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Serial Monitoring of Endogenous Neuroblast Migration by Cellular MRI

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Abstract

Endogenous neural progenitor cell migration *in vivo* can be monitored using MRI-based cell tracking. The current protocol is that micron sized iron oxide particles (MPIOs) are injected into the lateral ventricle proximal to the neural stem cell niche in the brain. MPIOs are endocytosed and incorporated into the neural progenitor cell population, making them visible by gradient echo MRI. Here this new method is extended to serially quantify cell migration. Initially, *in vivo* cell labeling methodologies were optimized, as high susceptibility effects from the MPIOs generate substantial signal loss around the injection site, masking early migratory events. Then, using improved labeling conditions, a longitudinal study was conducted over two weeks to quantify the migration of labeled progenitor cells towards the olfactory bulb (OB). By 3 days following injection, we calculated 0.26 % of the volume of the OB containing labeled cells. By 8 days, this volume nearly doubled to 0.49% and plateaued. These MRI results are in accordance with our data on iron quantification from the OB and with those from purely immunohistochemical studies.

INTRODUCTION

A neural stem cell (NSC) niche resides in the subventricular zone (SVZ) in the adult mammalian brain (Abrous et al., 2005; Doetsch et al., 1997). Briefly, NSCs residing behind the ependymal cells give rise to transit amplifying progenitor cells, which differentiate into migrating neural progenitor cells (NPCs) (Doetsch et al., 1997). NPCs migrate from the SVZ through the rostral migratory stream (RMS) to the olfactory bulb (OB) (Lois et al., 1996), where, they differentiate into olfactory interneurons in the granule cell and periglomerular layers (Lledo et al., 2008; Lois and Alvarez-Buylla, 1993). At 15 to 45 days after birth in the SVZ, approximately 50 % of the newly generated granule cells die, while the rest can survive up to a year (Petreanu and Alvarez-Buylla, 2002; Winner et al., 2002). In recent years NSC niches have been identified in primates (Kornack and Rakic, 2001; Pencea et al., 2001) and in humans (Baer et al., 2007; Curtis et al., 2007; Quinones-Hinojosa et al., 2006; Sanai et al., 2004).

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MRI has recently been used to monitor endogenous cell migration. Initially, 50 μ l 1.63 μ m micron sized particles of iron oxide (MPIOs) were injected into the lateral cerebral ventricles of rats and imaged at weekly intervals for four weeks (Shapiro et al., 2006a). Contrast was detected in the OB at 1 week, increasing by 2 weeks and maintaining through three weeks. MPIOs were attractive for *in vivo* labeling of NPCs due to a combination of efficient iron loading of the particle, as well as high r_2^* molar relaxivity (Shapiro et al., 2005).

This paradigm has been evaluated further in both rats and mice. MRI of endogenous NPC migration in rats by Sumner, et al, demonstrated that multiple cell types are labeled at the ventricle, including astrocytes, oligodendrocytes, neurons and microglia (Sumner et al., 2009). This study also confirmed the requirement for NPCs for detection of contrast along the RMS into the OB. Yang, et al, lowered the injected volume of MPIOs in a neonatal rat model (10 μ l 0.96 μ m MPIOs), and accomplished detection of migratory events close to the SVZ, yet failed to show significant contrast in the OB at day 14 (Yang et al., 2009). This is likely due to the lower volume which becomes too dilute as cells migrate away from the SVZ and choice of particle, with 0.96 μ m MPIOs consisting of 10 times less iron than a 1.63 μ m MPIO (Shapiro et al., 2004; Shapiro et al., 2005). Panizzo, et al, used SPIO for *in vivo* magnetic cell labeling of NPCs, and while migration near the SVZ was detected, similar to Yang, et al, no migration was observed within the RMS or in the OB, even out to 28 days (Panizzo et al., 2009).

