An optical imaging method to monitor stem cell migration in a model of immune-mediated arthritis

Elizabeth J. Sutton1,2, Sophie E. Boddington1,*, Alexander J. Nedopi1, Tobias D. Henning1, Stavros G. Demos3, Rick Baehner4, Barbara Sennino5, Ying Lu1,6, and Heike E. Daldrup-Link1

1 Department of Radiology, University of California, San Francisco, 185 Berry Street, Suite 350, San Francisco, CA, 94107-0946, USA
2 Department of Radiology, Mount Auburn Hospital, Harvard University, Boston, 330 Mount Auburn St, Cambridge, MA 02138, USA
3 Lawrence Livermore National Laboratory, Livermore, 7000 East Avenue, Livermore, CA 94550, USA
4 Department of Pathology, University of California, San Francisco, 505 Parnassus Avenue, San Francisco, CA 94143-0511, USA
5 Department of Anatomy and the Cardiovascular Research Institute, University of California, San Francisco, 505 Parnassus Avenue, San Francisco, CA 94143-0511, USA
6 Biostatistics Core Facility, Comprehensive Cancer Center, University of California, San Francisco, CA 94143-0981, USA

Abstract

The objective of this work is to establish an optical imaging technique that would enable monitoring of the integration of mesenchymal stem cells (MSC) in arthritic joints. Our approach is based on first developing a labeling technique of MSC with the fluorescent dye DiD followed by tracking the cell migration kinetics from the spatial distribution of the DiD fluorescence in optical images (OI). The experimental approach involves first the in vitro OI of MSC labeled with DiD accompanied by fluorescence microscopy measurements to establish localization of the signal within the cells. Thereafter, DiD-labeled MSC were injected into polyarthritic, athymic rats and the signal localization within the experimental animals was monitored over several days. The experimental results indicate that DiD integrated into the cell membrane. DiD-labeled MSC localization in the arthritic ankle joints was observed with OI indicating that this method can be applied to monitor MSC in arthritic joints.

Introduction

Mesenchymal stem cell (MSC) based approaches for arthritis treatment integrate immune modulation and tissue regeneration and lead to improvement of arthritic disease in the short and long term [1,2]. MSCs are a realistic and readily available therapeutic tool for patients with arthritis as they are well characterized, easily and safely harvested from bone marrow and efficiently expanded in vitro [1]. However, MSC based arthritis therapies would benefit from
an imaging technique that could monitor successful cell engraftment or diagnose early treatment failure by direct depiction of the migration of the transplanted cells.

Classical methods of cell tracking based on vector transfection and cell specific antibodies have the disadvantage of requiring post mortem immunohistologic staining thus impeding temporal monitoring [3,4]. Radiotracer-based cell labeling techniques have demonstrated high sensitivity and potential for whole-body evaluation. However, drawbacks include short isotope half-life, radiation exposure and high cost [5,6]. Magnetic Resonance Imaging (MRI) provides near-microscopic anatomical resolution, but limited sensitivity for in vivo cell tracking studies [7]. In recent years, optical imaging methods have provided new approaches for noninvasive real-time monitoring of stem cell transplants allowing repetitive, longitudinal studies of the engraftment process [8–11]. Optical Imaging (OI) is an effective means of tracking stem cells in experimental models as it is rapid (<5 minutes), inexpensive, noninvasive, provides single cell sensitivity and does not involve radiation exposure [8,10]. In addition, the information obtained from OI can be directly correlated with fluorescence microscopy as a standard of reference.

Stem cell labeling with exogenous labels provides several advantages over endogenous labels including an easy, inexpensive and non-toxic labeling procedure and absence of gene transfer, which would limit translational applications [12,13]. NIR cyanine dyes, such as the DiD dye, provide efficient labeling through simple incubation, a tolerable toxicity profile, a strong signal and photo stability [3,14,15].

The purpose of this study was to evaluate if optical fluorescence imaging can be used to track the migration of mesenchymal stem cells labeled with the fluorochrome DiD to arthritic joints. To the best of our knowledge, this is the first study to track fluorochrome-labeled human stem cells with OI in an arthritis animal model.

Methods and material

Cells and labeling procedure

The study was approved by the committee of human research at our institution. Primary human mesenchymal stem cells (hMSC) were obtained from bone marrow (BM) of a patient with no known bone marrow pathology, who was admitted to our institution for trauma surgery and provided consent for intraoperative donation of hMSCs for research purposes. BM cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) High Glucose media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FMB, Hyclone, Logan, UT, USA) and 1% Penicillin-Streptomycin. All experiments were performed between passages 10–16 to avoid senescence.

