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A Biological Global Positioning System: Considerations for Tracking Stem Cell Behaviors in the Whole Body

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Abstract Many recent research studies have proposed stem cell therapy as a treatment for cancer, spinal cord injuries, brain damage, cardiovascular disease, and other conditions. Some of these experimental therapies have been tested in small animals and, in rare cases, in humans. Medical researchers anticipate extensive clinical applications of stem cell therapy in the future. The lack of basic knowledge concerning basic stem cell biology-survival, migration, differentiation, integration in a real time manner when transplanted into damaged CNS remains an absolute bottleneck for attempt to design stem cell therapies for CNS diseases. A major challenge to the development of clinical applied stem cell therapy in medical practice remains the lack of efficient stem cell tracking methods. As a result, the fate of the vast majority of stem cells transplanted in the human central nervous system (CNS), particularly in the detrimental effects, remains unknown. The paucity of knowledge concerning basic stem cell biology—survival, migration, differentiation, integration in real-time when transplanted into damaged CNS remains a bottleneck in the attempt to design stem cell therapies for CNS diseases. Even though excellent histological techniques remain as the gold standard, no good in vivo techniques are currently available to assess the transplanted graft for migration, differentiation, or survival. To address these issues, herein we propose strategies to investigate the lineage fate determination of derived human embryonic stem cells (hESC) transplanted in vivo into the CNS. Here,

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we describe a comprehensive biological Global Positioning System (bGPS) to track transplanted stem cells. But, first, we review, four currently used standard methods for tracking stem cells in vivo: magnetic resonance imaging (MRI), bioluminescence imaging (BLI), positron emission tomography (PET) imaging and fluorescence imaging (FLI) with quantum dots. We summarize these modalities and propose criteria that can be employed to rank the practical usefulness for specific applications. Based on the results of this review, we argue that additional qualities are still needed to advance these modalities toward clinical applications. We then discuss an ideal procedure for labeling and tracking stem cells in vivo, finally, we present a novel imaging system based on our experiments.

Keywords Stem cells · Tracking system · Biological Global Positioning System (bGPS)

Introduction

Stem cell therapies hold promise for the treatment of various human diseases. Emerging data indicate that transplanted stem cells have the potential to be beneficial or detrimental to patients [1–3]. Future clinical trials will require determining the location and number of transplanted stem cells in vivo, over a life time [4, 5]. It is very difficult to track small numbers of cells in the body with current technologies. Current imaging modalities available for in vivo tracking of the biological fate of stem cells have been proposed, including Magnetic Resonance Imaging (MRI), Positron Emission Tomography (PET), Bioluminescence Imaging (BLI), and Fluorescence Imaging (FLI) with quantum dots (QD). None of these modalities, however, are optimal. Here, we discuss the advantages and disadvantages of current imaging technologies with respect to stem cell tracking applications, define the characteristics of an ideal imaging technology, and propose a new system specifically for stem cell imaging during clinical trials.

Magnetic Resonance Imaging (MRI)

MRI is a widely used medical imaging technique in which magnetic fields are used to detect the nuclear spin of molecules [6]. MRI is the most readily accessible tracking method. Under proper conditions, it is safe and reliable as well as available in most hospitals. MRI has been successfully to detect and track stem cell migration, particularly in cardiac, liver, [7–11] and brain disease models [12–23]. MRI techniques have been utilized to detect the presence and/or migration of transplanted stem cells in various animal models [24]. Surprisingly, MRI can detect a single hepatocyte grafted into a transplanted liver upon perfusion of the primary hepatocytes with double-labeled, green fluorescent 1.63-microm iron oxide particles and red fluorescent endosomal labeling dye, injected into the spleens of recipient mice [10]. The ability to detect single transplanted cells in vivo raises the interest in monitoring stem cell behavior for early detection of transplanted stem cell initiated tumorigenesis (teratoma formation), which may result from the use of stem cells outside of their "therapeutic window" [25].

The benefits of MRI include high resolution and 3-D anatomical imaging capability. Drawbacks include low sensitivity and difficulty in quantifying a labeled cell population [26]. MRI generally has lower sensitivity, but produces high soft-tissue contrast and provides spectroscopic information and functional MRI (fMRI) [27, 28]. However, dilution of intracellular markers occur with each cell division, and false signals may occur due to shedding and subsequent sequestration of iron particles [29].

There are two general categories of cellular MRI markers, T1 and T2/T2*, with T2 contrast being more commonly employed in MR imaging. Gadolinium (Gd^{3+}) contrast enhanced imaging is employed for T1 systems. Apart from Gd, Manganese is also a T1 agent [30, 31]. T2 contrast agents include superparamagnetic iron oxides (SPIO) [32], ultrasmall superparamagnetic iron oxides (USPIO), and magnetodendrimers [27], micron sized particles of iron oxide (MPIOs) [33]. These magnetic markers are sequestered into stem cells. They may exhibit long-term health problems due to endocytosis of magnetic particles with CNS (Fig. 1). The clinically transferable properties of superparamagnetic iron oxide nanoparticles (SPIO) requires modification in MR for tracking stem cells in living organisms [4]. MRI can be used to track cells labeled with iron particles in damaged tissues for at least 16 weeks after injection and to guide tissue sectioning by accurately identifying regions of cell engraftment. The magnetic properties of iron-labeled donor cells can be used for their subsequent isolation from host tissue to enable further characterization [34].

Bioluminescence Imaging (BLI)

Bioluminescence imaging is a technique in which a charged-coupled device camera detects light emitted from the substrate of the enzyme luciferase. The luciferase gene is incorporated into for example a lentiviral vector, and this vector transfects the luciferase indicator transgene into the defined stem cell populations. Firefly luciferase, in the presence of substrate D-luciferin, oxygen and ATP, emits light at about 560 nm [35] (Fig. 2). Bioluminescence imaging is safe, permitting the repeated tracking of small numbers of labeled cells. Bioluminescence imaging is typically not as sensitive to a small number of cells as MRI or PET. This technique has been little used in humans due to concern about immuno-

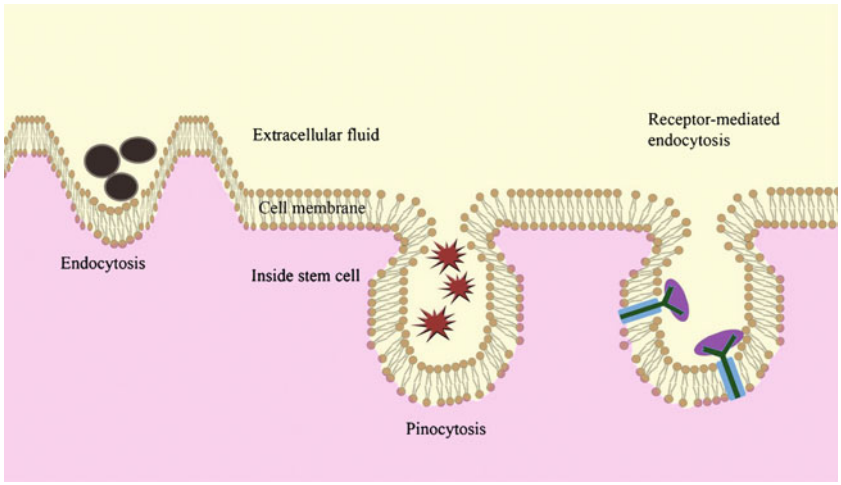

Fig. 1 Direct label of stem cells with markers. *Left*: by the process of phagocytosis of SPIO (usually coated with dextran). *Middle*: USPIO are taken in by pinocytosis [47]. *Right*: for cells without phagocytic ability, receptor-mediated endocytosis is used to facilitate labeling

genicity of the proteins and products involved. In addition, human clinical applications are limited because of the high absorbance and scattering of luminescence in living tissue. As such, BLI is likely to be limited to its current use in imaging transfected stem cells in small animals.

