of ferumoxtran-10, has particles that range from 15 to 30 nm in size; Resovist[®] (Bayer Schering Pharma, Berlin, Germany), which is a ferucarbotran, consists of particles that are 60 nm in size. Iron oxide particles can be classified into superparamagnetic iron oxide particles (SPIO), such as Feridex[®] and Endorem[®] (Guerbet, Gorinchem, the Netherlands) [35, 49–53]; ultrasmall superparamagnetic iron oxide particles (USPIO), such as Combidex[®] (Advanced Magnetic Industries, Cambridge, Maryland, USA) and Sinerem[®] [54–57]; very small superparamagnetic iron oxide particles (VSOP) [58–60]; ferrofluids [61, 62]; monocrystalline iron oxide nanoparticles (MION) [45, 63]; micrometre-sized iron oxide particles (MPIO) [16, 19]; and cross-linked iron oxide particles (CLIO) [64–66].

All these different particles have their specific characteristics [67–69]. Variations in size, surface charge, coating properties etc. influence the pharmacokinetics, biodistribution, uptake efficiency, metabolism and biocompatibility of the particles. For example, the smaller dextran-coated USPIO have a longer blood half-life than SPIO. SPIO are clinically used for liver imaging; Kupffer cells (liver macrophages) efficiently and quickly take up SPIO as opposed to tumour cells, thus facilitating detection and staging of liver tumours. USPIO are mostly used in clinical

trials for metastatic lymph node imaging, for imaging inflammatory tissue and as a blood pool agent. Furthermore, SPIO have a higher magnetic susceptibility and T2 relaxivity. Although SPIO are easily internalised by macrophages, uptake by nonphagocytic cells or slowly dividing cells is low. Therefore, high, potentially toxic concentrations are needed for efficient cell labelling of nonphagocytic cells. When labelling cells with paramagnetic particles, it is essential to determine the toxic effects of the contrast agent or the labelling procedure on the cells. Adverse effects in terms of cell viability, proliferation capacity, differentiation capacity and cell function need to be avoided. The lack of significant adverse effects of labelling of cells with iron oxide particles has been reported in a large number of studies; however, some adverse effects or changes in behaviour of the labelled cells have been reported: these include reduced differentiation capabilities of labelled mesenchymal stem cells [70], altered expression of proteins [71] and changed in vivo behaviour [53]. Although only few studies exist that report on potential adverse effects of paramagnetic labelling on cells, further studies on potential adverse effects of labelled cells in vivo need to be performed.

The particles consist of an iron oxide core and are usually coated with dextran or siloxanes [68] encapsulated by a polymer [16], or modified in other ways to enhance internalisation by cells and to prevent aggregation. The coating on the outer surface allows further biochemical manipulation. For example, dextran-coated iron oxide particles can be chemically conjugated to proteins such as monoclonal antibodies [72]. In general, monoclonal antibody-guided magnetic particles can provide a stronger contrast enhancement of specific target cells than particles without antibody guidance.

It is beneficial if two different imaging techniques can visualise the same contrast agent particle. For example, from the bacteria *Magnetospirillum gryphiswaldense* magnetic nanoparticles can be harvested and coupled covalently to a fluorescence dye [73]. These fluorochrome-coupled magnetosomes can be imaged using MRI as well as optical imaging methods. The use of this bimodal contrast agent benefits from the high spatial resolution of the MRI and the high sensitivity of fluorescence imaging. However, the possibility of using these magnetosomes in vivo needs to be investigated, especially since the protein shell of these magnetosomes may give rise to immunological reactions. Two imaging techniques can also be combined using other particles [73–76].

Gadolinium compounds

Gadolinium compounds can also be used for cellular labelling. In general, they induce a predominant T1 shortening and thus high signal on T1-weighted MR images, which is favoured whenever the surrounding tissue

has low signal intensity. However, detection of Gd compounds by MRI is much less sensitive than for SPIO [40, 77] and the contrast behaviour or relaxivity of Gd chelates may depend on the cell compartment in which these Gd compounds end up in. The cellular localisation of Gd-based probes depends on the size of the contrast agent used and/or the labelling method [77].

Internalisation of gadolinium can be accomplished by exposure of cells to Gd chelates [78, 79] or by using liposomes [23]. Liposomes are double-layered lipid spheres that can incorporate gadolinium inside the water or lipid phase. Gadolinium nanoparticles coupled to a fluorophore can also be used for cellular labelling [23]. These particles are constructed from lipid monomers with diacetylene bonds that are sonicated and photolysed to form polymerised nanoparticles. These nanoparticles allow efficient cell labelling. Another application of gadolinium is the conjugation of ionic gadolinium (Gd^{3+}) chelates to proteins that bind to dying cells [80]. This enables targeted MRI detection of cell death (apoptosis) in tumours after treatment with a chemotherapeutic agent. It is currently not clear how long gadolinium-labelled cells can be tracked in vivo and if there are any toxic side effects.

New developments for contrast manipulation

A new approach to cell tracking for MRI has recently been developed, in which genes encoding expression of proteins involved in iron metabolism, e.g. ferritin and transferrin receptors, are used as cellular MR reporter genes [81–84]. In this approach a gene, encoding the protein of choice, is introduced into the cell using molecular techniques [31]. These molecular techniques can be employed such that the newly introduced gene continuously (over)expresses the protein [85], or such that the protein is expressed simultaneously with another specific gene of choice, e.g. the endothelial cell adhesion molecule VE-cadherin [81], or that the protein is only expressed after induction of expression [81]. The (over)expression of proteins involved in iron metabolism then result in accumulation of iron in the cell leading to altered signals on MRI.

There are some potential pitfalls of using MR reporter genes to monitor transgene expression such as the low sensitivity of detection as well as difficult and sometimes delayed interpretation of signal changes, meaning that it may take some time before accumulated iron has reached detectable levels. However, MR reporter genes have the potential to provide information on the biodistribution and viability of cells after injection. A further major advantage of using MRI reporter genes over labelling of cells with paramagnetic particles is that when cells divide, the newly introduced gene is propagated in the daughter cells, and will give similar signal effects as the parent cell. For exogenously introduced particles, the particle content per cell will become diluted through subsequent cell divisions

with ultimate loss of signal [86, 87]. Further development of more sensitive and selective reporters, combined with improvements in detection sequences, will also help in the understanding of intracellular biological processes and the molecular bases of diseases.

Other novel contrast agents have also recently been explored for use in cell imaging. These include labelling of cells with manganese-based contrast agents, fluorine-based contrast agents [88–90] and chemical exchange saturation transfer (CEST) agents [91–93].

