Delivery of fluorescent probes using iron oxide particles as carriers enables *in-vivo* labeling of migrating neural precursors for magnetic resonance imaging and optical imaging

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Abstract. Iron oxide particles are becoming an important contrast agent for magnetic resonance imaging (MRI) cell tracking studies. Simultaneous delivery of fluorescence indicators with the particles to individual cells offers the possibility of correlating optical images and MRI. In this work, it is demonstrated that micron-sized iron oxide particles (MPIOs) can be used as a carrier to deliver fluorescent probes to cells in culture as well as to migrating neural progenitors *in vivo*. Migrating progenitors were tracked with MRI and easily identified by histology because of the fluorescent probe. These data suggest that using MPIOs to deliver fluorescent probes should make it possible to combine MRI and optical imaging for *in vivo* cell tracking. © *2007 Society of Photo-Optical Instrumentation Engineers.* [DOI: 10.1117/1.2800294]

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1 Introduction

Iron oxide particles are important contrast agents for magnetic resonance imaging (MRI). In particular, there is rapid growth in their use for experimental cell tracking studies (for recent reviews see Frank et al.[1] and Bulte and Kraitchman[2]). Most cell tracking studies by MRI involve labeling cells in culture with an iron oxide contrast agent and transplanting these labeled cells into an animal model.[3–5] Recently direct, *in-vivo* labeling of macrophages[6] and migrating neural progenitors[7] have been demonstrated. The advantage of iron oxide particles as contrast agents is their ability to disrupt the homogeneous magnetic field of nearby water molecules, thus producing a hypointense signal on T2 or T2* weighted MRI. A challenge to producing significant contrast is that there must be efficient loading of iron into a cell to be able to visualize it with MRI.[8] Micron-sized particles of iron oxide (MPIOs) have proven useful for labeling cells for MRI both *in vitro* and *in vivo* because they contain high iron oxide content, up to 10 pg of iron per particle, as compared to 0.5 attograms/particle for commonly used dextran-loaded iron oxide particles. MPIOs are readily endocytosed by many cell types and do not affect their viability or function.[9–11] Indeed, iron labeling of cells with MPIOs is so efficient that recent reports have demonstrated that single cells can be localized with MRI *in vivo*.[10–12] Thus, MPIO labeled cells can provide MRI contrast to map cell location, within the overall anatomy of the tissue.

A challenge for MRI cell tracking studies is to identify the cell that harbors the MRI contrast agent. Histology is performed usually with iron staining to identify the labeled cell. Alternatively, iron oxide particles can be made fluorescent using a variety of labeling strategies.[3,8] Fluorescent MPIOs are commercially available and can be easily identified with histology.[7] A fluorescent iron oxide labeled cell can be phe-

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notyped with simple histology or immunohistochemistry (IHC) using the appropriate antibody. However, often the cell morphology is difficult to delineate in iron oxide labeled cells, and immunofluorecence cannot be performed in living tissue. It would be preferable if the same cell that is labeled with iron oxide for MRI could simultaneously be labeled with a fluorescent probe that delineated the boundaries and morphology of the labeled cell. Recent work by Gan et al.[13] and Jiang et al.[14] have shown that fluorescent dyes can be adhered to gold, tungsten, and iron oxide particles, and biolistically delivered to cells *in vitro*. These procedures have not been extended to labeling cells *in vivo*. Bharali et al. has demonstrated the delivery of a GFP vector *in vivo* to brain cells using silica particles.[15] This has the advantage that only cells loaded with the silica particles and vector express the GFP but suffers from the inefficiency of transfection and shortcomings of transient expression.