Positron Emission Tomography (PET)

PET uses positron emitting radioisotopes as probes for imaging cells in vivo [36, 37]. The decay of a radioisotope produces two high-energy (511 keV) gamma photons that travel in two opposite directions, which are collected and verified by a ring of scintillating crystals. PET scanners can be used in conjunction with CT scanners to produce more detailed, fused, 3-D anatomical images [38]. To date, most clinical PET-CT studies have used radioactive metals such as ^{18}F-fluoro-2-deoxyglucose (^{18}FDG) and ^{64}Cu, as imaging markers [39, 40]. These markers need a chelator such as PTSM to label cells [41–43], and have been used to track mesenchymal stem cells [38]. Alternatively, the reporter gene method, using herpes simplex virus type 1 thymidine kinase (HSV1-tk), is a stable labeling method (Fig. 2) [44]. However, the latter method poses a dilemma because it has the potential to alter the genetic properties of labeled stem cells. Although the value of PET lies in its high-sensitivity tracking of biomarkers in vivo, it lacks the ability to resolve morphology.

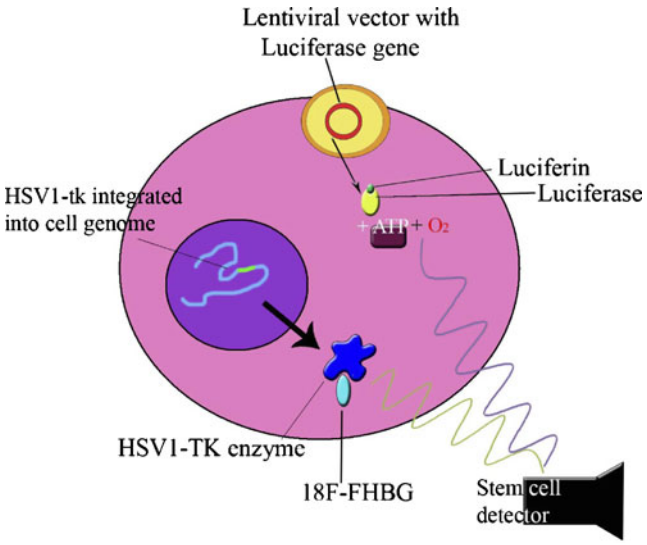

Fig. 2 Reporter gene methods appear to be the most effective way to stably integrate markers into cells for bioluminescence imaging and PET imaging. By inserting a gene into the cell, the marker becomes stem-cell specific and will not transfer to surrounding non-stem cells. The use of a reporter gene allows for serial transgene expression along with key cellular properties (differentiation, proliferation and cell viability). This tracking strategy can be integrated to track stem cells with other modalities (BLI, PET, and MPM)

Using PET and CT fused images, Love et al. were able to locate and detect as few as 10,000 mesenchymal stem cells (MSC) for over a two-month period in mice [38]. Potential disadvantages of PET include repeated injection of radioactive materials into an organism with the potential for radioactive damage to normal tissue associated with cumulative radiation and exposure of surrounding tissues [26]. Additionally, most currently available radiotracers have short half lives which makes them less suitable for long-term tracking. Imaging by positron emission tomography may generate stem-cell specific signals only if the marker is restricted to the stem cell and is the sole source producing a signal of radioactive decay. However, PET detects only the radioisotope. If the radiometals is released from the stem cell, the signal will reflect the location of isotope but not the stem cell. In fact, we know that transchelation is common.

Engineering cells with receptors for imaging is unlikely to find its application in human patients. Furthermore, PET imaging requires quite a bit of post-processing of images to get an image and it is therefore unlikely to be of great use immediately after implantation to track and guide the injection of stem cells.

Quantum Dots (QDots or QD)

Quantum dots (QDots or QD) represent one of several types of fluorescent imaging agents. (QDots are semiconductor particles with physical properties that enable them to emit fluorescent light from 525 to 800 nm (Fig. 3). Quantum dots consist of an inorganic core, a shell of metal and an outer organic coating with a total diameter of 2–10 nm [45]. Quantum dots are capable of entering stem cells through passive loading, receptor-mediated endocytosis or transfection [46]. Passive loading has been found to be the most effective method because it is efficient at labeling, limiting damage to surrounding stem cells. Quantum dots are capable of multiplex imaging [47], single quantum dot tracking [48], and 3-D imaging reconstruction [46].

Quantum dots represent a fairly new advance for tracking stem cells. However, light scattering for quantum dots limits the applicability of this approach, especially to the brain in humans. This scattering property makes it very difficult for use in 3D localization or quantitative estimation of cell survival, which is clearly desired properties in vivo imaging. The long-term biological health effects are unknown, and the method has not yet been used in vivo to track stem cells in animals larger than mice.

Multimodal Imaging

Multimodal imaging is becoming more popular because of its improved sensitivity, high resolution, and morphological visualization. More than a decade ago, Simon Cherry at the University of California, Davis, began working on small animal imaging system combining modalities with crucial qualities: MRI, with its excellent spatial resolution and high-contrast morphologic imaging of soft tissues; and PET, which can track the distribution of biologically targeted radiotracers with high sensitivity, but lacks anatomic context and spatial resolution. Physical constraints (i.e., no metal can get inside the MRI magnet) have frustrated these efforts. Cherry's group has recently revolutionized the identification of cancer biomarkers by creating an MRI-compatible PET scanner, allowing data from both modalities to be acquired simultaneously [49]. Judenhofer and colleagues reported that they have developed a three-dimensional animal PET scanner that is built into a 7-T MRI for a combined molecular imaging system [50], simultaneously acquiring functional and morphological PET-MRI data from living mice. Such a PET-MRI provides a powerful tool for studying biology and pathology in preclinical research and has great potential for clinical applications. Shen et al. combined MRI and PET methods to create multimodal imaging for infarcted myocardium in rats [51]. This technique is integrated for coregistration of PET (with higher sensivitity) or SPECT images with MRI (with 3-D anatomical details of MR scanning) and allows mapping of the location and distribution of stem cells on detailed myocardium structures. A new framework for how whole body imaging using different techniques can be integrated needs to be proposed and tested for stem cell tracking.