For manganese-based contrast agents proposed advantages include signal enhancement on MR images, instead of signal loss as seen with iron oxide particles, and the fact that cells can be efficiently labelled without the need for additional compounds. Disadvantages on the other hand may arise from toxic effects of manganese, low MRI sensitivity for detection and rapid clearance of manganese from cells [86, 87]. For fluorine-based cell imaging the main proposed advantages are the highly specific imaging of labelled cells, since there is no native background signal as seen with proton imaging used with regular T1- or T2-type contrast agents, and that quantification of the contrast agent and thus indirectly the number of cells is more straightforward than with iron oxide particles [89]. Limited sensitivity for MRI detection may, however, also be a disadvantage for fluorine-based cell imaging. CEST agents function by reducing the water proton signal through a chemical exchange site on the agent via saturation transfer [91, 92]. MR signal arising from protons tightly bound to a specific class of molecules (proteins, lipids etc.) could be selectively presaturated, and this saturation could then be transferred to the signal of the bulk water, producing a decrease in bulk water signal intensity. Major advantages of CEST agents arise from the fact that the contrast effect only arises after the saturation pulse is administered at the specific frequency. Therefore, the contrast can be switched on and off and multispectral imaging can be performed, meaning that various cell populations labelled with different contrast agents can be visualised [94]. Furthermore, CEST agents can be used to monitor clinically relevant physiological properties such as in vivo temperature mapping [94], pH mapping [95] and monitoring of enzyme activity [96]. Furthermore, the combination with liposomes offers the possibility of liposome-based chemical exchange saturation transfer (lipoCEST) [97]. The size of liposomes can be exploited to enhance the lipoCEST contrast. The size of the liposome determines the amount of water protons available in the liposomal vesicle and the exchange rate of water molecules over the liposomal membrane [98, 99]. Through these effects, smaller liposomes are more efficient in saturation transfer but larger liposomes perform better on a lipid molar-based comparison.

Another technical development for monitoring cell biological and physiological processes with high clinical potential is ^{13}C labelling of molecules [97]. Depending on the molecules selected, MR imaging or spectroscopy can

be used for in vivo monitoring of metabolic processes [100, 101], mapping of tissue perfusion [102] and pH mapping of tissue spaces [103].

Cell labelling methods

Association of cells with contrast agents can be achieved by nonspecific as well as specific cell labelling approaches (see below). For each of these approaches several biological or biochemical principles can be employed. In most cases, however, a rather complex biological process called endocytosis, which encompasses a variety of pathways, is central to the uptake of material from the extracellular environment into the cell [103–107]. Depending on the cell type, the contrast agent used and the specific cell labelling method used, contrast agents can be incorporated into the cell by phagocytosis, pinocytosis, receptor-mediated endocytosis, and caveolin-dependent and -independent endocytosis. In general, cell labelling methods are aimed at incorporation of the label into the cell (Fig. 1a–c). Various strategies are possible and are either mainly suitable for ex vivo labelling of cells or can be used for both in vitro and in vivo labelling strategies.

Nonspecific cell labelling

Nonspecific cell labelling approaches can in principle be used for all kinds of cells as they are based on general biological or biochemical principles. Because of the lack of specificity of these approaches, nonspecific cell labelling is generally suitable for ex vivo cell labelling, but can also be used in vivo. Nonspecific cell labelling can be performed on mixed, undefined as well as on defined and purified cell populations depending on the labelling method used. The efficacy by which this can be done strongly depends on the cell type [63], the specific labelling method [108] (see below) and the type of contrast agent used [14, 56, 109].

Nonspecific cell labelling approaches can roughly be divided into direct and indirect approaches.

Nonspecific direct cell labelling

The most straightforward approach to cell labelling is the direct cell labelling method, where the label is added to the culture dish of cultured cells for “spontaneous” uptake of the label by the cells. The efficacy of this labelling method is strongly dependent on cell-specific properties. Cells that normally fulfil a function as scavenger or antigen-presenting cell (e.g. dendritic cells) generally show good incorporation of the label via the direct labelling method. Other types of cells, however, show a large variation in incorporation efficiency [108]. The advantage of direct cell labelling is that there are no additional agents or