At least two studies have translated the rat experiment to mice. Vreys, et al injected mice with either 10 or 1.5 μ l volumes of MPIOs or MPIOs mixed with transfection agents (Vreys et al., 2010). Taking into account the 10 times smaller size of mice versus rats, these injection volumes are similar to those used for rats. MRI contrast was observed only at five weeks post injection, significantly delayed versus rat experiments, and incongruous with known NPC migration time frame. Nieman, et al, delivered 50 nl MPIOs directly into the RMS, rather than into the ventricle. Robust migration was detected along the RMS at day 1 and into the OB by day 4. Migration rates calculated using MRI showed excellent corroboration with histology (Nieman et al., 2010).

A critical requirement for using MRI-based cell tracking in therapeutic monitoring is a reliable quantification method. Various approaches to quantify numbers of magnetically labeled cells have been reported (Bos et al., 2004; Brisset et al., 2010; Bulte et al., 1997; Dahnke and Schaeffter, 2005; Liu et al., 2009; Politi et al., 2007; Rad et al., 2007), and are reviewed in Liu and Frank (Liu and Frank, 2009). In short, these techniques correlate R_2 or R_2^* relaxometry with iron concentrations. However, there are drawbacks to their utility. The first is that the spatial distribution of particles greatly influences the r_2 and r_2^* molar relaxivity of the particles (Tanimoto et al., 1994). Indeed, this is the principle behind magnetic relaxation switches (Perez et al., 2002a; Perez et al., 2002b). Secondly, the intactness of the particle coating influences molar relaxivity, and so, as particles degrade within the lysosomes, relaxivity will change (Arbab et al., 2005; LaConte et al., 2007). Lastly, particularly for R_2^* measurements, *in vitro* and *in vivo* relaxation rates are challenging to equate due to differences in magnetic susceptibilities between biological tissues and glass tubes containing contrast agent.

The use of MPIOs as the contrast agent provides an alternative mechanism for quantifying cell number in an MRI experiment. Studies have investigated the signal behavior of labeled individual cells, at various resolutions and imaging parameters (Shapiro et al., 2005), culminating with the detection of single cells, *in vivo* (Heyn et al., 2006; Shapiro et al., 2006b). Therefore, the use of MPIOs presents an opportunity to quantify cell number based on spot detection within a three-dimensional volume.

Initial experiments using MRI to track endogenous NPC migration in rats injected large quantities of MPIOs (Shapiro et al., 2006a). While this produced robust contrast of particle-laden cells in the OB, the large susceptibility effect around the ventricles obscured the SVZ and the early portion of the RMS. Thus, early migratory events were challenging to observe and quantify. Furthermore, a coarse temporal sampling was employed, missing early migration events. Here, we labeled the endogenous population of rat NSC/NPCs with lower volumes of materials and quantified the early migration within the first 2 weeks after injection. MRI results were confirmed with both immunohistochemistry and iron quantification.

MATERIALS AND METHODS

Animals and surgical procedure

Animal experiments were approved by the Yale Animal Care and Use Committee. Generally, rats were anesthetized with 3% isoflurane, orally intubated and mechanically ventilated at 65 breaths/minute. Respiratory patterns and end tidal CO₂ were monitored and body temperature was maintained at 37° C by use of a circulating water bath. Within this work, five specific experiments were carried out (summarized in Table 1). The first was optimization of the injection site. We hypothesized that placing injections more anterior and medial than previous studies would avoid particles becoming stuck in the choroid plexus. Green fluorescent MPIOs (1.63 µm, 45% magnetite, styrene/divinylbenzene encapsulated, Bangs Laboratories, Fishers, IN) were stereotactically injected into the right anterior lateral ventricles of adult Sprague-Dawley rats (6 weeks old, 150 g; Harlan, Indianapolis, Indiana) (Shapiro et al., 2006a). MPIOs (20 or 50 µl) were injected at either 1.5 mm caudal, 1.5 mm medial lateral from bregma, 3 mm into the brain, or 2 mm for both transverse axes, 3 mm into the brain (n=3 for each group).

The second study was a dose dependent study, to understand minimal required concentration of particles necessary for robust detection. MPIOs were injected at 5, 10, 20 or 50 µl (corresponding to 1.5×10^7 , 3×10^7 , 6×10^7 and 1.5×10^8 particles, n=6 for each group). The third study was a finely sampled temporal resolution study during early migration. 20 µl MPIOs were injected and rats were scanned at days 1, 2-3, 7-8 and 14 post injections (n=8). Daily ip injections of Bromodeoxyuridine (50mg/kg, BrdU) were delivered during the course of the experiment for immunohistochemical analysis. To corroborate MRI findings, iron content in the OB was measured by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, Bodycote, California) on a separate set of rats following sacrifice at day 1 (n=3), 3 (n=6) and 8 (n=5) post injections of 20 µl MPIOs.