In the present work, it is demonstrated that cells can be directly loaded with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) coated on MPIOs via simple incubation. The hydrophobic MPIOs enabled strong adherence of the hydrophobic DiD. The DiD was transferred from the particle to cellular membranes, both plasma and intracellular, after endocytosis, enabling delineation of the morphology and boundaries of the labeled cell. Additionally, it is demonstrated that dye-coated particles could be delivered *in vivo* to neural precursor cells and used to follow their migration from the subventricular zone (SVZ) through the rostral migratory stream (RMS) to the olfactory bulb using MRI. This method was able to identify cell morphology of labeled cells at each stage of migration, with fluorescence resulting from the DiD that had leaked off the MPIO. Finally, fluorescent calcium indicators could be loaded into cells using MPIOs, indicating that a variety of fluorescent probes can be utilized to label cells via iron oxide particles. This approach should lend itself to combined MRI and optical imaging of cell migration and function *in vivo* using *in-vivo* optical approaches based on optical catheters[16] or microlenses.[17]

2 Materials and Methods

2.1 *Dye Coating*

MPIOs were purchased commercially (Bangs Laboratories, Incorporated, Fishers, Indiana). These iron oxide particles have a hydrophobic, polystyrene coat that was on average 1.63 μm in diameter and contained 42.5% iron oxide. A fluorescein derivative, dragon green (ex: 488 nm, em: 520 nm) is trapped in the polystyrene coat. A lipophilic tracer (25 mg/mL), DiD (ex: 630 nm, em: 670 nm; Invitrogen, Carlsbad, California), was added to 2.8×10^9 particles/mL suspended in deionized water to make a 0.5 to 5% (v/v) dye-to-water suspension. The suspension was mixed 2 to 24 h, then rinsed three times, and the particles were resuspended in phosphate buffered saline (PBS) prior to labeling cells or stereotactic injection. The particle concentration was kept constant at 2.8×10^9 particles/mL throughout. The calcium dyes, Rhod 2 (ex: 550 nm, em: 580 nm) and Fura 2 (ex: 335 nm, em: 505 nm; Invitrogen) were coated onto the MPIOs similarly. In addition to1.63-μm MPIOs, Rhod 2 was coated onto 0.5-μm iron oxide silica particles (G.Kisker GbR, Steinfurt, Germany).

2.2 *Cell Culture*

Cell lines were purchased from ATCC (Manassas, Virginia). C6 glioma and PC-12 pheochromocytomas were grown in Ham's F12K medium and supplemented with 2-mM glutamine, 1.5-g/L sodium bicarbonate, 15% horse serum, and 2.5% fetal bovine serum. B35 neuroblastomas were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4-mM L-glutamine, 1.5-g/L sodium bicarbonate, 4.5-g/L glucose, and 10% fetal bovine serum. Cells were incubated at 37°C and 5% CO_2. Approximately 50,000 dye-coated particles were added to 250,000 cells 2 to 24 h prior to imaging. After imaging, cells were fixed using 4% paraformaldehyde/PBS solution (Fisher Scientific, Fairlawn, New Jersey). For calcium dye studies, images were taken every 200 μs as a 100-mM glutamate solution was added to the media (DMEM/F12). Fluorescence images were obtained on a Zeiss LSM 510 confocal microscope (Carl Zeiss Incorporated, Germany).

2.3 *Animal Injections*

19, 6-week old Sprague-Dawley rats (Charles River Laboratories, Incorporated, Wilmington, Massachusetts) were stereotactically injected with the DiD-coated MPIOs. Coordinates for injections into the lateral ventricle were based on work by Shapiro et al.[7] and were 2-mm caudal from Bregma, 2 mm to the right, and −3 mm from the dura. 50-μL (1.4×10^8 MPIOs) were injected into the ventricle of each rat. All the experiments with animals were in compliance with protocols approved by the National Institutes of Neurological Disorders and Stroke Animal Care and Use Committee.