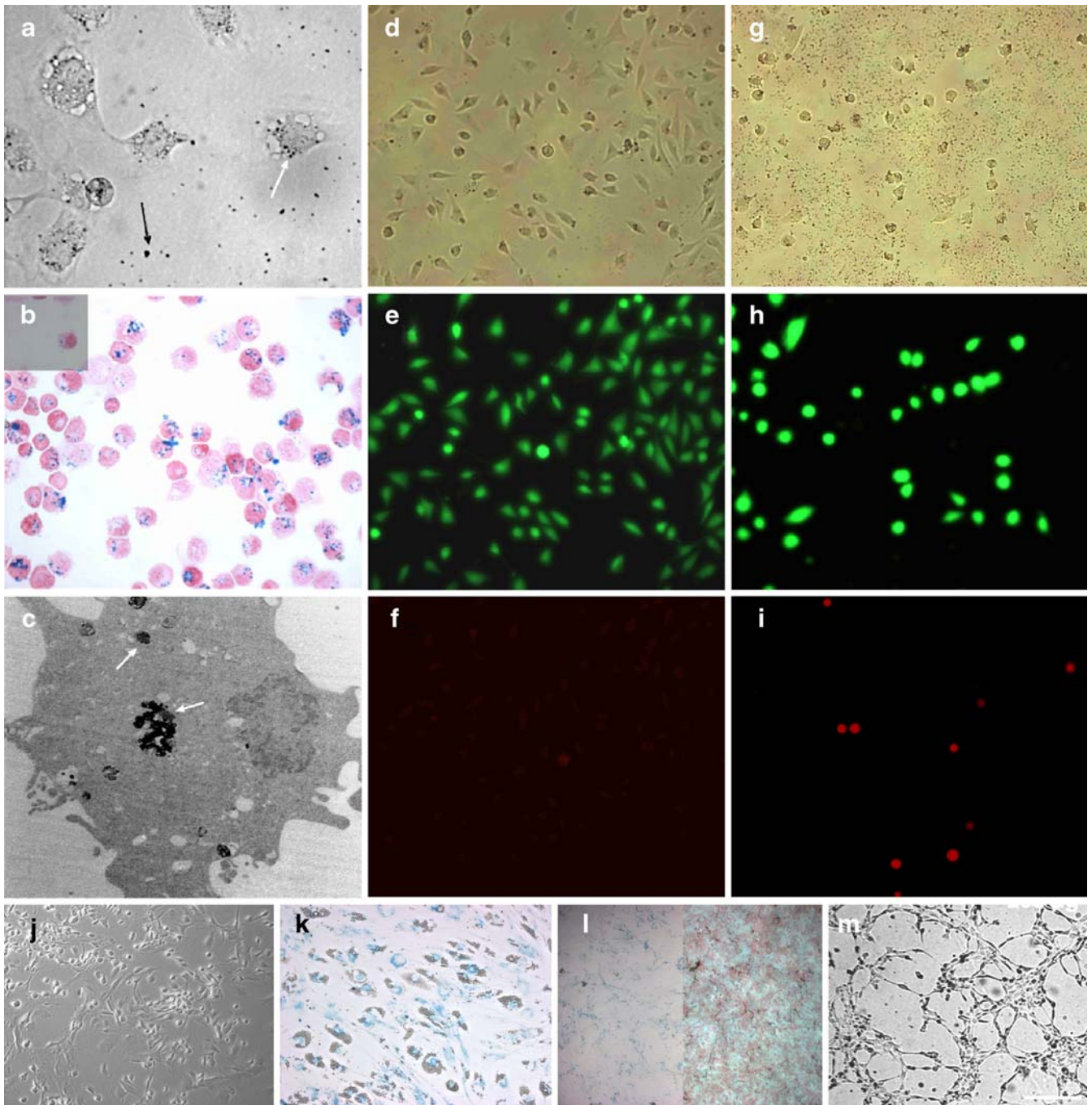


Fig. 1 Labelling efficiency of cells with iron oxide. **a** Stem cells in a culture dish with iron oxide–Lipofectamine complexes sedimented on the bottom of the culture dish (*black arrow*) and inside cells (*white arrow*). **b** Prussian blue-stained cell showing presence of iron oxide particles in the cell. **c** Electron microscopy image showing iron oxide particle complexes in intracellular vesicles. **d–i** LIVE/DEAD assay, using calcein and ethidium homodimer-1, of iron-labelled cells at two labelling doses with iron oxide of 200 µg (**d–f**) and 800 µg (**g–i**). Panels **d** and **g** show regular bright field microscopy images; **e** and **h**, *green* probe for living cells; and **f** and **i**,

red probe for dying cells. Note the decrease in the number of cells at the higher labelling dose and the increase in dying cells. **j** Mesenchymal stem cells in culture. **k** Labelled mesenchymal stem cells after differentiation into fat cells stained for iron content with Prussian blue. Note the clearly visible fat vacuoles. **l** Labelled mesenchymal stem cells after differentiation into bone cells: *left panel* stained for iron by Prussian blue, *right panel* stained for bone-specific markers. **m** Tube forming of iron oxide-labelled endothelial cells on Matrigel

manipulations needed. Furthermore, this technique can not only be used in vitro but also in vivo for phagocytic cells, macrophages, dendritic cells and cells from the reticulo-endothelial system. These cells easily incorporate the contrast agents as they are capable of phagocytosing large particles easily or they are capable of incorporating small particles through pinocytosis. In vivo direct nonspecific cell labelling is currently used to visualise Kupffer cells in liver tissue, for lymph node imaging and for inflammatory cells [57]. It allows cell-specific imaging in different organs, but provides nonspecific labelling of all cell types that take up the agent.

Nonspecific indirect cell labelling

To improve the efficacy of cell labelling with contrast agents for various cell types, different so-called transfection reagents have been used. Transfection-mediated endocytosis is suitable for all cell types and has a high efficiency of label incorporation. This method is more laborious than spontaneous endocytosis and there can be toxicity of the transfection agent. There are many transfection agents available, each with their own potential benefits and drawbacks. The transfection reagents will form complexes with the contrast agents.

Lipofectamine (Invitrogen, Carlsbad, CA, USA) is a commonly used lipid-based transfection reagent, with high transfection efficiency for a wide range of cell lines [110]. The positive charge on the cationic lipids helps to bind the complexes to the cell membrane, after which the complexes enter the cell via endocytosis. Lipofectamine has been used as an additive in cell labelling studies for a large variety of cell types and successful protocols for over 20 different cell lines are available (Table 1). However, lipofectamine can only be used for cells that adhere to culture dishes. Therefore, other transfection reagents are needed to label cells in suspensions, such as the transfection agents DMRIE-C (Invitrogen, Carlsbad, CA, USA) or FreeStyle™ MAX (Invitrogen, Carlsbad, CA, USA). The DMRIE-C transfection agent gives a good performance for transfection of cells in suspension such as lymphoid cell lines. The FreeStyle™ MAX Reagent is a proprietary, animal-origin-free formulation for the highly efficient transfection of eukaryotic cells in suspension [111].

The transfection agent poly-L-lysine (PLL) is also a commonly used agent for intracellular labelling [112]. The positively charged PLL is used to allow the cells to adhere with the cell membrane to negatively charged glass slides. This mechanism of charge-related binding is similar to the encapsulation of PLL–iron oxide complexes that will bind

Table 1 Methods for improved efficacy of contrast agent uptake

| Name | Cell type | Efficiency | Disadvantages | Advantages |
|--|-----------------------|------------|--|---|
| 1 Lipofectamine™ [9, 22, 53, 171] ^a | Adherent ^b | High | Slightly toxic to cells | Widely used Simple protocol |
| 2 Poly-L-lysine [18, 51, 77, 172, 173] | All ^c | High | Slightly toxic to cells Large variation in formulations available | Widely used Simple protocol |
| 3 Magnetodendrimer [12, 13, 174, 175] | Adherent | High | Slightly toxic to cells Not widely available | Uptake is similar for different cell types |
| 4 Protamine sulfate [7, 21, 49, 176] | Adherent | High | Heparin anticoagulant Tendency to form large aggregates | FDA-approved agent Simple protocol |
| 5 HIV-1 tat [17, 66, 177–179] | Adherent | High | Possibility of undesirable immune response Potential nuclear localisation of contrast agent Prior chemical linkage to contrast agent necessary | Fast labelling procedure |
| 6 Electroporation [11, 24, 180, 181] | All | Low | Technically challenging Specialised equipment needed | Can be used for difficult-to-transfect cells Cytoplasmic localisation of small molecules |
| 7 Gene gun [119, 120, 182] | All | Low | Specialised equipment needed | None |

^aReference papers

^bCells growing in culture adhered to the bottom of the culture dish

^cCells growing in culture adhered to the bottom of the culture dish as well as in suspension

to the target cells resulting in uptake of iron oxide nanoparticles (Table 1).

Magnetodendrimers [13] (Table 1) can enter the cell through a nonspecific membrane adsorption process after which the contrast agent will be localised in intracellular (non-nuclear) endosomes. Regardless of which cell type is used, transfection with magnetodendrimers shows a comparable degree of particle uptake. A high level of transfection is accomplished by using the heat-activated dendrimer SuperFect (Qiagen, Valencia, CA, USA) [12].

Protamine sulfate [49] (Table 1) can be used to get a contrast agent into the cell by endosome formation. The advantage of protamine sulfate is that it is an FDA-approved agent used as an antidote to heparin. However, in gene therapy protocols, protamine sulfate is commonly used for *in vitro* transfection [113]. Protamine sulfate combined with the FDA-approved Feridex shows potential for clinical application.

The peptide HIV-1 tat (derived from the trans-activating protein of the human immunodeficiency virus type-1) can also be used to internalise contrast agents (Table 1) [114]. This method is not dependent on a receptor, but translocates exogenous molecules into cells. Cross-linked with iron oxide particles the HIV-1 tat particles allow cell detection with MRI. The HIV-1 tat peptide is the only transfection agent that has been detected within the cell nucleus [17].