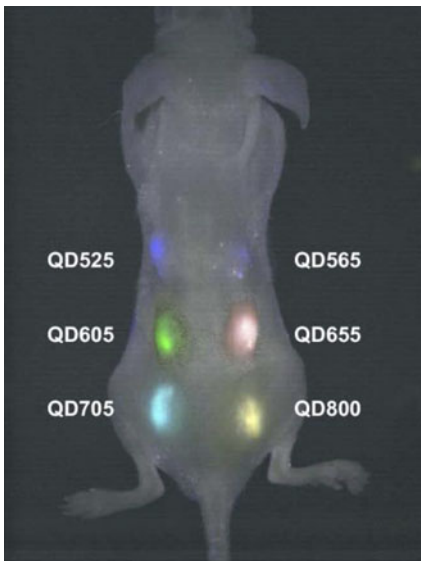

Fig. 3 Quantum dots (QD) are directly loaded into stem cells and used in multplex imaging. Multiplex Imaging (Stacked Image) based on the energy absorbed is different. Some QDs absorb less energy than others because QDs can produce different light levels at the same excitation wavelength QD 525, 565, 605, 655, 705, and 800 were used to label stem cells. QDs were excited by the same wavelength, which is why longer wavelengths are brighter [47]

Whole body imaging using different techniques has been tested in small animals for feasibility. I (Intravenous) injections of the ^{111}In-oxine-labeled human embryonic stem (ES) cell-derived neural progenitors and rat hippocampal progenitors accumulated primarily into internal organs, instead of in the targeted brain [52] while intra-arterial injection of the labeled stem cells showed a weak signal in the ischemic hemisphere detected using SPECT/CT device. The limit of detection sensitivity of SPECT/CT device was approximately 1,000 ^{111}In-oxine-labeled cells.

Requirements for Clinical Applications

*Definition of a Biological Global Positioning System (***bGPS***) for tracking stem cells.* The ability to observe targeted stem cell behaviors using a system capable of scanning for transplanted stem cells throughout the whole body, at any given time is essential to determining whether or not transplanted stem cells in any given clinical intervention is beneficial or harmful in vivo. An advanced bGPS system could be employed to record viability,

differentiation state and the functionality of targeted stem cells in vivo. Specifically, bGPS can be defined as a unit device to locate and record at regular intervals the precise location of a transplanted stem cell in tissue to which it has migrated within the body. Additionally, a bGPS could be designed to record and track the differentiation state, physiological or pathological state, direction and movement of a transplanted stem cell through the body during the time in which it remains in vivo. The work flow for labeling and tracking stem cells is described in Fig. 4. Critical elements in bGPS include:

(1) Sensitivity for single cell detection.
(2) Real-time positioning: Ideally, targeted stem cells could be monitored as they travel throughout the body over any given time period in order.
(3) An inducible system: Signal retrieval under the control of an inducing mechanism to minimize the impact of intervention on the host.
(4) Retractable: Ability to inactivate stem cells should effects prove deleterious.
(5) Targeted and durable: Label detectable for the lifetime of the cell and not transferable to host cells.
(6) Monitoring cell fate: Ability to track cell viability, proliferation, and differentiation state in vivo. More specifically, monitoring physiological changes in the stem cells, e.g., the growth rate (size of the cell), the proliferation rate (the number of the cell), and the differentiation state (the functional transformation). i.e., the label should last for the life time of the stem cell in vivo.
(7) Compliant with the FDA GMP guidelines for clinical applications.

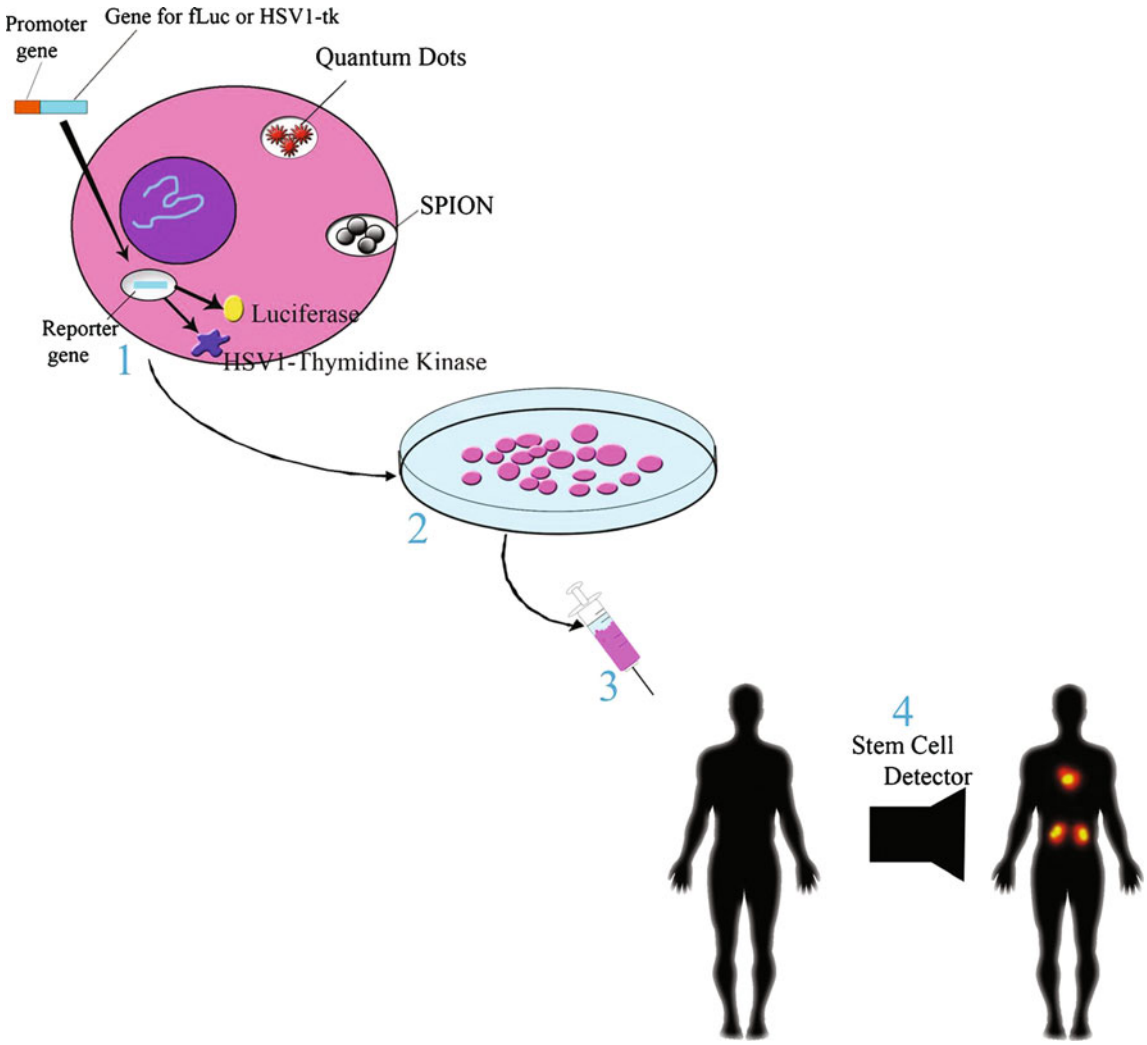

Fig. 4 Multimodality of imaging can be applied for tracking stem cell behavior. A work flow chart for labeling cells and introducing labeled cells into the human body include: (*1*) Cell is labeled using a marker for magnetic resonance imaging (MRI), bioluminescence imaging (BLI), positron emission tomography (PET) or fluorescence imaging (FLI). (*2*) Cells are cultured in vitro and ex vivo (i.e., OTS), and then (*3*) Injected intravenously into the human body. (*4*) Stem cells are then tracked in the body with a camera or scanner

An ideal procedure for labeling and tracking stem cells in vivo is illustrated (Fig. 4). Optimal qualities for cell tracking strategies were proposed by Fragioni [26]. These include biocompatibility, nontoxicity, quantification, no genetic modification, no perturbation of stem cells, and noninvasive imaging. Additional qualities were posited, including (1) specificity of stem cell labeling, (2) continuous in vivo tracking, (3) imaging in a greater depth, and (4) specificity of stem cell imaging. These four requirements are addressed respectively with four currently used tracking methods (Table 1).