2.4 *Magnetic Resonance Imaging*

Two weeks following postinjection, MRI data were acquired on an 11.7 T animal MRI system (30-cm 11.7 T horizontal magnet, Magnex Scientific, Oxford, England, MRI Electronics, Bruker Biospin, Billerica, Massachusetts, and 9-cm 3-D gradients, Resonance Research Incorporated, Billerica, Massachusetts) using a volume transmit coil and a custom-built, 2.5-cm-diam, receive-only surface coil. Animals were placed in a MRI compatible cradle with a stereotactic head frame. The animals were continually under anesthesia using 2 to 3% isofluorane in 75% oxygen/25% medical air throughout the duration of the experiment. Animals were mechanically ventilated at a rate of 66 breaths/min after intubation, and their steady-state CO_2 was monitored. Body temperature was maintained at 37°C by using a circulating water bath with a rectal temperature feedback probe. The following parameters were used to acquire 3-D gradient echo images: TE 8 ms, TR 30 ms, FOV 1.92 cm^3, and 256^3 matrix (75 μm isotropic resolution).

After the *in-vivo* MRI acquisitions, animals were transcardially perfused with PBS, followed with 10% formalin that contained 1-mM Gd-DTPA. Brains were removed with the olfactory bulb intact and post-fixed for at least 6 h in the same formalin/Gd-DTPA solution. Brains were placed in saline and imaged using a 35-mm volume coil with the following pa-

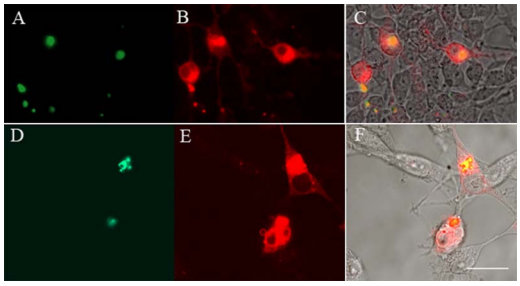

Fig. 1 (a), (b), and (c) B35 neuroblastoma cells and (d), (e), and (f) PC-12 pheochromocytoma cells labeled *in vitro* with MPIOs that have been coated with a lipophilic tracer, DiD. (a) and (d) Dragon Green fluorescence from the MPIOs. (b) and (e) Fluorescence from the DiD that has stained the cell morphology. (c) and (f) The fluorescent images overlaid with the bright field image. The scale is constant throughout the panels with the scale bar in (f) representing 20 μm.

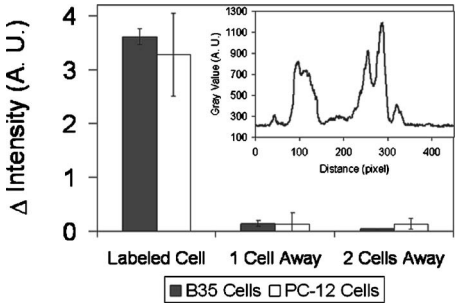

Fig. 2 Change in intensity compared to background (no cells) of both B35 cells and PC12 cells (n=3). Inset: plot of the gray value profile from DiD-labeled cells in Fig. 1(b), which passes over five cells, two of which were colabeled.

rameters: FOV 3.08 cm^3, 256^3 matrix, TE 8 ms, TR 30 ms, and 20 averages.

2.5 *Histology*

Brains were placed in a 30% sucrose solution. After 2 to 3 days, samples were processed for frozen sections (Histoserv, Incorporated, Germantown, Maryland). 16-μm sagittal sections were cut that included the olfactory bulb, RMS, and SVZ. No further processing was done to the tissue sections.