Lastly, to confirm that NPCs were required for the contrast observed in the RMS and OB, experiments were carried out where Ara-C, a potent anti mitotic agent, was delivered to the brain during the experiment (Sumner et al., 2009). Ara-C (Sigma-Aldrich, St. Louis, MO) was infused into the anterior lateral ventricles at a rate of 0.5 µL/h for 2 weeks, via ALZET mini-osmotic pumps (model 2002, Durect Co., Cupertino, CA). Pumps were prepared to deliver either 5% or 10% Ara-C through a cannula extending into the anterior lateral ventricles at the following coordinates: 3.5 mm caudal from bregma, 3.5 mm medial lateral, and 3 mm dorsal. For control rats of sham operations, pumps were loaded with 0.9% saline. One week following pump insertions and without removal of the pumps, the rats underwent a second surgery to inject 20 µl of MPIOs (n=3 each group). Rats were scanned 1 week post injections.

MRI experiments

MRI was performed on an 11.7T Bruker Biospec, Billerica, MA, Paravision software 3.0.2. Separate transmit-only volume (70 mm birdcage coil) and receive-only surface coils (35 mm diameter ring) were used. Under 3% isoflurane anesthesia in 90% oxygen/10% medical air, animals were placed in a custom-built MR compatible cradle. The surface coil was placed directly on the head of animals. Animals were orally intubated and mechanically ventilated at 65 breaths/min. Respiratory patterns and end tidal CO₂ were monitored and body temperature was maintained at 37° C by use of a circulating water bath. 3D gradient echo MRI was performed using the following imaging parameters: FOV 2.56 cm³, matrix 256³ (100 µm isotropic voxel size), TR = 30 ms, TE = 8 ms, 15 kHz acquisition BW. Following MRI, animals were revived and returned to the animal facility. Subsequent MRI scans were performed on days 1, 3, 8 and 14 post injections.

Data analysis

3D data sets were imported into BioImage Suite software (Duncan et al., 2004). First a rigid linear registration was applied to all brains according to a predetermined template. Neuronal precursor cell migration from the SVZ, along the RMS to the OB was serially quantified by detection of dark signal voids in the OB. Using phantom samples we previously demonstrated that single cells labeled with 1.63 µm MPIOs produce a 30% drop in signal intensity in a gradient echo image (Shapiro et al., 2005). Thus, we have defined pixels containing labeled cells by applying a threshold of > 30% decrease in signal intensity relative to the background signal. The background signal in this case was the signal intensity of non-contrast enhanced OB from the same animal. For each 3D data set we created a segmented map of the dark pixels detected in the OB. The occupancy of labeled precursor cells in the OB (in mm³ volume), was normalized to the volume of each OB and presented as volume percentage as a function of time following MPIO injection. Statistical analysis for the significance of change in the occupancy of dark pixels in the OB was performed using the Student's t-test (1 tail, paired).

Immunohistochemistry

At the end of the MRI sessions, rats were transcardially perfused with 0.1 M PBS, followed by 10% formalin. Brains with intact olfactory bulbs were excised and processed for frozen sections. After 2 days of post-fix, brains were transferred into 30% sucrose for 3 days, embedded in TissueTek® embedding compound and frozen. Sagittal sections (16 µm) were cut and stained for neuronal markers. Sections were washed twice in 0.1 M PBS and non specific epitopes were blocked by 1 hour incubation at room temperature with 10% goat serum and 0.1% Triton X-100. Sections were then incubated with the following primary antibodies: rabbit anti doublecortin (1:200 Millipore); rabbit anti IBA1 (1:500, Wako, Richmond, VA). After overnight incubation at 4°C, sections were washed 3 times and incubated for 1 hour at room temperature with secondary antibodies (1:200 goat anti rabbit conjugated to Cy5, Chemicon). Sections were washed with PBS and mounted with Prolong anti fade containing Dapi (Invitrogen). For BrdU staining, sections were pretreated with 2% HCl for 20 minutes at 65 °C, followed by washing with PBS. Sections were blocked with 10% goat serum and 0.3% triton-100 at room temperature for 1 hour. Sections were incubated overnight at 4°C with rat anti-BrdU (1:500, Accurate) followed by secondary antibody 1 hour at room temperature. Fluorescence images were obtained with a Leica TCS SP5 Spectral Confocal Microscope.