Specificity of Stem Cell Labeling

The confusion between specificity and sensitivity can be clarified as follows: Specificity reflects the ability to differentiate transplanted cells from a host cell or an artifact. In this application, sensitivity describes the minimum number of labelled cells necessary for detection; ideally this would be one cell. For example, MRI is sensitive enough to detect transplanted cells (even a single one), but not specific enough to distinguish it from ferritin deposits in host cells. For example, the blooming effect of iron oxide particles poses a potential obstacle to locating cells by diffusing a signal across a much larger area than where a transplanted stem cell is actually located. There is a thorough study on fluorine imaging by MRI to selectively and quantitatively detect transplanted cells and avoid many of the artifact traps associated with ferritin labeling [53].

Specific labeling of targeted stem cells could be accomplished through genetically labeling using a reporter gene (Fig. 2). This reporter gene allows for detection of serial transgene expression in conjunction with key cellular properties (differentiation, proliferation and cell viability) [54–57]. This tracking strategy can be used with other modalities including PET, BLI, and MPM. Labeling specificity is an important requirement for tracking stem cells because it ensures that the label is present only in the stem cells of interest. Furthermore, the label should not interact with or transfer from stem cells to surrounding non-stem cells. Stem cells would ideally retain the label for life in order to maintain specific targeting in vivo. After a labeled cell dies, the label should spontaneously dissipate or be eliminated to prevent re-uptake by surrounding non-stem cells and to indicate that the labeled stem cell has been cleared.

Luciferase, used for bioluminescence imaging, remains specific to transduced stem cells because the marker gene is incorporated into the DNA of the labeled stem cell. Although the marker is retained within the cell, bioluminescence imaging can still be distorted because the surrounding living tissue absorbs and scatters the light given off by the luciferase marker [26].

The magnetic markers used in MRI are not neccessarily specific to stem cells. Iron oxide nanoparticles can potentially transfer from stem cells to non-stem cells, such as macrophages. Moreover, these iron particles may shed or spill from stem cells into the surrounding living tissue [6, 26, 29]. To address these issues, Modo et al. (2002) established that the Gd particles remained inside labeled cells in a co-culture system [58] and the uptake of USPIO in macrophages was demonstrated in an in vitro system [59].

Depending on the mechanism used to insert the marker into the stem cell, positron emission tomography may or may not specifically detect only labeled stem cells. When a radiotracer is directly loaded into a cell, the possibility exists that non-stem cells will uptake the marker [1]. Alternatively, we can design an enzymatic conversion/retention of radiotracers for stable incorporation of markers into the stem cell, eliminating the possibility of transferring markers from labeled stem cells to non-labeled cells.

Quantum dots might be used as markers but, in comparison, are not stem cell-specific. While passive loading of bare quantum dots into mesenchymal stem cells provided a very effective method of delivery [46], the dots demonstrated no specificity for stem cell binding, and therefore, the possibility that quantum dots can spill out of the labeled stem cells, resulting in a loss of fluorescent signal. Quantum dots, however, do not appear to be transferable from labeled stem cells to non-stem cells. When QD-labeled human mesenchymal stem cells

Table 1 Comparison of currently used methods for tracking stem cells

	BLI	QD	MRI	PET
Specificity for stem cell labeling	●	×	×	●
Marker is nontoxic to stem cell	●	●	●[a]	●
Marker does not alter stem cell properties	●	●	●	●
Non-invasive tracking	×	●	●	×
Continuous in vivo tracking for up to one month	●	×	●	●
Tracked at >3 in depth from skin	×	×	●	●
Specificity for stem cell imaging	●	●	×	●
Quantification capability	●	×	×	●

● Indicates that tracking method fulfills this capability

× Indicates that tracking method does not fulfill this capability

[a] There are reports to show that the marker particles are toxic for certain functions for stem cells (see the text for details)

were perfused in animals, adjacent cardiac cells failed to take up the released quantum dots in vivo [46].

Marker is Nontoxic to Stem Cells

An essential requirement for cell labels is that they are not toxic over time. Markers for bioluminescence labeling, small doses of radiotracers for PET, and small amounts of quantum dots have generally not shown to be toxic to labeled cells. Still, Lin, et al. demonstrated for fluorescent imaging that although quantum dots are generally nontoxic, escalating concentrations (>20 nmol/L) of quantum dots prove to be cytotoxic over time [47]. A recent study found that commonly used iron oxide nanoparticles used for MR imaging can have toxic effects on neuronal cells [48]. The possibility that iron oxide nanoparticles can transfer from labeled cells to neuronal cells poses a hindrance to clinical applications of tracking stem cells through MR imaging. Modo et al. (2008) followed Gd-labeled cells over one year and found that although there was no in vitro effect, cells labeled with Gd did not recover as they exerted deleterious effects on repair [60]. Microscopy studies revealed lysosomal compartmentalization of iron particles in human mesenchymal stem cells up to 14 days after labeling [61]. With subsequent loss of the compartmentalization of iron oxide particles, resulting in release of iron oxides into the adjacent environment clearly, clinical safety standards for the application of labels remain to be defined.

Marker does not Alter Stem Cell Properties

While searching for ideal labeling strategies, it is essential that imaging must not alter the behavior or fate of marked stem cell populations. No major adverse effects of the four imaging techniques have been shown to interfere with stem cell life span or the ability to proliferate over the time period observed [62–66]. Indeed, in vitro labeling of human central nervous system stem cells with magnetic nanoparticles does not adversely affect survival, migration, and differentiation or alter neuronal electrophysiological characteristics [67]. Amazingly, these labeled human central nervous system stem cells transplanted either to the neonatal, the adult, or the injured rodent brain responded to cues characteristic for the ambient microenvironment. They survive long-term and differentiate in a site-specific manner identical to that seen for transplants of unlabeled cells. However, it was reported that a reporter gene is a better marker for monitoring cell viability, whereas iron particle labeling is a better marker for high-resolution detection of cell location by MRI [68]. No significant difference has been reported thus far in stem cell growth and development when a marker is added to the cell [47]. Time-lapse microscopy successfully captured a moment of dramatic change in chromosome positioning during the transition between two differentiation stages of pluripotent mouse ES cells [69]. These images following the epigenetic markers showed unique nuclear organization in that methylated centromeric heterochromatin coalesced to form large clusters around the nucleoli. Upon differentiation, the organization of these heterochromatin clusters changed dramatically.

However, Kostrua et al. (2005) showed that using iron oxide particles on mesenchymal cells impaired their ability to differentiate into chrondrocytes [70, 71]. The inhibitory effect of iron oxide compounds on stem cell differentiation was a major issue in using labeled stem cells for infarcted myocardium repair [11]. There is, however, no demonstrable effect on stem cells originating from the CNS including viability, proliferation, or multipotency [72]. Moreover, it is becoming increasingly clear that one of the major effects of stem cells (regardless of their niche of origin) is mediated through the paracrine release of various cytokines or growth factors [73–77]. It should be noted that probe labeling of stem cells might alter this "paracrine effect." There is currently little data to prove or disprove the impact of tracking agents on this important (in vivo) stem cell "pro-healing" effect.