3 Results

3.1 *Fluorescence of 1,1′-Dioctadecyl-3,3,3′, 3′-Tetramethylindodicarbocyanine Perchlorate/Micron-Sized Particles of Iron Oxide*

MPIOs, with a polystyrene matrix, were coated with a lipophilic tracer, DiD, to test whether passively delivering MPIOs to cells could result in fluorescent agents being transferred to the cell. In PBS, greater than 90% of the DiD remained coated on the polystyrene coated particle after 5 h of suspension (data not shown). The coated MPIOs were added to cell cultures where the particles were allowed to incubate with the cells for several hours in a manner that only labeled approximately 30% of the cells. One to five particles were found to be internal to the cells, presumably due to endocytosis by the cells after the incubation period. Cells that contained the dye-coated MPIOs are shown in Fig. 1; MPIOs appear as green fluorescence and DiD shows up as red fluorescence. For the cells that are labeled, the entire cell is labeled with DiD, enabling the boundaries and morphology of the labeled cells to be visualized. To investigate the cell specificity of this colabeling process, a few different cell lines were investigated. Rows 1 and 2 in Fig. 1 are images of B35 neuroblastomas and PC-12 pheochromocytoma cells, respectively, that have been colabeled with the MPIO and DiD. These data indicate that the mechanism for colabeling is cell line independent.

Neighboring cells that had not incorporated MPIOs showed no evidence of fluorescence from the DiD, even at greater than 90% confluence. This indicates that MPIOs can act as a dye carrier, which is specific to the individual cells that get labeled with MPIOs. The specificity of DiD labeling is illustrated graphically in Fig. 2. The intensity of the MPIO containing cells was compared to the intensity of the neighboring cells. The DiD fluorescence intensity is at background intensity levels for cells that surround the MPIO labeled cells. Based on these results, it is clear that DiD comes off the MPIO only into the specific cell that has been labeled with the MPIO. Therefore, DiD labeling is specific for MPIO labeled cells.

3.2 *Magnetic Resonance Imaging and Fluorescence of 1,1′-Dioctadecyl-3,3,3′, 3′-Tetramethylindodicarbocyanine Perchlorate/ Micron-Sized-Particles of Iron Oxide*

Migrating rodent neuroblasts have recently been labeled with MPIOs for MRI.[7] These cells are known to change their morphology from a spindle-shaped cell to a fully differentiated neuron as they migrate.[18] DiD-coated MPIOs were injected into the rat lateral ventricle to test whether the particles would be endocytosed by the migrating neural progenitors *in vivo* and whether the neuroblast morphology could be identified by the DiD in subsequent histology. Serial MRI of a rat brain that was injected with dye-coated MPIOs is shown in Fig. 3. The hypointense signal in the MRI is a well-known effect of the iron oxide particles and is exploited to observe the neural progenitor cell migration *in vivo*. The large dark region in the ventricles is a result of the large number of MPIOs remaining around the ventricular region. The hypointensities detected from the subventricular zone to the olfactory bulb along the rostral migratory stream are due to migrating neural progenitors that have been labeled with MPIOs (arrows in Fig. 3). MRI of fixed brain tissue confirmed the presence of MPIOs in the RMS (data not shown). These data are consistent to those previously reported, which used standard MPIOs that were not adhered with DiD.[7] Therefore, DiD labeling of MPIOs did not block their uptake into neural progenitors and subsequent migration.

Figure 4 shows the merger of MRI slices through the RMS taken two weeks postinjection, and the corresponding fluorescent images that were taken at separate points along the migration pathway of the neuroblasts. Figure 4(b) confirms that there is a high density of MPIOs around the ventricle. Many cells in this region are colabeled with DiD. In this figure, there appears to be many DiD-labeled cells that do not contain MPIOs; however, many of the MPIOs are out of plane and visualized with deeper z sectioning with the confocal micro-