The cells were labeled with DiD (C_{67}H_{103}CIN_{2}O_{3}S, Vibrant cell labeling solution, Molecular Probes, Oregon, USA), a lipophilic, cyanine near-infrared fluorochrome with a molecular weight of 1052Da and excitation and emission maxima of 644nm and 665nm respectively, as confirmed by spectrometry. The cells were incubated for 20 minutes with a labeling solution, consisting of 5μl of DiD and 1ml of serum free media per 1.0*10^6 MSC. The cells were washed, counted and viability tested by the trypan blue exclusion assay (Sigma Aldrich, St. Louis, MO, USA). Representative samples of DiD labeled hMSC and unlabeled controls were imaged using a Zeiss-LSM 510 confocal fluorescence microscope.

Optical Imaging System

All studies were performed using the IVIS 50 small animal scanner (Xenogen, Alemeda, CA) using the Cy5.5 filter set (excitation filter passband: 615–665 nm, emission filter passband: 695–770 nm, background filter passband: 580–610 nm) to match the absorption and emission

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characteristics of the labeling fluorophore. Specifically, while DiD exhibits its maximum emission intensity at about 665 nm, the emission spectrum extend to about 800 nm with about half of the emitted photon flux at wavelengths longer than 695 nm, which allows them to be captured by the imaging system using the Cy5.5 filter set. A detailed description of the imaging system is provided by Troy et al. [16].

Identical illumination parameters (exposure time = 2 seconds, lamp voltage = high, f/stop = 2, field of view = 12, binning = 4) were selected for each acquisition. Gray scale reference images were also obtained under low-level illumination. For in vitro studies, cell samples were placed in a non-fluorescing black container. Whole-body real-time OI scans of anesthetized rats were acquired in prone and supine position pre-injection and, 4, 24, 48 and 72 hours post injection.

**OI of DiD labeled stem cells**

For all in vitro studies, the DiD-labeled hMSC were suspended in DMEM (isotonic solution) to preserve viability during imaging. Two sets of control experiments were performed to establish a basic understanding of signal localization and behavior. In the first experiment we evaluated the fluorescence intensity of a decreasing concentration of labeled cells \(2 \times 10^6, 1 \times 10^6, 5 \times 10^5, 2.5 \times 10^5, 1.25 \times 10^5\) and \(6.25 \times 10^4\) unlabeled controls in 0.5 ml of DMEM. In a second experiment, we evaluated the fluorescence intensity arising from a set of concentrations of cells \(2 \times 10^6, 1 \times 10^6\) and \(5 \times 10^5\) and \(5 \times 10^5\) unlabeled controls in 0.5 ml of DMEM at day 0, 2, 4, 6 and 8 after labeling.

**Animals and arthritis induction**

The Committee on Animal Research at our institution approved this study. Fourteen 4- to 6-week-old female homozygous athymic nude rats (Harlan, Indianapolis, Indiana, USA: 150–250g) were used in this study as they permitted the administration of allogenic MSC. Standard rodent chow caused significant production of autofluorescence thus the rats were fed a manganese-free diet (ssniff R/M-H, ssniff Spezialaeten GmbH, Soest, Germany) throughout the study. An immune mediated polyarthritis was induced under isofluorane anesthesia by an intra-peritoneal (960μl) injection of 1.0ml (5.2mg) of Peptidoglycan-Polysaccharide (PG PS 10S) (Fischer Scientific, Pittsburg, PA, USA), a compound composed of fragments of streptococcal cell walls. Although the animals have a well-known impaired immune response, previous studies have shown that the rats nonetheless develop an immune mediated arthritis with this agent [17]. The animals were observed daily for clinical signs of arthritis (joint swelling and limping) and underwent cell injection and OI when a polyarthritis of the ankle joints had developed, which occurred on day three post-injection (p.i.). The medio-lateral diameter of the ankle joints was measured with a caliper under anesthesia before PGPS10S injection and directly before the optical imaging studies, when clinical signs of arthritis had appeared. At this point, \(n = 11\) anesthetized rats received an intra-peritoneal injection of \(3 \times 10^6\) DiD-labeled hMSC in 0.5ml of serum free DMEM. Three additional control animals received an intra-peritoneal injection of 15μl of DiD in 0.5ml of serum free DMEM (\(n = 1\)), PGPS arthritis induction but no cell or dye injection (\(n = 1\)) and no arthritis induction and no dye injection (\(n = 1\)).