RESULTS

Detection of MPIOs in the RMS and Olfactory Bulb is dose and temporal dependent

Previous studies using MRI to monitor endogenous NPC migration injected large quantities of MPIOs into the ventricles. While that protocol enabled detection of cell migration along the RMS and into the OB, the migratory events that occurred adjacent to the injection were masked due to the high susceptibility effect of the particles. In order to improve visualization of early RMS, we first optimized the injection site. MPIOs were injected at either the previously published coordinates (2 mm caudal and 2 mm medial to bregma) (Shapiro et al., 2006a) or at 1.5 mm caudal and 1.5 mm medial to bregma. We evaluated the effect of the two different injection sites on the degree of contrast within the RMS both at high dose of 50 μ l injection (2 weeks post injection), as well as at lower dose of 20 μ l (1 day post injection). In T_2^* gradient echo images of rat brains that were injected at 1.5×1.5 mm more contrast was detected within the RMS (Figure 1). Therefore, we continued our study using these new coordinates for injection of MPIOs.

Next, a dose dependent study to find the optimal injection volume was conducted, taking into account both the need to preserve enough of the blooming effect that enables cell detection, while reducing the susceptibility artifact at the ventricles that masks the specific migration. 3D T_2^* weighted MRI experiments that were performed 1 day after injections revealed that while contrast in the RMS could not be visualized after injections of 5 or 10 μ l MPIOs, dark contrast was detected following injection of either 20 or 50 μ l of MPIOs. Moreover, injection of 20 μ l enabled visualization of more of the posterior RMS compared with the high dose of 50 μ l MPIOs (Figure 2 c and d, arrows). Thus, the optimal labeling dose was found to be 20 μ l (6×10^7 MPIOs total). Interestingly, migration within the RMS could be detected as early as 1 day post injection of MPIOs. The observed signal was tight and reached the entrance to the OB. After 1 week, signal voids were not only detected within the OB, but were also spread out to outer layers (Figure 2 g and h, white circle). The binary quality of the data between 10 and 20 μ l injections, where MRI detection of migrating neuroblasts is largely absent (10 μ l) or robust (20 μ l) is noted and suggests that interventricular pressure from the injection may have some role in *in vivo* labeling efficiency.

Labeled progenitors reach the OB within 3 days and by a week occupy the outer layers: Temporal resolution during early migratory events

In order to resolve early migratory events, a temporal study was performed to determine the rate of NPC migration that can be measured using cellular MRI. MPIOs (20 μ l) were injected into the lateral ventricles of the rats and serial 3D T_2^* gradient echo images were acquired between 1 day and 14 days post injection. At 1 day the contrast from the MPIOs reached the OB and as early as 2-3 days signal voids were detected in the center portion of the OB (Figure 3). By 7 days, signal voids were spread out and could be detected in the outer layers of the OB.

This accumulation was quantified over a period of 14 days following injection (Figure 4). Figure 4a displays the parallel layout of three orthogonal views, allowing anatomical verification of the selected pixels, thus reducing false positive selections. Figure 4b is an overlay of three time points extracted from 3D data sets acquired from an individual rat at days 1, 3 and 8 post injection. The overlay presentation illustrates the progressive spread of dark pixels during 1 week migration. It is apparent that the entry into the OB was rapid, initially occupied the inner layers of the OB and gradually spread into the outer layers. This architectural spread correlates with the anatomical architecture of BrdU positive cells that migrate to the OB (Figure 4d). Furthermore, at 1 day post injection green fluorescent