Non-invasive Tracking

Stem cell tracking in humans ideally should be non-invasive. Both magnetic resonance scanners and fluorescent detectors for quantum dots can image stem cells without procedures more invasive than stem cell injection. Once these cells are labeled in vitro and transplanted into the body, imaging for tracking the fate of these cells is non-invasive.

Bioluminescent imaging and PET imaging require a slightly more complicated process in which specific substrates must also be injected in order to insert detectable substrates into transplanted stem cells. These substrates, when attached to the enzymes produced by labeled stem cells, produce a signal which can be used to identify stem cells in vivo. However, there have been concerns about the effect of iron oxide compounds on stem cell differentiation, a pivotal cell property [11].

Continuous Tracking In Vivo

In order to determine the accuracy and efficiency of stem cell therapy in vivo, stem cells must be continuously traceable from the time of injection to the time that they reach a final destination. For example, stem cells should be tracked for months at a time to monitor the long-term engraftment and function of stem cells migrated to a damaged region.

However, most experiments tracking stem cells have been conducted in small animals and last only days or weeks. For clinical applications, stem cell labeling must remain intact for months in order to monitor their viability, proliferation, and integration into the targeted host microenvironment.

J.M.W. Bulte et al. tracked magnetically labeled stem cells traveling into the brain parenchyma for up to six weeks, illustrating clinical potential of MRI [27]. However, MRI technology still calls for improvement because each cell division of a labeled cell reduces the concentration of superparamagnetic iron oxide nanoparticles within progeny cells. Such a progressive dilution of the cell marker through cell division limits the capability of this method for long-term tracking of stem cells. A study by Daldrup-Link et al. demonstrated that with concentrations of less than 20 nmol/ml, individually labeled cells cannot be detected by MRI [78]. A threshold concentration must be maintained in order for cells to be imaged by MRI. This threshold limits the length of time that MRI can be used to track stem cells in vivo.

Whereas the MRI technique can track magnetically labeled cells for over a month, studies employing bioluminescence imaging have successfully tracked cells for up to three months [38]. Positron emission tomography tracking has also been successfully employed for over two months using the HSV1-tk reporter gene [38]. Transfecting the stem cell with reporter genes appears to be a promising method for stable integration of a marker into the target cell permitting longer periods of tracking.

The capability for continuous tracking of quantum dots in vivo has not been tested as rigorously as that for MRI, bioluminescence imaging, or PET imaging. Although current technology generally enables continuous in vivo tracking in mice for only a few weeks [47], Rosen et al. were able to identify stem cells within a canine heart labeled by quantum dots 8 weeks after injection of mesenchymal stem cells by ex vivo imaging of these cells [46]. In order for quantum dots to be clinically useful, larger test subjects must be used to determine the maximum depth that emitted fluorescence can be reliably detected in vivo and repeatedly.

Tracking and Depth

MRI, BLI, PET and QD techniques can successfully image stem cells within small rodents. However, these same tracking capabilities can not be currently translated into imaging larger organisms, such as humans. Ideally, labeled stem cells should be traceable at depths greater than 3 in. from the skin in order to image internal organs, such as the heart or the brain.

MRI and PET medical imaging routinely explore internal regions of the human body, creating 3-D renditions of the anatomy of interest. As such, MR and PET scanners are advantageous for this desired characteristic of in-depth detection.

A major impediment to the current use of bioluminescent imaging in clinical applications is that luciferase and other BLI markers can only be tracked to depths of 2–3 cm from the skin [79]. While this does not pose a problem in laboratory experiments with small animals, such superficial imaging will not be practical with human patients. Imaging of deeper regions, such as the human brain or heart, is impractical with current BLI markers. There is a possibility for deep tissue visualization with FLI and “stealth” near infra-red fluorescent probes (See details in the later sections).

Quantum dots may have great potential for tracking stem cells in vivo at greater depths than conventional fluorescent markers because of its increased intensity of light emission. Ballou et al. have reported that fluorophore signals can be detected deep from within the liver and bone marrow of a mouse using whole-body imaging [80]. Additionally, Larson et al. have been able to image quantum dot emission signals in capillaries hundreds of millimeters deep in the blood vessels from the skin of mice [81]. However, experiments tracking stem cells in vivo in larger animals must be conducted to determine the extent to which quantum dot fluorescence can be tracked.

Specificity for Stem Cell Imaging

Specificity, the ability of an imaging method to accurately discriminate between labeled stem cell and surrounding non-specific signal noise, is a fundamental requirement for stem cell tracking.

MRI has poor specificity because signal loss or gain does not solely correlate with superparamagnetic iron oxide (SPIO) MRI imaging contrast agent from a labeled cell. With a negative contrast, it may become difficult to distinguish between a signal loss from a labeled cell or the innately low signal in tissue [51]. Additionally, Mani et al. describes how cardiac imaging is difficult with MRI because signal loss may not only be attributed to magnetically labeled stem cells, but to motion, partial volume averaging effects, and necrosis [29]. MRI is also not sensitive enough to discriminate between a labeled stem cell signal and products of ferritin deposition [29]. Positive contrast, on the other hand, is not necessarily better than negative contrast [82]. In neural imaging, hemorrhages, extracellular SPIO or other iron-enriched brain regions can be mistaken for a positive signal.

Bioluminescent markers are advantageous for producing stem cell-specific images. There is an inherently low

background luminescence from tissue [35], making bioluminescent markers easy to identify in vivo. In comparison, negative or positive contrast signals from MRI may be attributed to activity in the surrounding tissue, which makes it difficult to distinguish stem cell presence.

Quantum dots, like bioluminescent markers, have useful qualities for producing stem cell-specific images. Their fluorescent signals have very narrow emission spectra, so the wavelength is specific to a particular-sized quantum dot. In some studies, there was no background autofluorescence to interfere with the quantum dots signal, and when there was autofluorescence, programs can be used to eliminate it from the image. Furthermore, their fluorescence is much stronger than organic fluorophores [6].

Quantification Capability

Ideally, a linear relationship should exist between the number of stem cells bearing a label and the generated intensity or concentration of the emitted signal. This relationship should follow a predictable course over the lifespan of the label in order to derive absolute values. Quantification is important to differentiate where the majority of stem cells are located in comparison to where minor populations of stem cells are found within an organism. Daldrup-Link et al. found no significant correlation between the number of labeled cells and MR imaging data between 1×10^7 cells and 3×10^8 cells [78]. They proved that quantification with MR imaging is limited for stem cell tracking and a refinement is needed.

Quantification with bioluminescence imaging has been performed. Togel et al. has found a linear relationship between photon emission by the cell markers and the number of stem cells [83]. PET is the only other tracking method of the four in which there is a correlation between signal intensity and population of labeled stem cells.

A Novel Practical Device Complementary to Establish a GPS-like Tracking System for Tracking Stem Cells In Vivo

An estimated 18,820 new cases of brain and other CNS cancers will be diagnosed in the United States each year, and more than 12,000 will die from the disease (data from the US National Cancer Institute). Brain tumors are now the leading cause of cancer-related deaths in children under age 15 [84]. Malignant brain tumors represent a major pediatric and adult health care threat with long-term prognosis remaining dismal [85]. New approaches to the treatment of brain tumors are desperately needed. Public enthusiasm has fueled interest in stem cell research as a promising approach such that stem cell therapy has been regarded as an eternal wonder medicine of "cure-it-all," and novel applications for stem cell therapy have been proposed for the treatment of malignant gliomas as they "surround" the invading tumor border while "chasing down" infiltrating tumor cells [86, 87].