**OI Image analysis**

Images were acquired and analyzed using Living Image 2.5 software (Xenogen, Alameda, CA, USA) integrated with Igorpro (Wavemetrics, Lake Oswego, OR, USA). The digitized image intensity is expressed in arbitrary units as the fluorescent image is divided by a reference image (image of a reference object) to account for the spatial distribution of the excitation light. It must be recognized that the as recorded images contain three main image components arising from a) the fluorescence of DiD, b) the native fluorescence (autofluorescence) of the cells or

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experimental animals and c) a scattering image of the object arising from a small leakage of light through the excitation filters, an inherent limitation of the imaging system [16].

For the in vitro experiments, image analysis involved the designation of regions-of-interest (ROI) as the circular area of the well containing the cell concentrations to extract the average intensity (sum of the intensity of all pixels within this ROI divided by the number of pixels).

For the in vivo experiments, image analysis involved the definition of the ROI in each location of enhanced fluorescence as the area having intensity larger than 50% of the peak signal intensity. This ROI was automatically selected by the imaging software. The operator also defined regions of interest where there is no detectable fluorescence from DiD and served as reference representing the background (autofluorescence and leakage) components. The average normalized intensities from these ROIs of the background were also recorded using a second set of excitation filters, referred to as background filter in the Optical Imaging System section. This second filter provides excitation of the target with light that is out of resonance with the absorption spectrum of DiD, thus generating an image where the autofluorescence and leakage components are the dominant contributors. The ratio of the average intensity from the background ROIs under the two excitations provides the means to monitor the stability of the background components in time and can be used to further normalize the data against such changes [16].

**Histological evaluation**

After the last imaging procedure, the animals were sacrificed and the ankle joints, liver, lymph nodes, heart and lungs were harvested. Additionally, the ankle joints were decalcified. The samples were then bisected parasagitally, dehydrated, paraffin embedded and sectioned into 5µm transverse slices. These sections were stained with hematoxylin and eosin (H&E) for evaluation of cell morphology, with diamidino-2-phenylinole (DAPI) for core-counterstaining and localization of DiD fluorescence, and with CD44 immunostains for detection of hMSCs.

**Statistical analysis**

The statistical analysis was performed using SAS software (SAS Institute Inc, V6.9, Cary, NC, USA). Measured raw fluorescence signal intensity was described as means and standard deviations for the various experimental groups. A two-way analysis of variance (ANOVA) was used for in-vitro experiment with the dependent variable of the optical intensity and the two categorical dependent variables of days and the concentrations. A linear mixed random effects model was used for the in-vivo experiments and applied to ROI of each location separately. The animal identity was the random factor. The signal intensity was the dependent variable. The observation time was treated as a categorical variable. All three controlled animals were labeled as one control group. Interaction between treatment group and observation time were used to assess the treatment difference at different time points. Linear contrasts were used to determine the significance of change from baseline due to treatment effect at a specific observation time. P-values were adjusted for multiple comparisons using Tukey-Kramer’s method. Statistical significance was assigned for a p < 0.05.

**Results**

**Optical Imaging of DiD dye and DiD labeled stem cells**

To analyze the signal intensity of labeled hMSCs compared to non-labeled controls samples of hMSCs were imaged in 500 µl of serum free media. The imaging system was used to acquire a fluorescence image of the entire set of samples [see Fig. 1(a)] and the average intensity within the entire image area of each well was calculated. The results demonstrated that the DiD labeled hMSC exhibited a significantly stronger fluorescence signal compared to non-labeled controls.
The DiD signal of labeled hMSCs increased linearly with cell concentration as shown in Fig. 1(b) for day 0 (with $R = 0.993$), and remained significantly elevated for eight days, although it slightly decreased over time ($p = 0.0063$).

To confirm labeling and localization of the dye in the cells, fluorescent microscopy experiments were performed. The images demonstrated integration of DiD in the hMSC cell membrane as shown in Fig. 2. Trypan blue tests were performed on all cells before and after labeling to verify viability. The trypan blue test revealed no significant differences in cell viability before (97 ± 3%) and after DiD labeling (97 ± 3%; $p > 0.05$).

### Tracking transplanted hMSC's migration in vivo

Whole-body fluorescence OI baseline images before hMSC injection revealed a level of background (autofluorescence and excitation leakage) image intensity (signal per image pixel) that is about two orders of magnitude lower than the signal observed after intra-peritoneal injection of DiD labeled hMSC into animals with arthritis. Specifically, the anterior OI images showed an initial significant increased fluorescence of the whole abdomen, followed by a slow decline as demonstrated in Fig. 3. Few, small areas of persistent focal fluorescence were noted in the mid abdomen and inguinal regions, which corresponded to lymph nodes on postmortem examinations as shown in Fig. 4.