particles are detected along the RMS extending just beyond the elbow of the RMS (Figure 4d point1). By 14 days post injection these green fluorescent particles are not only detectable farther into the RMS (Figure 4d point2) but also within the OB (Figure 4d point3). Quantification of this dynamic migration (Figure 4c) revealed linear accumulation of dark pixels in the OB during the first week after MPIOs injection. By 3 days following injection, 0.26 % of the volume of the OB contained labeled cells. By 8 days, this volume nearly doubled to 0.49%. At 14 days post injection this accumulation plateaued and no difference was measured in the occupancy in the OB. A similar elevation in iron content was measured by ICP-MS (Figure 5a). Noticeably, the increase in dark spots volume fraction in the OB and the observed accumulation of iron in the OB were well correlated (Figure 5b, $R^2=0.96$). ICP-MS was utilized as it is 1000 times more sensitive than ICP optical emission spectrometry (ppb vs ppm sensitivity) and small differences in iron content between animals were expected.

Progenitor cells are essential for early detection of MPIOs in the Olfactory Bulb

Given that the early detection of dark pixels in the RMS and OB could be a false positive signal that is related to non specific leakage of the label via the CSF, it was investigated whether NPCs were required for detection of contrast. To do this, Ara-C, an anti-mitotic agent, was delivered to the ventricle for 1 week prior to injection of MPIOs, with infusion continuing for one additional week following the injection. This would substantially reduce the number of migrating NPCs capable of carrying the label through the RMS to the OB. In sham rats that did not receive Ara-C, we could detect dark contrast within the RMS as well as dark pixels in the OB (Figure 6a and d, arrow and asterisk), as expected. In rats that received Ara-C, very minimal dark contrast was observed, both at 5 % and 10 % dose. This very minimal contrast was due to the fact that Ara-C cannot fully halt NPC proliferation and migration (Breton-Provencher et al., 2009). Other work has shown that the distribution pattern of MPIOs qualitatively matched that of BrdU labeled cells in the RMS, and MPIOs were found predominantly in astrocytes and migrating neuroblasts (Nieman et al., 2010). Furthermore, immunohistochemical staining of the RMS revealed fluorescent particles within doublecortin positive cells as early as 1 day after injection (Figure 7). Thus, early detection of particles in the RMS is associated with the existence of migrating NPCs and intracellular internalization. This is continued into the OB, where IHC confirms the intracellular localization of MPIOs within doublecortin and BrdU double-positive cells (Figure 8).

DISCUSSION

New paradigms in cellular therapies aim to employ reserves of endogenous cell populations (Leker and McKay, 2004). While the use of MRI for tracking migration of transplanted cells is well established, methodologies for monitoring and quantifying endogenous cell migration are only recently emerging. In this study we have tuned an *in vivo* cellular labeling protocol to enable visualization of early migration of NPCs into the OB, facilitating quantification of this phenomenon. During the first week following labeling, a linear accumulation of newly arriving cells in the OB was measured. Initially, at day 1, cells were confined to the RMS; the contrast in the image was spatially compact and reached the entrance to the OB. This resembles the migratory pattern of a tight chain structure that characterizes neuroblasts migration, and is referred to as tangential chain migration (Lois et al., 1996; Rousselot et al., 1995; Whitman et al., 2009). By day 3, cells are observed entering the central portion of the OB; the granule cell layer, where most cells are destined to reside. At day 8, many cells can be detected not only in the central portion of the OB, but also in the outer edges, where some cells can migrate to (Luskin, 1993). The volume fraction of dark spots in the OB was well correlated with pure ICP-MS determination of iron content,

emphasizing the reliability of using the MRI and BioImagesuite software to measure migration of MPIO labeled progenitor cells. This migration rate matches nearly identically to classical neurogenesis studies employing immunohistochemical means, which monitored 70 - 80 $\mu\text{m}/\text{h}$ (Luskin, 1993; Nam et al., 2007). MRI detection of migration plateaus from week 1 to 2, indicating that the MPIO injection acts like a bolus, not a sustained slow release label. Recently, Sumner et al. published that only 10 - 30 % of the purified cells in the SVZ/RMS region harbored the MPIOs, of these approximately 50 % were found to be GFAP and vimentin positive astrocytes (Sumner et al., 2009). Thus, the total percentage of labeled neural stem cells can be estimated to range between 5 – 17 %.