One major obstacle is to better understand how stem cells access the brain tumor and move within it during tumor initiation and progression. Aboody and colleagues visualized and quantitatively analyzed the spatial distribution of tumor-tropic NSCs in a mouse model of orthotopic glioma in order to predict the therapeutic efficacy of a representative NSC-based glioma therapy [88]. However, this single-color-dye-loaded and confocal microscopy-based method is limited. Multiphoton excitation fluorescent microscopy (MPM) is a laser-based technology that allows subcellular resolution of native tissues in situ [89, 90]. As compared with other in vivo imaging techniques, multiphoton microscopy is uniquely capable of providing a window into cellular and subcellular processes in the context of the intact, functioning animal. In addition, the ability to collect multiple colors of fluorescence from the same sample makes in vivo microscopy uniquely capable of characterizing up to three parameters from the same volume, supporting powerful correlative analyses [91, 92].

We have developed and applied this technology previously to the structural and photochemical imaging of human pathological conditions [93–96]. This system has most recently been applied to cultured glioma cells and experimental gliomas ex vivo [97–100]. These studies demonstrated that high microanatomical definition of the tumor, invasion zone, and normal adjacent brain can be obtained down to single-cell resolution in unprocessed tissue blocks. More intriguingly, they can use multiphoton excitation and four-dimensional microscopy to generate fluorescence lifetime maps of murine brain anatomy, experimental glioma tissue, and biopsy specimens of human glial tumors. In murine brain, cellular and noncellular elements of the normal anatomy were identified. Distinct excitation profiles and lifetimes of endogenous fluorophores were identified for specific brain regions. Intracranial grafts of human glioma cell lines in mouse brain were used to study the excitation profiles and fluorescence lifetimes of tumor cells and adjacent host brain [99]. These studies demonstrated that normal brain and tumor could be distinguished on the basis of fluorescence intensity and fluorescence lifetime profiles. Human brain specimens and brain tumor biopsies were also analyzed by multiphoton microscopy, which demonstrated distinct excitation and lifetime profiles in glioma specimens and tumor-adjacent brain. This study demonstrates that multiphoton excitation of autofluorescence can distinguish tumor tissue and normal brain based on the intensity and lifetime of fluorescence [99]. Wilson et al. (2009) used

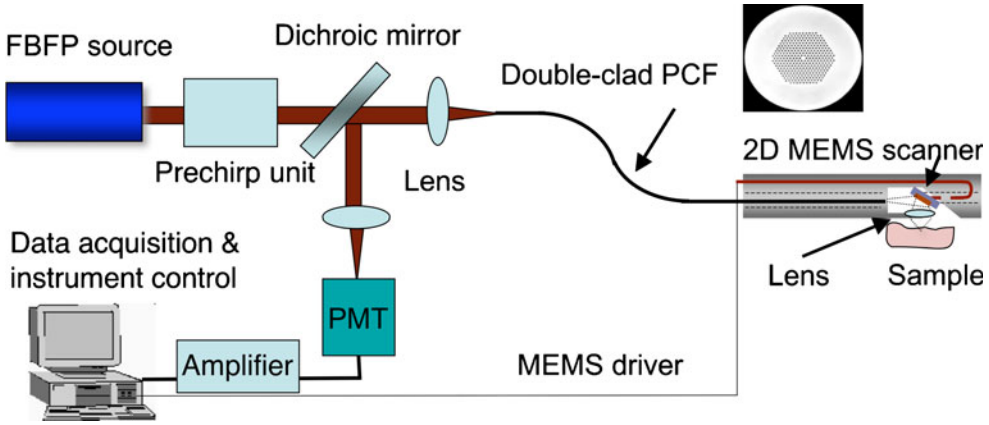

Fig. 5 Schematic diagram of the fiber-based MPM system. The double-clad PCF has a core with a diameter of 16 μm and an inner cladding with a diameter of 163 μm. Because of the large inner cladding and multimode propagation with a large numeric aperture, a much higher collection efficiency can be achieved in comparison to the conventional single mode fiber. In addition, the use of a large core double clad PCF minimizes the pulse broadening due to the nonlinear effect. The scanner and A/D board are controlled and synchronized by a PC

MPM to visualize the behavior of infiltrating CD8+ T cells in cerebral cortex by using reticular fiber conduits to move within the brain [101]. We and others have worked on technical developments in this technology that may provide a means for in situ tissue analysis, which might be used to detect a residual tumor at the resection edge.

We have devised a comprehensive GPS-like tracking system for stem cell delivery and tracking in vivo with combination of genetically labeled stem cells and in vivo imaging technology (Figs. 5, 6, 7, 8, 9). These genetically labeled stem cells are tagged with constitutive or developmentally regulated viable fluorescent markers for tracking a transplanted stem cell, in vitro or in vivo by the innovative GPS-like tracking system [93, 94, 102–104]. In vivo tracking relies upon nanotechnology coupled with an optical fiber detector camera probe surgically implanted, providing continuous circumferential visualization within a radius of 500 nm.

The miniaturized probes are the most critical components for the in vivo multiphoton microscope (MPM) imaging system (Figs. 7, 8, and 9). In the past few years, we have developed a number of miniaturized probes for OCT and MPM imaging [93, 94, 104–106]. Figures 7, 8 and 9 show MEMS-based and needle-based miniature probes. The fiber-based MPM system offers three advantages over the current prototype system. The fiber-based femotosecond pulse (FBFP) source is much more compact and robust than the Ti:sapphire laser, which is ideal for in vivo imaging [107, 108]. In addition to the MPM system and probe development, the fiber based MPM imaging system can be combined with the optical coherence tomography (OCT) system to enable simultaneous monitoring and evaluating of the therapeutic effect of stem cell treatment [107, 109].

Imaging and tracking stem cell distribution in in vitro, ex vivo and in vivo systems is essential for defining basic stem cell behavior and for optimizing stem cell therapy. MPM, which includes multiphoton excited fluorescence and harmonic generation, has been widely used for biological imaging with molecular contrast and sensitivity [110–112]. Several groups have shown in ex vivo studies that MPM can be used to identify the tumor, invasive zone, and normal adjacent brain with a single-cell resolution [99]. It has been found that normal and brain tumors can be distinguished by either fluorescent intensity or lifetimes profile from endogenous fluorophores. The intrinsic contrast of MPM imaging technology, combined with fluorescent-tagged stem cells, would allow imaging, identifying, tracking and evaluating interactions of tumor cells, and transplanted stem cells.