Contrast agents can also enter the cell via electroporation [115] (Table 1). This can work well for cell types that are difficult to transfect with commonly used transfection agents. An electrical pulse creates a potential difference across the cell membrane and induces temporary pores in the cell membrane for the entry of the contrast agents. Depending on the type of contrast agent used, this will result in endosomal entrapment of the contrast agent (nanoparticles) in the cell [115] or localisation of the contrast agent in the cytoplasm (small molecules) [116]. Although no additional reagents are necessary for electroporation, specialised equipment is needed and the procedure is technically challenging. It requires optimisation of electrical pulse and field strength parameters [117]. Furthermore, there is a large variation in cell tolerance as high toxicity levels are observed after electroporation. It can irreversibly damage the membrane and lyse the cells. Cell survival after labelling is usually not above 70% [118].

Another method of nonspecific cell labelling is by using a gene gun (Table 1) [119, 120]. Gene guns were developed to introduce genetic material into tissues, cells or organelles [121]. It can also be used to introduce nanoparticles, including MRI contrast agents, into cells. The material of interest is “shot” into cells of the recipient using pressurized helium that launches a disk carrying the nanoparticles. This disk travels at the speed of a rifle bullet, and hits a screen, which detains the disk, but launches the microscopic particles towards the target cells.

There are no specific benefits described that favour the use of a gene gun for cell labelling. The technique needs specialised equipment and the efficacy of cell labelling is difficult to manipulate. Furthermore, the potential adverse effects are not yet known.

Specific cell labelling *in vitro*

The major advantage of specific cell labelling is that it allows labelling of specific cells in a mixed cell population. *In vitro* labelling can be established by using a targeting vector, such as antibodies, peptides, aptamers, amino acids and lectines conjugated to the contrast agent [14, 72, 122–124]. These vectors will bind specifically to target molecules/receptors on the cells of interest. Compared with regular endocytosis, there is increased efficiency [19]. However, it needs previous knowledge of the expression of the target molecule. Furthermore, there needs to be chemical linkage of the label to the target molecule and attachment of the probe to the receptor. This chemical linkage of the ligand to the receptor could lead to unintended triggering of biochemical processes. Furthermore, depending on the effect of receptor–ligand binding, internalisation of the probe may not always occur. Without internalisation, it could be possible that the contrast agent might be rapidly cleared from the cell.

Other particles that can be used for cellular labelling are microbeads. Microbeads used for magnetic cell sorting can be used as a contrast agent linked to an antibody that is bound to the target cell [125]. The advantage of this labelling method is that the particles do not internalise, and therefore it is assumed that no toxic effects of the particle are exerted. Furthermore, it is not required to culture the cells, as labelling can be performed in a cell suspension. However, the attachment to the outer cell membrane is likely to interfere with cell–surface interactions, and unwanted activation of biochemical pathways, including apoptosis, can result. Furthermore, cell membrane labelling often does not result in sufficient signal for *in vivo* MR imaging, and as the particles do not internalise, the antibody might also easily detach and attach to other cells.

However, when labelling a complete cell population *in vitro*, an additional probe to select a subpopulation of cells is not needed and nonspecific labelling is preferred [126].

Specific cell labelling *in vivo*

The major advantage of specific *in vivo* cell labelling [127] is that there are no preceding cell harvesting procedures or any *in vitro* manipulations needed. Furthermore, one can choose to label cells at any given time point. However, the method faces an extra challenge because of the delivery

barrier. There are several prerequisites for cell/tissue-specific contrast enhancement *in vivo*. First, there should be sufficient binding of contrast agent to the target cells. This can be achieved, for example, by antibodies [128]; however, there should not be any steric hindrance on the outside of the cell membrane if internalisation of the antibody does not occur. It should also always be kept in mind that the probe may be passed to other cells if the primary labelled cell dies and is endocytosed by macrophages [129]. Second, for organ imaging, overcoming the delivery barrier can be challenging, especially for neural imaging when the blood–brain barrier has to be crossed. Finally, most of the contrast agents that are nowadays used in clinical practice have a fast clearance after injection [130]. This does not allow a long follow-up with MRI.

Labelling protocol

A large variety of labelling methods have been described [131]. There are so many different protocols because each differs depending on which transfection agent is being used, as well as which contrast agent is chosen, and furthermore, which preferred labelling method is used *i.e.* specific, unspecific, *in vitro* or *in vivo*. Moreover, the relative dosage of contrast agents used to label cells, as well as the incubation time, varies strongly between studies. For example, the dosage for labelling cells with iron particles may vary from 1 to 2,800 µg/ml. Furthermore, labelling times of between 1 and 72 h have been described [114, 132, 133]. The combined effect of labelling dose and labelling time determines labelling efficiency and/or toxicity, and depends on the cell type used. The labelling method used may influence the compartmentalisation of the probe within the cells, and consequently the fate/behaviour and toxicity of the probe. *In vitro*, it seems important to quantify the amount of label per surface area instead of the concentration of label in the fluid above the cells.

Not all contrast agents that are used to label cells are commercially available. Studies that use non-commercially available probes have their own manufacturing protocols. Therefore, not only the protocol used to label the cells may vary a lot, but also the method of preparing the desired paramagnetic nanoparticles. What should be kept in mind is that the preparation protocol of iron-coated particles, for example, will lead to different physicochemical properties.

Labelling efficiency

It is important to check the efficiency of cell labelling, as more label will in general lead to better visualisation with MRI. Labelling efficiency can be assessed by two different approaches, by evaluating how many cells are labelled or by assessing how much label is incorporated within the cells. It is very easy to look at the percentage of labelled

cells of the population of interest. This can be done by counting the labelled cells under light, fluorescent or electron microscopy. However, to quantify the amount of contrast agent inside the cell, more laborious measurements need to be performed. It should be kept in mind that the labelling efficiency is dependent on many parameters such as cell type, labelling method and labelling protocol.

To visualise iron in the cells/tissue after labelling, Prussian blue [35] staining is a widely used and easy method. Using potassium ferrocyanide in acetic acid or by dilute mineral acid hydrolysis, ferric ions are released from protein-bound tissue deposits. The non-haemoglobin-bound iron reacts with potassium ferrocyanide to form potassium ferric ferrocyanide ($FeCl_3 + K_4Fe(CN)_6 \rightleftharpoons KFe[Fe(CN)_6] + 3KCl$). This is an insoluble, blue compound known as Prussian blue (Fig. 1b). The intensity of the colour gives some indication as to the amount of iron (Fe_{3+}); however, other sources of iron besides Fe_{3+} will also have to be demonstrated. For example, Fe_{2+} ions do not produce a coloured reaction product and will therefore not be visualised by Prussian blue staining [134].

Another similar staining assay that can be used to quantify the amount of iron in the cells is the QuantiChrom iron assay [135]. This method utilises a chromogen that forms a blue complex specifically with Fe^{2+} . The Fe^{3+} in the sample is reduced to Fe^{2+} , and therefore the assay will measure total iron concentration. The intensity of the colour, measured by light spectrophotometry, is directly proportionate to the iron concentration in the sample.