Super paramagnetic iron oxide particles are extensively used in cellular MRI for *in vivo* cell tracking and several methodologies have been developed for quantification of transplanted labeled cells, including T_2^* relaxometry (Dahnke and Schaeffter, 2005; Politi et al., 2007), or pixel intensity histograms. Other non direct methods such as flow cytometry (Williams et al., 2007) or atomic absorption spectrometry (Raschok et al., 2009) can only be performed on *ex vivo* samples. Phase map cross correlation is a new post processing data analysis technique that was recently developed to quantify localization and accumulation of SPIO particles (Mills et al., 2008). This method was successfully applied on phantom agar gels with implanted SPIO labeled macrophages, and awaits *in vivo* implementation.

The quantification strategy employed here uses a different paradigm, more akin to spot-detection. Due to the T_2^* blooming artifact generated by the MPIOs, labeled cells can be detected as dark contrast at resolutions larger than their physical size (Shapiro et al., 2004). Thus, identification of dark spots is indicative of a pixel containing labeled cells. Prior calibration of the contrast characteristics from labeled cells aids in thresholding. For example, a 30% drop in signal intensity has been measured for single cells harboring 1.63 micron MPIOs imaged at resolutions used in this study (Shapiro et al., 2005). Therefore, any pixel which is more than 30% lower signal intensity than the local background was counted in this study as containing labeled cells. Indeed, voxels were detected with contrast changes of less than 30%, but they were not counted as having labeled cells, even though they may well have. In that regard, the analysis used herein was very conservative and some labeled cells likely were not included in the enumerations, however the exact number of cells is not possible to compute currently.

There are still some challenges using spot detection for quantification of iron oxide labeled cells. One fundamental hindrance is that signal voids generated in the gradient echo image could result from intrinsic anatomical properties and not necessarily relate to labeled cells. Furthermore, since the generated susceptibility effect is independent on the cell type, no method yet exists to discriminate the signal arising from different cell types. Thus, it cannot be determined whether the label resides in the original cell population or arises from a different cell through secondary transfer of the label. In addition, we still cannot resolve the differences between signal voids produced by extracellular or intracellular particles. Considering these limitations, we applied several constraints to our quantification approach. First, we quantified the early migratory rates during the initial 2 weeks post injection. During this restricted time frame we assume low percentage of apoptosis of migrating cells, as it was reported that the high mortality rate occurs 15 to 45 days after the cells were born (Petreanu and Alvarez-Buylla, 2002; Winner et al., 2002). According to Sumner et al, 4%–13% of the labeled cells in the OB are microglia (Sumner et al., 2009), thus the prominent population of labeled cells in the OB lineage is from migrating progenitor cells. Second, in order to avoid false positive selection of anatomical structures, every dark pixel was manually investigated on three orthogonal MRI views. Thus, we have used semi automated quantification and have chosen only those dark pixels that could arise from migrating cells. In addition we have avoided selection of dark pixels that are located in the proximity of the

edge of the brain, an area in which by fluorescence microscopy imaging (unpublished data), we often observe fluorescent particles that non-specifically attached to the rim of the brain.

The challenge to achieve accurate numbers of migrating cells following endogenous labeling of precursor cells is still in its infancy. The *in vivo* labeling efficiency has yet to be determined and amounts of iron to cell numbers have yet to be correlated. For that goal, Magnetic Particle Imaging (MPI) could potentially be applied to correlate magnetic field maps to amount of iron and presumably cell numbers (Weizenecker et al., 2009). Critical for this might be methods for modulating intracellular versus extracellular differences in relaxivity of the particles, thus determining whether the signal arises from labeled cells. Work in this direction is currently underway.

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Figure 1. Determination of co-ordinates for intra-ventricular injection

MPIOs (20 μ l) were injected into the anterior ventricle proximal to the SVZ in adult rats. Co-ordinates for injection: 2 mm caudal and 2 mm medial lateral from bregma 3 mm dorsal (a); 1.5 mm caudal and 1.5 mm medial lateral from bregma 3 mm dorsal (b). 3D gradient echo images were acquired at 11.7T at 1 day post injection. Arrow points to early RMS detection.