To study the in vivo interaction of stem cells with brain tumors, two fluorescent dyes can be simultaneously infused intravenously, one of high molecular weight (fluorescein-labeled dextran, 70 kDa, green fluorescence) for labeling the stem cells and one of low molecular weight (sulforhod-

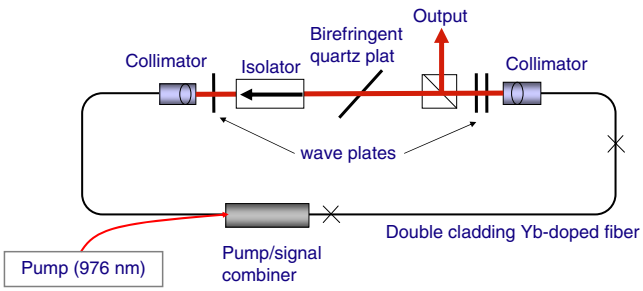

Fig. 6 Schematic of the femtosecond fiber laser oscillator. The pump laser has a central wavelength of 976 nm and maximum power of 20 W. The pump power is delivered to the cladding of the DC gain fiber through a home-built pump-signal combiner. The Yb-doped double cladding gain fiber has a core diameter of 10 mm and inner cladding of 105 mm [116]

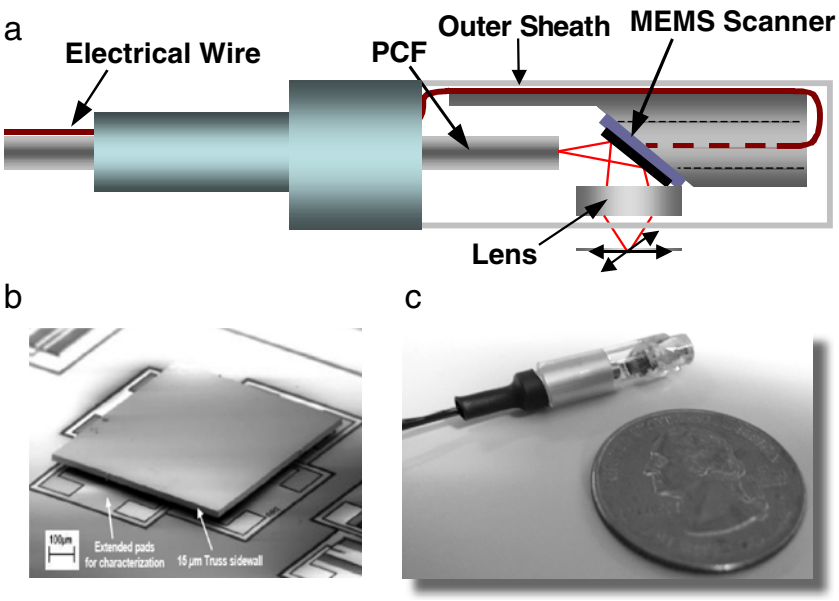

Fig. 7 **a** Schematic of the MEMS-based miniature probe; **b** picture of the MEMS scanner; **c** picture of packaged probe

amine B, 559 Da, red fluorescence) for labeling the tumor cells. A two-photon microscope can be directed through a cranial window, and obtain separate images of the two dyes in the cortex. The gains of the two channels may be adjusted so that the signals coming from within the vessels are equal. Subtraction of the image of the fluorescein-dextran from that of the sulforhodamine B gives images in which the vasculature is invisible and the sulforhodamine B in the parenchyma will be imaged with high resolution as described previously [113].

However, current commercial MPM has limited capability for in vivo imaging because of the lack of a compact and flexible miniature probe. We have developed a fiber based MPM system that is compact and robust for in situ imaging and tracking stem cell migration, differentiation, and survival in animal and human subject. There are a number of obstacles in translating the MPM technology for clinical applications, including the compact and robustness of the femtosecond source, efficient excitation light delivery and signal collection, and probe miniaturization. Our fiber based MPM addresses these limitations: (1) the development of a fiber-based femtosecond-pulse (FBFP) sources as a stable and compact light source, (2) design and development of a MPM system that integrates a FBFP source, a double clad fiber, and a MEMS probe, and (3) design and development of a miniature scanning probes.

Fiber-Based MPM System Design

The schematic diagram for the endoscopic MPM is shown in Fig. 5. A short pulse beam generated from the FBFP source first goes through a grating-based pulse stretcher

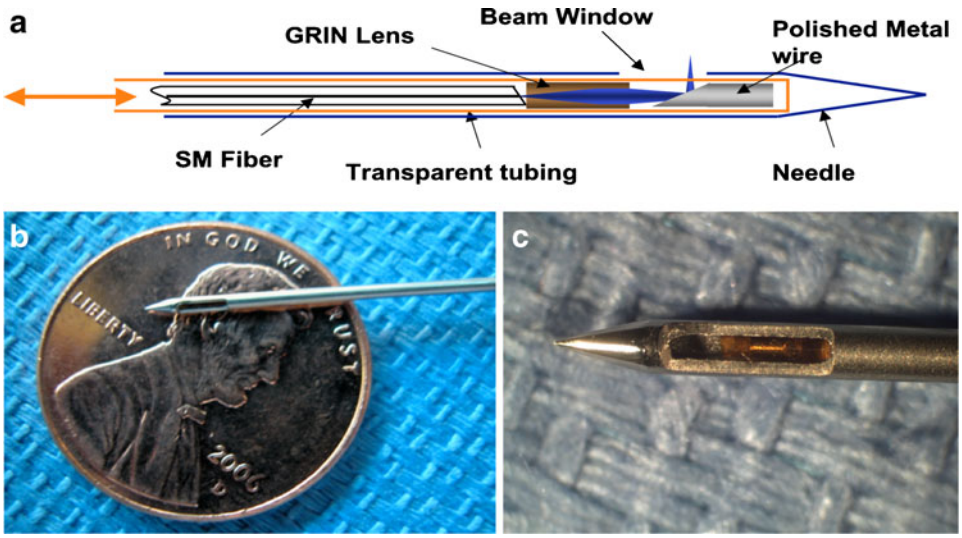

Fig. 8 **a** Schematic of the needle-based miniature probe; **b** picture of the probe; **c** picture of the tip of the probe

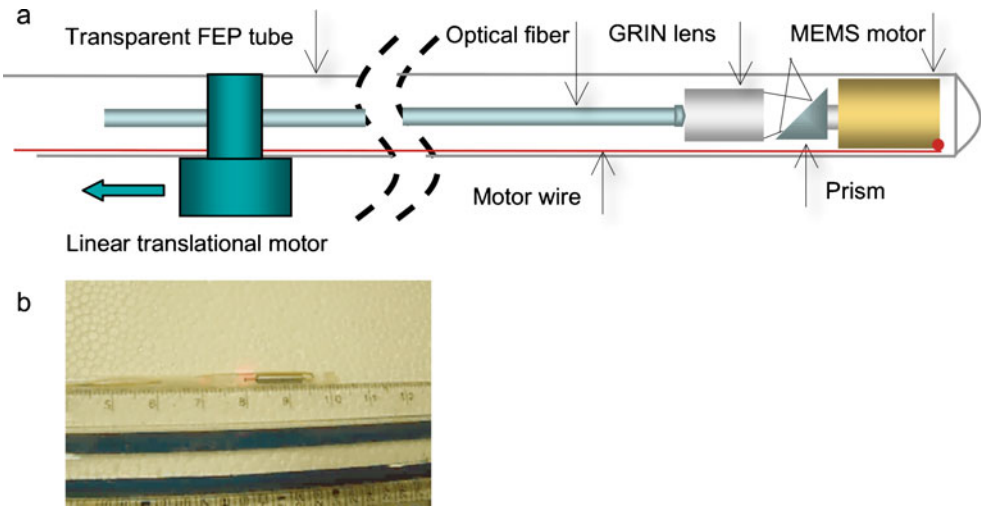

Fig. 9 **a** Schematic of the endoscopic MEMS probe; **b** picture of the probe

which can negatively pre-chirp the femtosecond pulses in the pre-chirp unit to compensate for pulse broadening caused by positive dispersion in the double clad PCF core. The pre-chirped pulse passes through a dichroic mirror and is coupled into the double clad PCF. The short pulse light coupled out from the double clad PCF may be focused on the tissue through a miniature probe. The fluorescent or harmonic generation signal can be collected through the lens in the miniature probe and coupled into both the core and inner clad modes of the fiber. The collected MPM signal propagates through the double clad fiber and passes through the dichroic mirror that removes the excitation beam. The MPM signal detected by the photomultiply (PMT) is amplified and digitized.