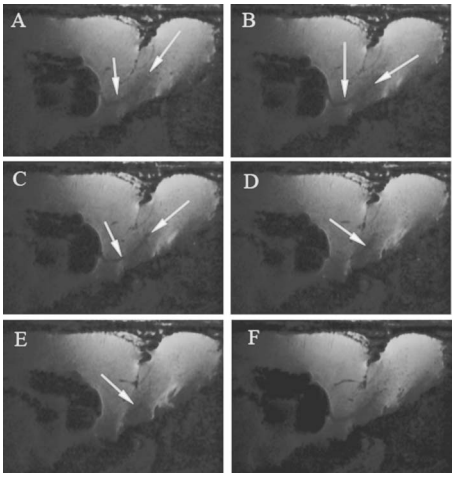

Fig. 3 MRI through the RMS in an animal that was injected with the dye-coated MPIOs. (a) through (e) are 75-μm serial images encompassing the RMS. (f) is the merge of serial images. MRI was performed at 11.7 T using 3-D gradient echo images at 75-μm isotropic resolution. Images were taken two weeks after injection of DiD-labeled MPIOs. Arrows indicate loss of signal due to migration of the MPIO-labeled neural precursors throughout the RMS.

scope (data not shown). Nonetheless, this does not discount the possibility of having DiD-only cells. In this region, the cell morphology is difficult to delineate, because many cell types are labeled including neural precursors; however, only neural progenitor cells will migrate rostrally from the ventricle to the olfactory bulb.[19] The well-defined lines of hypointense signal in the MRI adjacent to the ventricle are a result of MPIO-labeled neuroblasts in the white matter track known as the rostral migratory stream (RMS). Lois, Garcia-Verdugo, and Alvarez-Buylla have described these neuroblasts in RMS as having a spindle shape and moving in closely spaced chains.[18] Fluorescent images taken from sections of the RMS are shown in Fig. 4(c). This figure shows colabeled cells that are in close proximity to one another and

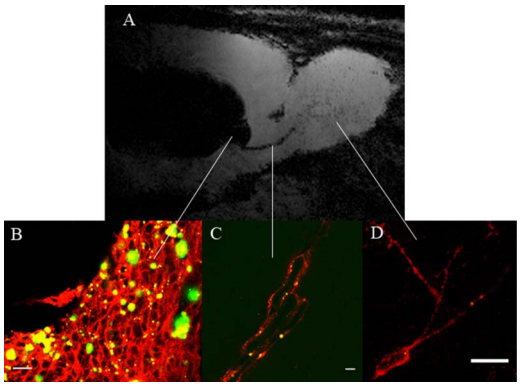

Fig. 4 MRI of endogenous neural progenitors in the RMS and olfactory bulb, and the corresponding fluorescent images from the histology slices. (a) Adjacent MR slices along the right RMS were merged to create this figure. (b), (c), and (d) are fluorescent images from histological sections showing cells in the rat brain labeled with the MPIOs (green) and stained from the DiD (red). Fluorescent images show (b) the SVZ, (c) spindle-shaped cells in the RMS, and (d) an individual neuron in the olfactory bulb. Scale bars represent 20 μm.

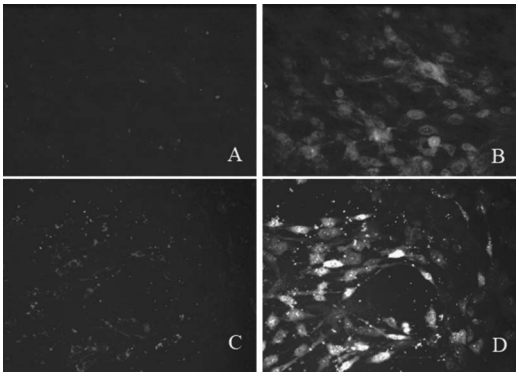

Fig. 5 Fluorescent images of C6 glioma cells that contain calcium-sensitive dyes that were delivered by MPIOs. (a) and (b) show response of Fura-2 labeled cells to a glutamate-induced calcium wave, and (c) and (d) show the response of Rhod-2 labeled cells to a glutamate-induced calcium wave. The polystyrene particles were coated with Fura-2, and silica particles were coated with Rhod-2. The image pairs were acquired using the same field of view and acquisition times.

have the characteristic closely spaced, spindle shape. Additionally, this figure shows that the DiD and MPIOs are confined to the RMS pathway. Once the neuroblasts reach the olfactory bulb, they migrate radially and become fully differentiated neurons.[20] Figure 3(d) shows a fluorescent image taken in the olfactory bulb. This cell's morphology is defined by the DiD and is colabeled with the MPIOs. This distinct morphology is that of a neuron with many processes being extended, indicating that labeling with MPIOs did not affect morphological transitions in neural progenitors.