Figure 2. Detection of MPIO in the RMS and OB is Dose and Temporal Dependent
MPIOs (5 μ l, a&e; 10 μ l b&f; 20 μ l c&g and 50 μ l d&h) were injected into the anterior ventricle proximal to the SVZ in adult rats. 3D gradient echo images were acquired at 11.7T at 1 day (upper row) and 7 days (lower row) post injection. Arrows point to early RMS detection, circles show the spread of dark spots in the OB.

Figure 3. Temporal resolution during early migratory events

MPIOs (20 μ l) were injected into the anterior ventricle proximal to the SVZ in adult rats. 3D gradient echo images of two injected rat brains were acquired at 1 day (a & d), 2-3 days (b & e) and 8-9 days (c & f) post injection. Circles show the entrance to the OB (b,e) and the spread of dark spots in the OB (c,f).

Figure 4. Labeled progenitors reach the OB within 3 days and by a week occupy the outer layers
a) Data analysis using BioImage Suite Software. Data set from MRI experiment, imported into the BioImage Suite Software. <http://www.bioimagesuite.org>. b) Overlay of serial progression of cell migration into the OB - red, 1 day; yellow, 3 days and green 8 days after injection of MPIOs. c) Quantification of volume increase of labeled progenitor cell population in the olfactory. p value < 0.05. d) Fluorescence microscopic image montage of BrdU labeling in rat neurogenic system, from RMS (left) to OB (right). Shown are animals at 1 and 14 days post injection of MPIOs. Insets are expansions of areas adjacent to numbering as indicated in D.

Figure 5. Correlation between iron content in the OB and dark spot volumes

a) Graphic presentation of the Fe content in the OB as measured by ICP-MS at various time points post MPIO injection. Shown are mean Fe $\mu\text{g/gr OB} \pm$ SEM. b) correlation between iron content in the OB and the volume fraction of dark spots as measured by 3D gradient echo MRI.

Figure 6. Progenitor Cells are Essential for Detection of MPIOs in the Olfactory Bulb

Progenitor cell population was diminished by infusion of Ara-C (14 days, 12 μ l/day; sham (A & D), 5% (B & E) 10% (C & F)). Ara-C was infused 1 week prior to injection of MPIOs (20 μ l) and continued throughout the experiment. 3D gradient echo images were acquired at 7 day post injection of particles. Arrow points to RMS detection, asterisk shows dark spots in the OB and circle shows the migration of dark spots into the OB.

Figure 7. Early Detection of Particles in the RMS is Associated with Intracellular Internalization

Immunohistochemistry staining of the RMS was performed on rat brain sections (16 μm) at 1 day (upper row, a and b) and 2 weeks (lower row, c and d) post injection of MPIOs (20 μl). DCX, doublecortin, marker for migrating progenitor cells (a and c, red channel); IBA1, marker for microglia (b and d, red channel); DAPI, nucleus (blue channel a–d). Images were acquired on a spectral confocal microscope.

Figure 8. Particles are present within NPCs in the OB

Immunohistochemistry staining of the OB was performed on rat brain sections (16 μm) at 2 days (a), 7 days (b) and 2 weeks (c) post injection of MPIOs (20 μl). Doublecortin, red channel; BrdU, nucleus, blue channel; MPIO, green channel. Images were acquired on a spectral confocal microscope.

Table 1

Experimental designs

Experiment	MPIO	Caudal and ML co-ordinates from bregma	MRI (DPI)	# of rats
i) Injection site	20, 50 μ l	15 $\times$ 15 mm / 2 $\times$ 2 mm	1, 14	12
ii) Dose dependent	5–50 μ l	15 mm $\times$ 15 mm	1, 7	24
iii) Temporal resolution	20 μ l	15 mm $\times$ 15 mm	1, 2-3, 7-8, 14	8
iv) Iron content in OB	20 μ l	15 mm $\times$ 15 mm	1,3,8	14
v) Ara-C infusion	20 μ l	15 mm $\times$ 15 mm	7	9