Design and Development of a Fiber-based Femtosecond-pulse (FBFP) Source for MPM

One of the key components for MPM is the short pulse laser. Efficient generation of a nonlinear signal requires focus of the excitation laser in both space and time. Therefore, a short pulse source is essential for efficient MPM signals. Currently, most MPM systems use a Ti: sapphire laser. The Ti:sapphire laser offers short pulse width, high power, high repetition rate and wavelength tunability from 710 nm to 1,000 nm. However, it also has the disadvantages of being bulky, expensive and not maintenance-free. Recent advances in the development of fiber lasers have made an inexpensive, portable and maintenance-free laser source available.

We have collaborated with Dr. Wise's group at Cornell University in developing a fiber laser for in vivo optical imaging [114, 115]. We have recently developed a compact fiber laser that meets the requirement of MPM imaging. The schematic of the fiber laser is shown in Fig. 6 [116]. The pump laser has a central wavelength of 976 nm and a maximum power of 20 W. The pump power is delivered to the cladding of the double cladding gain fiber through a home-built pump-signal combiner. The Yb-doped double cladding gain fiber (Liekki DC1200-10/125) has a core diameter of 10 mm and inner cladding of 105 mm. The fiber laser can generate a 120 fs pulse with an average power larger than 2 W. The high output power of the fs pulse of the fiber laser is achieved by the following method.

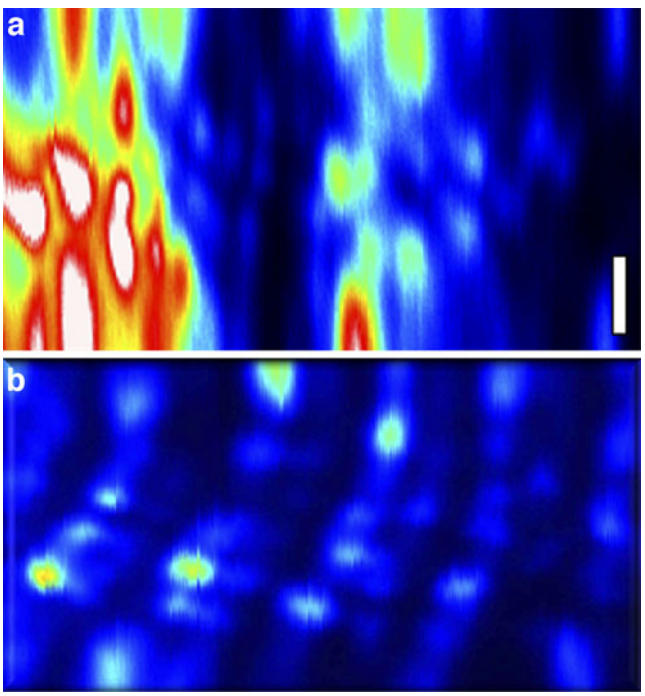

Fig. 10 MPM images of rat tail tendon (**a**) and fish scale (**b**) using rotational probes. The image is unfolded with the *horizontal direction* scanned by a translation stage, and the *vertical direction* scanned by the rotational probe. The images are expressed in 8-bit pseudocolor, and the *scale bar* is 25 μm [105]

First, we used a larger core (10 μm) single mode fiber instead of a 6 μm fiber. The 10 μm diameter core fiber matches the core diameter of the doped double clad gain fiber. Hence, the splice loss was reduced. Second, a more powerful pump laser was used. The pump laser used could provide a maximum power of 20 W. The desired output pulse had a pulse width of 120 fs and a wavelength bandwidth of 40 nm.

Design and Development of MPM Scanning Probes

The miniaturized probes are the most critical components for an in vivo MPM imaging system. A number of miniature scanner probes can be developed to meet the needs of in vivo MPM imaging for tracing stem cell therapy. In the past few years, Dr. Chen's group has developed a number of miniaturized probes for OCT and MPM imaging [93, 94, 104–106, 117]. MEMS-based and needle-based miniature probes developed in Dr. Chen's laboratory are shown in Figs. 7 and 8.

In addition to the 2-D linear scanner, deep tissue may be better imaged through a cranial window with a cylindered geometry. A rotational probe may be optimal to image a relative large area through the cranial window. We have developed a rotational probe based on a miniaturized rotational MEMS motor (Fig. 9) [105, 118, 119]. The MEMS micromotor is mounted in a backward configuration toward the proximal direction, and a 45° prism is used to deflect optical light toward the sample. We have built and tested a prototype device with a motor with a diameter of 2.2 mm. A GRIN lens with a diameter of 1 mm was used since it was readily available with 3° cleaved angles. Imaging was performed by pulling the whole probe with a linear translational stage while the MEMS motor rotated to create a 3-D helix scan. Figure 10 shows the preliminary results of the MPM signal acquired through such a miniature rotational probe [105].

Signal and Imaging Processing

In addition to the intensity measurement, appropriate spectral filter can be included in the MPM detection system so that multiple spectral channel images can be acquired simultaneously. Furthermore, time-correlated sing-photon counting can be developed to enable flourescence lifetime imaging.

To study the in vivo interaction of stem cells with brain tumors, two fluorescent dyes are simultaneously infused intravenously, one of high molecular weight (fluorescein-labeled dextran, 70 kDa, green fluorescence) for labeling the stem cells and one of low molecular weight (sulforhodamine B, 559 Da, red fluorescence) for labeling the tumor cells. A two-photon microscope can be directed through a cranial window, and obtain separate images of the two dyes in the cortex. The gains of the two channels are adjusted so that the signals coming from within the vessels are equal. Subtraction of the image of the fluorescein-dextran from that of the sulforhodamine B gives images in which the vasculature is invisible and the sulforhodamine B in the parenchyma can be imaged with high resolution as described previously [113].

Conclusion

(1) We have defined the problems of tracking the migration and fate of transplanted stem cells. (2) We have also defined the ideal qualities of a stem cell tracking system for clinical use. (3) None of the four stem cell tracking methods fulfill all of the requirements needed for clinical application at this time. Bioluminescence, despite its high sensitivity and specificity, proves problematic in animals bigger than mice. Studies with quantum dots provide benefits. This labeling system is in its early developmental stage, and further development is necessary if quantum dots were to be used in clinical function. To create the optimal in vivo imaging modality, multimodal markers will provide the benefits of each different labeling technique. MRI dual-imaging might prove to be the most favorable combination because MRI is widely used in hospitals. State of the art confocal and multiphoton microscopy (MPM) and optical coherence tomography (OCT) may be integrated to complement MRI in order to create a biological global positioning system to track stem cell migration, differentiation, and survival in vivo.

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Conflict of Interest All the authors declared no financial conflict of interest.

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