3.3 *Calcium Indicator Delivery with Micron Sized Particles of Iron Oxide*

Fura-2 and Rhod-2 are commonly used Ca^{2+} indicators, and these were tested to see if the process of passive dye delivery to cells could be mediated with MPIOs. Both indicators were applied using the same method as the DiD experiments. The Fura-2-coated MPIOs and the Rhod-2-coated MPIOs were separately incubated with C6 glioma cells. Only the culture incubated with the Fura-2-coated MPIOs labeled the cells, as shown in row 1 of Fig. 5. There was no evidence of Rhod-2 labeling the cells; instead, the dye remained on the MPIOs (data not shown). A silica-coated iron oxide particle was used in place of the polystyrene particles to test whether the MPIO coating material would make a difference in the ability of Rhod-2 to disassociate from the MPIOs. Figure 5 row 2 shows that the silica MPIOs were able to colabel C6 gliomas with Rhod-2.

The response of the calcium dyes was tested to determine whether the MPIO-mediated delivery enabled monitoring a cellular calcium transient. C6 cells were incubated with the Ca^{2+} dye-coated MPIOs long enough for the calcium dye to diffuse off the particle to stain the cell (2 to 6 h). A calcium wave was invoked by applying 100-mM glutamate[21] to the cell culture. The first column of Fig. 5 shows the fluorescent image prior to the addition of glutamate. There is little fluorescence from the cells at this gain setting, and only particles are visible because they still contained a high concentration of

dye. Once the glutamate is added, the fluorescence increases (Fig. 5 column 2) as the calcium wave propagates across the sample. Therefore, MPIO mediated dye delivery does not interfere with monitoring a cellular response to calcium.

4 Discussion

In this study, it is demonstrated that MPIOs can be used to deliver fluorescent dyes to individual cells. The hydrophobic properties of the MPIO coat and the fluorescent probes were used to adhere the probes to the particles by incubation. Once the MPIOs are taken up by cells, the fluorescent dye can come off, presumably due to the hydrophobic membrane that the particle comes in contact with during and after it is endocytosed. Evidence for the specificity of the dye diffusing off the particle onto the cell membrane of only the MPIO labeled cell is shown in Fig. 1 where MPIO+ cells are colabeled with DiD. Cells that did not have MPIOs also did not accumulate DiD. This process of dye delivery with iron oxide may be cell line independent, as multiple cell lines could be labeled with DiD. However, delivery of different dyes was found to be dependent on the iron oxide coating material. The results with Rhod-2 suggest that the dependence of dye delivery is probably due to the hydrophobicity of the dye and the MPIO coating. Further engineering of the MPIO coating should enable a broad range of fluorescent dyes to be delivered to cells.