To best represent the kinetics of the observed fluorescence signal, ROIs were defined in anterior images that represent the inguinal LN, mesenteric and injection site locations as well as the right and left ankle in the corresponding posterior images. The results are summarized in Fig. 5 as normalized intensity as a function of the post-injection time. The normalized intensity represents the ratio of the as measured average intensity of each ROI divided by the average intensity at 4 hours (for normalization) after the background signal was removed from both images via subtraction of the average intensity of the same ROI in the pre-injection image. The error bars represent one standard deviation of the results obtained from 11 rats.

The results of the statistical analysis of the average fluorescence signal from the five ROIs from each rat where fluorescence localization was observed are summarized in Table 1. The $p$ values were calculated by linear mixed random effects model. Significantly increased fluorescence compared to baseline is indicated by a $p$ value of less than 0.05. (NS = non-significant. The confidence intervals of the normalized intensities as shown in Fig. 5 are also provided. The posterior (dorsal) fluorescence images revealed a significantly increased fluorescence of arthritic ankle joints compared to baseline at all time points post injection (Table 1). This fluorescence signal of the arthritic ankle joints peaked between 24 and 48 hours post injection and persisted until the end of the study, 72 hours post injection as shown in Fig. 5.

Control experiments revealed different distribution pattern kinetics. Intra-peritoneal injection of free DiD (without cells) revealed a peak fluorescence in the abdomen at 4 hours post injection, followed by a rapid decline with baseline fluorescence at 24 hours post injection. Posterior views showed no significant fluorescence of the ankle joints. Additional controls with arthritis but no cell or dye injection as well as controls without arthritis and no dye injection also did not demonstrate any fluorescence of their ankle joints.

### Histological evaluation of transplanted hMSC’s

H&E stains of the right ankle joint showed a markedly thickened synovium with inflammatory infiltrates comprised of neutrophils, lymphocytes and foamy macrophages [Fig. 6(a)]. CD44 stains showed hMSC’s in lymph nodes and the synovium of inflamed ankle joints [Fig. 4, 6 (b)]. Ankle joints of control animals showed no CD44 positive cells in the synovium.
Discussion

Our findings using optical imaging to track the fluorescence arising from labeled human stem cells are in accordance with previous studies that have proven carbocyanine dyes to be highly sensitive labels for long term in vivo cell tracking [11,12,14,18,19]. A similar dye analog, DiI, was used to label and track MSCs in a scaffold after intramuscular implantation [19]. The DiD dye has been previously used to label and track systemically injected leukocytes [14] and CD4 + Th1 cells [18], but not stem cells.

Alternative approaches for cell tracking with OI have been performed in animal models of arthritis by using endogenous labels [20]. For example, Nakajima et al. transfected CD4 + T cells with a recombinant retroviral vector encoding green fluorescent protein and firefly luciferase reporter genes to evaluate the localization of the cells in a collagen induced arthritis model with OI [21]. The advantage of our technique over endogenous probes is the ease of labeling and the lack of genetic perturbation where the long-term consequences are unknown. Endogenous labels have the advantage of a stable signal that is not affected by cell division and disappears after cell death enabling confirmation of cell engraftment.

There remains no consensus as to the optimal delivery mode (intra-articular or systemic) or cell number of stem cell transplants [21–28]. Tang et al. reported preferential cell engraftment with neuronal progenitor cells after local injections, however, intravenous injection did have migratory capabilities [29,30]. A similar result was found with MSC injection in a model of myocardial ischemia [31]. Intra-peritoneal injection was selected in our study based on current practice and clinical relevance in that multiple affected joints could be treated with one cell administration. However local injection would have the advantage of a more targeted cell delivery without loss of cells to other organs [32].