By performing atomic absorption spectrophotometry the amount of iron can also be quantified [136]. In order to analyse for any given element, a lamp is chosen that produces a wavelength of light that is absorbed only by that element. The technique usually uses a flame to atomise the sample. The electrons of the atoms in the flame can be promoted to higher orbitals by absorbing energy. This amount of absorbed energy is specific to the particular electron transition in a particular element. If any ions of the given element are present in the flame, they will absorb light produced by the lamp before it reaches the detector. As the quantity of energy put into the flame is known, and the quantity remaining can be measured, it is possible to calculate how many of these transitions took place. Therefore, the amount of light absorbed depends on the amount of the element present in the sample. The signal obtained is proportional to the concentration of the element that was measured.

Inductively coupled plasma mass or optical emission spectroscopy (ICP-MS or ICP-OES) can be used to quantify the amount of contrast agent (gadolinium or iron for example) in the cells/tissue after labelling [13, 40, 137]. Inductively coupled plasma spectroscopy is based on optical emission spectroscopy. Emission spectroscopy is a technique that examines the wavelengths of photons emitted by molecules during their transition from an excited state to a lower energy state. Each element emits a

characteristic set of wavelengths according to its atomic structure; by observing and quantifying these wavelengths the elemental composition can be determined.

With nuclear MRI relaxation, labelling efficiency can also be determined [13, 40, 137]. There are differences in relaxation times between for example tissue and labelled cells; therefore, with NMRI, the spin relaxation can be measured. T1 relaxation refers to nuclei that return to the thermodynamic state in the magnet. T2 relaxation refers to precessing nuclei that fall out of alignment with each other and stop producing a signal. The sensitivity of the technique depends on the strength of the magnetic field.

Toxicity assessment

For molecular and cellular imaging, nontoxic biocompatible contrast agents are needed to safely and effectively use MRI for cell tracking. To measure toxicity in vitro, cell survival, cell proliferation and/or cell function have to be monitored. A variety of assays are available to assess potential adverse effects.

Cell survival

Cell viability can be defined as the number of healthy cells in a sample. Whether the cells are actively dividing or functioning is not distinguished. Cell survival can be assessed in several different ways (Table 2). For example, using light microscopy by counting the number of cells and using trypan blue exclusion [138] (Table 2). Trypan blue is a negatively charged chromophore that only interacts with nonviable cells. In this assay, cell viability is measured by the ability of cells with uncompromised membrane integrity to exclude the dye. This assay is very simple

and cheap. However, each individual sample must be counted and it only stains necrotic or very late apoptotic cells.

Using spectrophotometry the conversion of tetrazolium salts into formazan can be measured (Table 2) [139]. Formazan is a light-absorbing product formed by reduction of tetrazolium salts in mitochondria. This reduction takes place only when mitochondrial enzymes are active, and therefore the amount of formazan formed can be directly related to the number of living cells. However, one has to keep in mind that under nonideal cell culture conditions, mitochondrial metabolism may vary greatly because of the metabolic state of the cells [139]. The measurement obtained should therefore always be compared with unlabelled controls. There are several commercial kits available for measuring metabolic conversion of tetrazolium salts into formazan, e.g. MTT assay, XTT assay and WST-1 assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is an inexpensive assay and MTT is metabolised by all cells into formazan crystals that have to be solubilised before measurement. However, cells with low metabolic activity must be used in high numbers. XTT and WST-1 are tetrazolium salts that form soluble formazan products; however, they are not metabolised by all cell types.

With fluorescence microscopy the exclusion of a fluorescent vital dye can be assessed. Fluorescent vital dyes, like ethidium homodimer-1 or propidium iodide, only enter nonviable cells [140] (Fig. 1d–i). The propidium iodide exclusion assay (Table 2) is a quick and cheap assay method to distinguish viable from dead cells by fluorescence microscopy and flow cytometry. The interaction of the fluorescent vital dye or apoptosis marker with the cell can be assessed. Only a small fraction of total cells from a cell population is required; however, each individual sample must be counted.

Table 2 Assays for assessing cell survival/cell viability

| | Name | Biological principle | Advantages | Disadvantages |
|----|---|--------------------------------|--|---|
| 1 | Trypan blue exclusion assay [8, 10, 114] ^a | Cytoplasmic membrane integrity | Simple Inexpensive | Laborious Stains only necrotic or late apoptotic cells |
| 2a | MTT assay [7, 21] | Metabolic activity | Detects living cells Suitable for all cell types High throughput | Signal is dependent on cell activation |
| 2b | XTT assay/WST-1 assay [5] | Metabolic activity | Detects living cells High throughput Fast assay | Signal is dependent on cell activation Not metabolized by all cell types |
| 3 | Propidium iodide exclusion [7, 9, 114] | Cytoplasmic membrane integrity | Simple Inexpensive High throughput | Toxic compound (health hazard) |

^aReference papers

Some of the assays used for the assessment of cell survival provide indirect measures of cell survival. For each assay, calibration/validation of the assay for the specific cell type or assay conditions is required, otherwise false positive or false negative results may be obtained, e.g. reduction of tetrazolium into formazan by mitochondria is used as a measure of cell number. The rate of mitochondrial metabolism may, however, change without a corresponding change in cell number.

Cell proliferation

Cell proliferation is the measurement of the number of cells that are dividing in a culture as well as the speed of cell division. Cell proliferation can also be assessed using different techniques (Table 3). One way of measuring proliferation is by performing clonogenic assays [141]. A defined number of cells are plated on culture dishes in low densities and the numbers of colonies that are formed after a period of growth are counted. Drawbacks to this method are that it is laborious and therefore not practical for large numbers of samples. In addition, if cells divide only a few times and then become quiescent, colonies may be too small to be counted and the number of dividing cells may be underestimated. Alternatively, growth curves could be established by repeated counting of the number of cells

over time by light microscopy (Table 3), which is also time-consuming and laborious [22].

A commonly used assay to measure proliferation is the MTT assay. As with the assessment of cell viability, the amount of formazan formed can be directly related to the number of cells. In order to assess cell proliferation, growth curves have to be generated from repeated measures over time. Again, one has to keep in mind that under nonideal cell culture conditions, the metabolic state of the cells and thus formazan formation may vary greatly [139].

Alamar Blue (Table 3) is a safe, nontoxic aqueous dye that is used to assess cell viability and cell proliferation [142]. Because the Alamar Blue assay requires a very simple measurement, it does allow continuous monitoring of cells even after the measurement. Alamar Blue consists of an oxidation-reduction (redox) indicator that yields a colorimetric change and a fluorescent signal in response to a metabolic activity. The extent of conversion is a reflection of cell viability and it can be quantified by its optical density or by fluorescence for greater sensitivity [142].