DiD-coated MPIOs were delivered *in vivo* to neural progenitors in the rat subventricular zone. Previous work demonstrated that MPIOs could be used to label migrating neuroblasts for MRI.[7] Furthermore, it has been demonstrated that DiI (an analog of DiD) could be injected into the subventricular zone to label migrating neuroblasts directly.[19] As was the case in culture, following injection into the ventricle, only cells with MPIOs were found to be labeled with DiD in the subventricular zone, along the RMS, and in the olfactory bulb. Both undifferentiated neuroblasts and differentiated neurons that were colabeled with DiD and the MPIOs [Figs. 4(c) and 4(d)] were identified based on morphology. Neighboring cells outside the RMS did not contain DiD or MPIOs. Despite a large network of neurons in the olfactory bulb, only the cell that contains the MPIO was labeled with DiD, verifying the specificity of the labeling. These data suggest that in these experiments, MPIOs can be used to mediate the delivery of fluorescent dye to migrating neurons *in vivo*. This demonstrates the advantage of this technique. First, MPIO-directed dye labeling of cells can be performed *in vivo*, where as immunofluorescence remains strictly an *ex-vivo* technique. Secondly, this technique derives some specificity as the dye delivery is MPIO mediated. The simplicity of MPIO-aided delivery allows for the possibly of using other probes to label cells *in vivo*. Adhering DiD to the MPIOs enables both MRI and optical imaging of the migration of this important class of neural progenitors. Indeed, it will now be possible to use MRI to guide high-resolution optical imaging of cell morphology and function, as measured by calcium probes, along the whole migratory pathway of these cells. The optical imaging will require new techniques based on catheters or microlens-based microscopes.[16,17]

There are several challenges associated with the MPIO-assisted fluorescence labeling of cells. In this work, only cells labeled with MPIOs were colabeled with DiD. However, the possibility remains that during mitosis of the neuroblasts, dye could be transferred to daughter cells without the transfer of MPIOs due to dilution (or vice versa, MPIOs could be transferred without DiD). Another issue is that the particle may only need to come in contact with the external side of the cell membrane for the cell to become labeled with the DiD, which could lead to a DiD-labeled cell without MPIOs. The fact that both in culture and *in vivo* only cells with MPIOs were DiD labeled indicates that endocytosis of the MPIOs likely occurs prior to release of DiD. There is also the possibility that the hydrophobic DiD might form small crystals that could dislodge from the MPIO before coming in contact with a cell. Evidence that greater than 90% of the DiD fluorescence remained with the MPIOs in solution mitigates this concern.

The largest problem that was encountered was that the absorption of DiD to the polystyrene particles caused the particles to aggregate. Given the relatively large size of the particles, this aggregation can affect the availability of the MPIOs for cell labeling. Cells may be less effective at endocytosing large aggregates of MPIOs. The aggregated particles were difficult to inject, and this may account for the relatively low success rate of labeling neural precursors with the DiD-coated MPIOs. Optimizing the DiD concentration minimized this problem to some extent; however, further optimization will be required to eliminate this aggregation. It may be that altering the coating material of the MPIOs will also help to minimize this problem. However, a balance must be met such that the dye's adherence to the particle surface is strong enough to remain on the surface but eventually diffuse off. This problem was encountered with the Rhod 2, which adhered strongly to the polystyrene particles. Replacing the polystyrene particles with silica-coated MPIOs enabled the Rhod 2 to be transferred. For *in-vitro* studies, the MPIO-mediated delivery of calcium probes provides no significant advantage compared to ester derivative calcium probes. However, the possibility of MR imaging to guide the *in-vivo* optical calcium imaging in select cell populations should provide significant advantages.

In conclusion, MPIOs could be used to carry DiD and calcium indicators into cells by simple incubation of the fluorescent probe with MPIOs prior to labeling cells. Using MPIOs as carriers of fluorescent probes leads to highly specific labeling of cells in culture and migrating neural precursors *in vivo*. This procedure should enable a number of new experiments. The ability to define neural connections made by these new neurons within the olfactory bulb can be accomplished, because the neuronal morphology can be defined with the DiD, which would not be possible with a single point source of fluorescence from an MPIO. MRI can be used to guide high-resolution optical imaging of these relatively rare cells, enabling more efficient characterization of their morphology and function both *in vitro* and *in vivo*. Colabeling cells with MPIOs for MRI and fluorescent probes for optical imaging should be applicable to a broad range of precursor cells or circulating stem cells, for example hematopoietic stem cells or embryonic stem cells. The simplicity and ease of use should enable combined MRI and optical imaging of cell migration and cell integration in a wide variety of tissues and in a number of animal models.

Acknowledgments

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