We recognize several limitations of our study. The streptococcal derivative, PGPS10S is a recognized experimental model for rheumatoid arthritis with comparable features such as synovial hypervascularization and cellular infiltration with macrophages and fibroblasts [33]. However, the transient nature of this arthritis model with spontaneous resolution of joint inflammations after about 10–14 days limits long-term evaluations. Furthermore, although the animals have a well known impaired immune response previous studies have shown that the rats nonetheless develop an immune mediated arthritis with this inducing agent [17]. The volume of cells chosen for injection was based on previously published reports and allowed for in vivo tracking of transplanted cells. Due to a non-linear correlation between DiD concentration and fluorescence signal, we were not able to estimate the number of transplanted cells that finally reached inflamed joints [34–36]. In contrast, recent studies have shown a robust correlation between fluorescence and bioluminescence signals and cell numbers, further attesting to the use of OI for following cell survival quantitatively and longitudinally [37,38]. General limitations of OI-based cell tracking techniques include limited depth of penetration, limited quantification and poor spatial resolution due to scatter [10,39]. However, these limitations are currently being addressed by the development of NIR fluorescence molecular tomography, as well as hybrid OI-radiography or OI-ultrasound techniques with the realistic possibility of imaging human joints in the future [40]. Despite these limitations, our data demonstrate that DiD labeling provides stable detection of stem cells with OI over several days and can be applied to trace the accumulation and integration of MSC in arthritic joints. OI techniques appear useful for expediting research on monitoring various factors that may influence transplanted stem cell survival and therapeutic progress.

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Fig. 1.
(A) In vitro fluorescence image of samples containing different concentrations of DiD-labeled hMSC’s in 500 μl of DMEM for decreasing cell concentration (cells/ml) and 500,000 cells/ml non-labeled control. (B) Normalized intensity of the fluorescence OI (in arbitrary units) of DiD-labeled hMSC’s and non-labeled controls immediately after labeling (day 0) and post labeling (day 2, 4, 6, 8). Data are displayed as means and SD of triplicate samples.
Fig. 2. High magnification fluorescence microscopy images of (A) DiD labeled hMSC (shown as red in left image) with DAPI counter stain (blue = nucleus in middle image) demonstrating the accumulation of DiD within the cell membrane and, (B) MSC labeled only with DAPI. Image showing the spatial overlap of the distribution of each label is shown on the right. Scale bar: 10μm.
Fig. 3.
Optical images before and after IP injection of DiD-labeled hMSC’s show fluorescence of the abdomen on early post-injection scans (indicated by blue arrow) and a progressively enhanced fluorescence of right ankle (red arrow). The corresponding color bars indicate the as recorded pixel intensity of the normalized fluorescence image (in arbitrary units).
Fig. 4.
(A) Post-mortem fluorescence image of DiD labeled MSC distribution to the inguinal lymph nodes (ROI 1) and mesenteric lymph nodes (ROI 2) demonstrates hMSC’s migrational Dynamics. (B) CD44 stain of a mesenteric lymph node confirms presence of CD44 positive hMSC’s (60x magnification, cells are indicated by arrows).
Fig. 5.
Normalized (at 4 hours post-injection) intensity of the fluorescence image within the ROI in the inguinal LN, mesenteric, injection site, right and, left ankle joints after background subtraction (obtained from pre-injection image) at different time points post injection (units in hours). Data are displayed as means and SD’s of 11 animals.
Fig. 6.
(A) H&E stain of right ankle confirms arthritis with inflammatory cells (10x, inset 40x). (B) CD44-stain confirms CD44 positive MSCs in the arthritic synovium (40x, arrow).
Table 1

Statistical analysis of the average fluorescence signal of various targeted locations before and after DiD labeled MSC administration. More details are provided in the text.

|                          | Pre-Injection | 4 Hours PI | 24 Hours PI | 48 Hours PI | 72 Hours PI |
|--------------------------|---------------|------------|-------------|-------------|-------------|
| **Anterior OI**          |               |            |             |             |             |
| Inguinal LN              | NS            | p = 0.0265 [0.84;1.16] | p = 0.0388 [0.79;1.11] | p = 0.0258 [0.94;1.26] | NS [0.59;1.01] |
| Mesenteric LN            | NS            | p = 0.0001 [0.81;1.19] | p = 0.0008 [0.71;1.09] | p = 0.0060 [0.48;0.92] | p = 0.0111 [0.46;0.94] |
| Injection Site           | NS            | p = 0.0148 [0.78;1.22] | p = 0.0093 [0.88;1.32] | p = 0.0999 [0.80;1.30] | p = 0.0329 [0.58;1.12] |
| **Posterior OI**         |               |            |             |             |             |
| Left Ankle               | NS            | p = 0.0013 [0.59;1.41] | p = 0.0196 [0.84;1.66] | p = 0.0163 [0.48;1.42] | p = 0.0442 [0.18;1.12] |
| Right Ankle              | NS            | p<0.0001 [0.56;1.44] | p<0.0001 [1.21;2.30] | p<0.0001 [0.87;1.93] | p<0.0001 [0.11;1.09] |