Cell proliferation can also be monitored by assessing DNA synthesis. The two most commonly used assays are [³H]thymidine incorporation [143] and (5-bromo-2'-deoxyuridine) incorporation [144] (Table 3). During the S phase of cell division the cell undergoes DNA synthesis and replicates its genome. Addition of [³H]thymidine or

Table 3 Assays for assessing proliferation

| Name | Based on | Advantages | Disadvantages |
|--|--------------------|---|---|
| 1 Cell counting [182] ^a | Actual cell number | Simple Inexpensive Actual cell numbers determined | Laborious Large number of cells needed |
| 2 MTT assay [7, 21] | Metabolic activity | High throughput Sensitive (requires only a few cells) | Signal is dependent on cell activation Separate samples per time point needed |
| 3 XTT assay/WST-1 assay [5] | Metabolic activity | High throughput Sensitive (requires only a few cells) Short period of incubation | Signal is dependent on cell activation Not metabolized by all cell types Separate samples per time point needed |
| 4 Alamar Blue® assay [183] | Metabolic activity | High throughput Sensitive (requires only a few cells) Allows continuous/longitudinal monitoring | High sensitivity to buffer conditions |
| 5 [³ H]Thymidine incorporation assay | DNA synthesis | High throughput Sensitive (requires very few cells) | Radioactive |
| 6 BrdU incorporation assay [184] | DNA synthesis | High throughput Sensitive (requires very few cells) | Radicalization may itself lead to sister chromatid exchanges |
| 7 CFSE assay | Protein binding | High throughput Longitudinal measurements possible Different populations within one sample can be monitored | Weak fluorescence after long culture time |

^aReference papers

bromodeoxyuridine (BrdU) to the culture medium provides a way to assess DNA replication. [³H]Thymidine is a radioactive compound and the amount of thymidine incorporated into DNA is determined either by measuring the total amount of labelled DNA in a population, or by detecting the labelled nuclei microscopically. It is a sensitive assay that allows linear measurement of cell proliferation over a broad, logarithmic range. However, a big disadvantage is that the radioactive isotope has a long half-life.

Incorporated BrdU can be visualised by using a monoclonal antibody against BrdU (Table 3). BrdU incorporation and/or distribution can also be measured by colorimetric or chemiluminescent immunoassays. The colorimetric measurement is less sensitive than a chemiluminescence measurement, and it does not allow linear measurement of cell proliferation. It should be noted that the ability to detect a minimum number of proliferating cells depends on the amount of BrdU incorporated into the cells and thus on the labelling period. In most cases, detection requires a labelling period of 2 to 24 h. However, the assay is quite simple.

Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a fluorescent molecule that divides equally over daughter cells, enabling visualisation of cell division [145] (Table 3). When CFSE-labelled cells divide, the intensity of their fluorescence is halved with each successive cell division and therefore a final fluorescence measurement corresponds to the number of divisions the cells have undergone.

When assessing cell proliferation there are potential pitfalls. Similar to assays of cell survival, most assays used are indirect measures of cell proliferation and calibration/validation of the assay is required. Therefore, different outcomes of toxicity assessment with identical cell labelling protocols can be obtained with different assays [9].

Cell function

Besides evaluating cell survival and cell proliferation after cell labelling, the third and very important requirement for assessing toxicity is measurement of cell function. To

assess primary cell function cytokine production [19, 71, 137] (Table 4), cytolytic activity or tube forming capacity [9, 146] (Table 4) can be evaluated. Furthermore, the differentiation potential [40, 49, 51, 53] (Table 4) and the capacity to migrate or home [15, 40] (Table 4) to appropriate target tissues can be tested. In general, although all the assays described provide complementary information about cell function, not all tests are relevant for every cell type and testing one of the assays described is enough to make a general statement about cell function after labelling.

Cytokines are proteins that are used extensively in cellular communication and play an important role in the development and functioning of the immune system, in various pathologic processes and are also involved in several developmental processes during embryogenesis. Cytokine production can be measured in the supernatant of cultured cells by using cytokine ELISA plates [129]. These are sensitive enzyme immunoassays that can specifically detect and quantify the concentration of soluble cytokine and chemokine proteins; however, they do not provide information concerning the biological potency of the proteins detected.

Cellular differentiation is a complex process involving growth arrest, exit from the cell cycle and expression of differentiated cell-type-specific functions. Especially when using stem cells, it is important that the labelling procedure does not affect their ability to differentiate after injection *in vivo* [53]. In differentiation assays cells are cultured in a special medium containing specific growth factors that induce cellular differentiation. After several days or weeks the differentiation of the cells into a specific lineage can be confirmed and/or assessed by looking at the cell phenotype, the morphological appearance and the expression of cell lineage markers or specific receptors, using lineage-specific dyes or antibodies (Fig. 1j–l).

Angiogenesis is altered as a result of certain diseases such as cancer, diabetic retinopathy and rheumatoid arthritis, which results in excessive or insufficient blood vessel formation. Tube formation is a multistep process involving cell adhesion, migration, differentiation and growth that is very important in angiogenesis. Tube formation capacity

Table 4 Assays for assessing cell function

| | Functional capacity | Most relevant for ^b | Limitations and pitfalls ^c |
|---|--|--|--|
| 1 | Cytokine production [19, 71, 137] ^a | Haematopoietic lineage cells | Biological potency of cytokines undetermined |
| 2 | Tube formation capacity [9, 146] | Stem cells Endothelial precursor cells | Quantification difficult |
| 3 | Cellular differentiation [49, 51, 53] | Stem cells | Lengthy assay (cell culture may take up to weeks) |
| 4 | Cell migration [15, 40] | Haematopoietic lineage cells Stem cells | No discrimination between chemotaxis or chemokinesis |

^aReference papers

^bCell types for which the listed functional capacity is most central or essential

can be measured with a so-called Matrigel assay [147]. Matrigel is a solubilised basement membrane preparation that is rich in proteins. The major components are laminin, collagen and growth factors. It is mainly used for cell invasion and tube formation assays. The tube formation assay is based on the ability of cells to form 3D capillary-like tubular structures when cultured on the gel of basement membrane extract (Fig. 1h). Quantification can be performed by manually counting the number of cell clusters and branching under the microscope or automatically with software. However, quantification is not easy because of the complex 3D structures that are formed.

Cell migration is crucial for several processes within the human body such as embryonic development, the inflammatory immune response, wound repair, tumour formation and metastasis. One of the most frequently used in vitro assays to assess cell migration capacity is the filter assay. The assay involves a two-compartment system where cells may be induced to migrate from an upper compartment through a membrane with small pores into a lower compartment [148]. The assay is based on a chemotactic response of cells. However, chemotaxis, which is directed movement, and chemokinesis, which is an undirected increase in migratory activity, cannot be discriminated with this assay. This is because an equilibrium of the chemo-attractant between the two compartments will eventually be formed. Cell migration through the membrane can be measured by staining and quantifying the cells that attach to the other side of the membrane.

Image acquisition

An MRI machine is a versatile imaging system that can integrate a broad range of specialised applications, providing a wide basis for the diagnosis, staging or monitoring of pathological conditions or biological processes, including cell imaging. Its versatility, however, also makes it complex and many factors may influence the information obtained during image acquisition.

MR field strength

In vivo imaging of iron-labelled cells has been demonstrated at magnetic field strengths ranging from 0.5 T to 17 T (Table 5). In MR imaging, signal-to-noise (SNR) is proportional to the magnetic field strength, as well as the spatial resolution or imaging time. MR systems with higher magnetic field strengths therefore offer advantages over MR machines with lower field strengths. Nonetheless, it must be kept in mind that higher magnetic field strengths require faster signal sampling to counteract the dramatically increased T2* shortening, e.g. at any air-tissue interface (heart-lung, bowel etc.).

For cell imaging, higher field strength also appears to be beneficial. Reported detection limits of iron-labelled cells are lower at higher magnetic field strengths because of increased susceptibility effects [149]. This higher detection sensitivity may, however, depend on the anatomical context of the labelled cells. At higher field strengths the T1 relaxation of tissue lengthens and T2* shortens and chemical shift effects may increase, each of which in turn may decrease the conspicuity of iron-labelled cells.

It is unclear whether or not higher magnetic field strengths are also beneficial for cell imaging using Gd-based contrast agents. Commercial, Gd-based contrast agents generally have low relaxivity, which becomes even less at higher magnetic field strengths, leading to reduced sensitivity [150]. Nonetheless, success with Gd-labelled cells at high magnetic field strength has been reported [5, 151].

Besides some potential benefits of high magnetic field strengths in cell imaging, the management of higher heat deposition may become problematic, especially if cell imaging/tracking is to be performed effectively in humans. The specific absorption rate (SAR) greatly limits acquisition speed in order to keep heat deposition limited at high magnetic field strengths. Even for applications in animals, there will also be limits that have to be respected. The most frequently used imaging strategies for iron-labelled cells involve T2*-weighted protocols employing gradient-

Table 5 In vivo cell imaging at various field strengths

| Magnetic field strength (T) | Cell type | Contrast agent | Target organ (species) | Reference |
|-----------------------------|--------------------|----------------|-------------------------|-----------|
| 0.5 | MSC | Ferumoxide | Heart (rat) | [52] |
| 1.5 | MSC | Ferumoxide | Kidney (rat) | [152] |
| 3.0 | MSC | Ferucarbotran | Kidney (rat) | [154] |
| 4.7 | Oligodendrocyte PC | MION | Spinal cord (rat) | [153] |
| 7.0 | Embryonic SC | Ferumoxtran | Brain (rat) | [10] |
| 9.4 | T cells | CLIO | Spleen (mouse) | [185] |
| 11.7 | Dendritic cells | SPIO | Skeletal muscle (mouse) | [137] |
| 17.6 | Embryonic SC | VSOP | Brain (rat) | [186] |

MSC mesenchymal stem cell, PC precursor cell, SC stem cell, MION monocrySTALLINE iron oxide nanoparticles, CLIO cross-linked iron oxide, SPIO superparamagnetic iron oxide, VSOP very small superparamagnetic iron oxide particles

recalled echo (GRE) sequences. Such imaging strategies are not radiofrequency (RF) intensive (low flip angle regime). However, steady-state free precession (SSFP) sequences (using high flip angles with short repetition times, TR) on the other hand, are SAR intensive. Also, T1-weighted protocols that may be used in the future with cells labelled with contrast agents providing short T1 relaxation times are SAR intensive.

MR imaging hardware

Generally, higher magnetic field strengths are being used to improve the balance among SNR, resolution and imaging time. Alternatively, SNR can be improved by using better performance (multichannel) receiver systems. Small loop coils with a diameter of less than 1 cm make it possible to image single labelled cells in vitro (single cell level detection with positive or negative contrast) at voxel sizes in the order of $40 \times 40 \times 100 \mu\text{m}^3$ and acquisition times of less than 30 min even at 3.0 T [9]. To provide optimal image quality over larger fields-of-view phased-array-combined small surface coils can be used. Generally, these are more effective than volume coils for providing optimal SNR. The gain in imaging speed that is possible with the smaller coils can be traded effectively for higher in-plane and through-plane resolution in molecular imaging applications. It must nonetheless be kept in mind that the smaller the loop coil diameter is, the less the sensitivity volume will be; signal reception (sensitivity volume) is limited to about the radius from the surface coil.

MR sequences

Different acquisition sequences allow manipulation of contrast mechanisms and possibilities for signal amplification strategies for cell imaging in conjunction with detailed anatomical and functional information. Tissue contrast can be generated mainly through differences in proton density and relaxation times (T1 and T2) by adjusting the MR pulse sequence timings to provide a suitable contrast to distinguish target structures. Imaging of iron-labelled cells is commonly performed using T2- and T2*-weighted imaging employing gradient echo or spin-echo sequences [7, 10, 15, 114, 132, 152–154] (Fig. 2d). For Gd-labelled cells T1-weighted spin-echo sequences are most commonly used [5, 14, 39, 116, 151] (Fig. 2); however, T2-weighted spin-echo sequences may also be employed [8, 155]. Which type of contrast weighting will be most beneficial for the imaging of Gd-labelled cells may depend on the local Gd concentration or potentially the intracellular compartmentalisation of the Gd [40, 116].

Improved detection sensitivity of labelled cells may be obtained with the more sophisticated steady-state free precession (SSFP) imaging techniques, also known as

FIESTA or True FISP [20, 48, 155–159]. However, it must be taken into account that with SSFP images, contrary to the more conventional spin-echo or gradient-recalled echo sequences, high resolution imaging at reasonable image quality is difficult to achieve (off-resonance effects on signal behaviour) on clinical imaging equipment unless more potent (customised) gradient insert coils are used.

Recently, new acquisition approaches have been pursued to generate positive contrast from traditionally ‘negative contrast’ materials such as iron oxide nanoparticles [160–163]. In *in vivo* applications it sometimes proved to be difficult to identify the presence of iron-labelled cells due to the presence of other hypointense regions. In order to facilitate the detection of iron-labelled cells in such regions, investigators have used spectrally selective RF pulses to excite and refocus water off-resonance in regions near the labelled cells to obtain positive contrast [160, 163]. Alternatively, so-called white marker imaging has been implemented, in which traditional GRE sequences are modified to include a variable dephasing gradient, also resulting in positive contrast [161, 162].

Unsolved issues

While cell imaging by MRI has already carefully entered the clinical arena, several challenges still need to be met in order to turn cell imaging by MRI into a robust technique either in experimental settings or clinical applications. These challenges comprise the ability to quantify the number of labelled cells, the ability to distinguish viable iron-labelled cells from other sources of iron or susceptibility artefacts in tissue and the assessment of long-term effects of intracellular contrast agents in terms of toxicity.

For the development and validation of cell-based therapeutic strategies, quantification of the number of cells, homing to or residing in the target organs, is of crucial importance. For iron-labelled cells quantification of cell numbers is feasible under controlled (*in vitro*) conditions, using T2- or T2*-mapping approaches [164, 165]. However, robust *in vivo* quantification using such MR relaxometry approaches is hampered by a variety of factors: variations in tissue T2 and T2* values as a result of field inhomogeneities, and physiological status [165], density distribution of labelled cells [166] and changes in intracellular iron loads as a result of cell division or iron metabolism [166]. Several potential solutions to overcome these complicating factors have been proposed and are currently under investigation [4, 107, 149].

The signal changes caused by labelled cells *in vivo* are unfortunately not unique features. Areas with signal loss or signal gain similar to that produced by labelled cells can also arise from various (patho)physiological conditions such as: deposits of haemosiderin, blood flow and air-tissue interfaces. Identification of labelled cells in or near such areas may therefore be impossible. The use of tailored

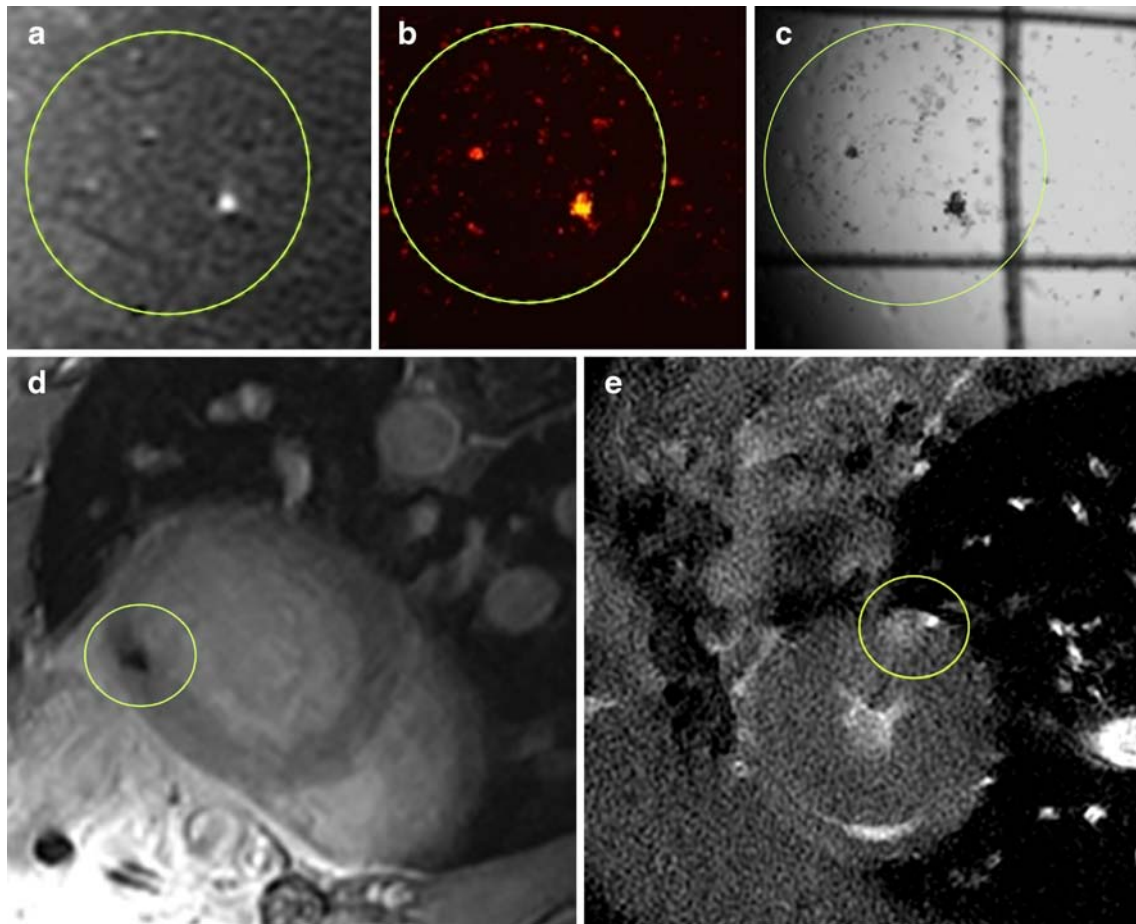


Fig. 2 Magnetic resonance imaging of paramagnetically labelled cells. **a–c** Stem cells in a culture dish labelled with fluorescent nanoparticles containing Gd-DTPA, imaged by MRI (**a**), fluorescent microscopy (**b**) and light microscopy (**c**). Acquisition parameters for MRI: 3D-SPGR sequence with TE=3.0 ms, TR=31.0 ms, $\alpha=50^\circ$ and resolution $142 \times 142 \times 100 \mu\text{m}^3$. **d** MR image of stem cells labelled with iron oxide particles, injected into the myocardium of a

rat heart. Acquisition parameters for MRI: 2D CINE GRE sequence with TE=4.5 ms, TR=18.0 ms, $\alpha=18^\circ$ and resolution $156 \times 156 \times 700 \mu\text{m}^3$. **e** MR image of stem cells labelled with fluorescent nanoparticles containing Gd-DTPA, injected into the myocardium of a rat heart. Acquisition parameters for MRI: 3D T1W GRE sequence with TE=1.5 ms, TR=11.1 ms, $\alpha=50^\circ$ and resolution $215 \times 215 \times 600 \mu\text{m}^3$

sequences [167], ultrashort echo times [168], off-resonance techniques [169] or other new developments in MR hardware or software may provide solutions in this respect. Whether these solutions will also help in distinguishing viable labelled cells from iron deposits released from dead or dying cells or macrophages that have engulfed dead cells is the question. A solution to the latter problem may be provided by MR reporter gene technology [84].

Most studies on cells labelled with contrast agents have demonstrated limited or no acute toxic effects on the labelled cells. It is, however, still unclear whether or not long-term toxic effects occur or whether or not the labelled cells might cause any toxic effects or show compromised function in vivo. In recent papers by Brekke et al. [40] and Modo et al. [170] the need for detailed, long-term, in vitro and in vivo assessment of the effects of contrast agent on cell function was clearly demonstrated. Using a Gd-based

contrast agent (GRID), no detrimental effect of the contrast agent on neural stem cell viability, migratory capacity or multipotency was observed. Nonetheless, proliferative capacity of the cells was reduced, and the in vivo functional capacity of the cells was largely annihilated.

Conclusions

Although cell tracking with the MRI of paramagnetic labelled cells is very promising, there are still many challenges to overcome. The field is moving forwards quickly, and newly developed contrast agents and imaging technologies are very exciting. However, many hurdles will still be faced in translating these new particles and imaging strategies to the clinic. Issues regarding potential cellular and systemic toxicity need to be resolved. For

instance, the recent discussion regarding the link between gadolinium-based contrast agents and nephrogenic systemic fibrosis (NSF) may hamper the licensing of newly developed Gd-based contrast agents. Imaging strategies developed on dedicated animal scanners may prove difficult to transfer to clinical scanners.

For further advancement of the field more systematic approaches and development of standardised (clinically applicable) protocols will be crucial. The focus should not only be on the ability to visualise cells with MRI, but also on methods of quantifying cells and for each (new) labelling approach detailed and thorough assessment of acute and chronic cell functionality should be performed. In order to facilitate comparison between different studies it

would be very useful if the assessment of toxicity (on cell survival, cell proliferation or cell function) could be assessed using similar assays.

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