Video Article

Multimodal Imaging of Stem Cell Implantation in the Central Nervous System of Mice

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Abstract

During the past decade, stem cell transplantation has gained increasing interest as primary or secondary therapeutic modality for a variety of diseases, both in preclinical and clinical studies. However, to date results regarding functional outcome and/or tissue regeneration following stem cell transplantation are quite diverse. Generally, a clinical benefit is observed without profound understanding of the underlying mechanism(s)[1]. Therefore, multiple efforts have led to the development of different molecular imaging modalities to monitor stem cell grafting with the ultimate aim to accurately evaluate survival, fate and physiology of grafted stem cells and/or their micro-environment. Changes observed in one or more parameters determined by molecular imaging might be related to the observed clinical effect. In this context, our studies focus on the combined use of bioluminescence imaging (BLI), magnetic resonance imaging (MRI) and histological analysis to evaluate stem cell grafting.

BLI is commonly used to non-invasively perform cell tracking and monitor cell survival in time following transplantation[2-7], based on a biochemical reaction where cells expressing the Luciferase-reporter gene are able to emit light following interaction with its substrate (e.g. D-luciferin)[8, 9]. MRI on the other hand is a non-invasive technique which is clinically applicable[10] and can be used to precisely locate cellular grafts with very high resolution[11-15], although its sensitivity highly depends on the contrast generated after cell labeling with an MRI contrast agent. Finally, post-mortem histological analysis is the method of choice to validate research results obtained with non-invasive techniques with highest resolution and sensitivity. Moreover end-point histological analysis allows us to perform detailed phenotypic analysis of grafted cells and/or the surrounding tissue, based on the use of fluorescent reporter proteins and/or direct cell labeling with specific antibodies.

In summary, we here visually demonstrate the complementarities of BLI, MRI and histology to unravel different stem cell- and/or environment-associated characteristics following stem cell grafting in the CNS of mice. As an example, bone marrow-derived stromal cells, genetically engineered to express the enhanced Green Fluorescent Protein (eGFP) and firefly Luciferase (fLuc), and labeled with blue fluorescent micron-sized iron oxide particles (MPIOs), will be grafted in the CNS of immune-competent mice and outcome will be monitored by BLI, MRI and histology (**Figure 1**).

Video Link

The video component of this article can be found at https://www.jove.com/video/3906/

Protocol

1. Cell Preparation

1. Experiments should be initiated using ex vivo cultured stem cell populations genetically engineered to express the Luciferase and eGFP reporter proteins. Here we use Luciferase/eGFP-expressing murine bone marrow-derived stromal cells (BMSC-Luc/eGFP) as previously described by Bergwerf *et al.*[2, 5].
2. Two days before cell labeling, plate BMSC-Luc/eGFP cells at a density of 8 x 10^5 cells per T75 culture flask in 15 ml complete expansion medium (CEM) supplemented with 1 µg/ml Puromycine.
3. Allow cells to grow for 48 hr at 37 °C and 5% CO_2.
4. Add 5 x 10^6 Glacial Blue micron-sized iron oxide particles (GB MPIO) per ml culture medium to the growing cell culture (total: 75 x 10^6 particles per 15 ml culture medium in a T75 culture flask).
5. Incubate for 16 hr to allow particle uptake via endocytosis.
6. Wash away remaining particles and allow cells to grow for an additional 24 hr to obtain cell confluency and homogeneous distribution of the GB MPIO.

7. Validate particle uptake visually using fluorescence microscopy by counting the ratio of eGFP+ and GB MPIO+ cells to the total amount of eGFP+ cells.
8. Wash GB MPIO-labeled BMSC-Luc/eGFP cells twice with 10 ml PBS and harvest cells following trypsin-EDTA treatment (5 ml per T75 culture flask for 5 minutes).
9. Wash harvested cells and resuspend at final concentration of 133 x 10^6 cells/ml in PBS.
10. Keep cells on ice until transplantation.

2. Cell Implantation in the CNS of Mice

1. Anaesthetize mice by an intraperitoneal injection of a ketamine (80 mg/kg) + xylazine (16 mg/kg) mixture.
2. Wait for 5 minutes to allow mice to fall asleep.
3. Shave the mouse head to allow for sterile manipulations.
4. Place the mouse in a stereotactic frame.
5. Desinfect the skin and wet the eyes of the mice to prevent dehydration.
6. Make a midline scalp incision to expose the skull, and drill a hole in the skull using a dental drill burr at the given coordinates: 2 mm posterior and 2 mm lateral to Bregma.
7. Vortex the cell suspension briefly and aspirate the cell suspension in the syringe.
8. Place the syringe needle at a depth of 2.5 mm under the dura and allow pressure equilibration for 1 minute.
9. Inject a 3 µl cell suspension (4 x 10^5 cells) at a depth of 2 mm under the dura and at a speed of 0.70 µl/min using an automated micro-injection pump.
10. Wait for an additional 5 minutes to prevent backflow of the injected cell suspension.
11. Slowly retract the needle and disinfect the skin borders before suturing the skin.
12. Inject 300 µl 0.9% NaCl solution subcutaneously in order to prevent dehydration, and place the mouse under a heating lamp to recover from anaesthesia. Administer post-operative analgesic in accordance with institutional standards.

3. *In vivo* Bioluminescence

1. Anaesthetize the mice by a mixture of 3% isoflurane and oxygen.
2. Place the mice in the Photon Imager and reduce anaesthesia level to 1.5% isoflurane and oxygen.
3. Inject 150 mg D-luciferin per kg body weight intravenously.
4. Acquire image for 5 minutes using the Photo Vision software.
5. Perform image processing using the M3vision software. Quantify the observed signal using fixed regions of interest.

4. *In vivo* Magnetic Resonance Imaging

1. Anaesthetize the mice with 3% isoflurane in a mixture of O_2:N_2O (3:7).
2. Place the mice in the restrainer of a horizontal 9.4T MR system and reduce anaesthesia level to 1% isoflurane in a mixture of O_2:N_2O (3:7).
3. Wet the eyes of the mice to prevent dehydration, attach a rectal probe to monitor the body temperature and monitor the breathing rate by placing a sensor underneath the mouse belly.
4. Maintain breathing rate at 110 ± 10 breaths per minute and keep body temperature constant within a narrow range of 37 ± 0.5 °C.
5. Place the surface RF coil on top of the mouse head and position the mouse in the middle of the magnet.
6. Acquire a set of 10 coronal T2-weighted spin echo (SE) images to obtain specific anatomical information and T2*-weighted gradient echo (GE) images in order to study stem cell migration with an in-plane resolution of 70 µm^2. Set sequence parameters as follows: repetition time (TR): 500 ms, echo time (TE): 8 ms (GE sequence) and repetition time (TR): 4200 ms, echo time (TE): 12.16 ms (SE sequence); field of view (FOV): 18x18 mm^2, matrix: 256x256, 1 mm slice thickness and 1 mm slice separation (Paravision 5.1 software).
7. Perform data processing using Amira 4.0 software.

5. Post Mortem Histology

1. Anaesthetize the mice very deeply by inhalation of an isofluorane (4%), oxygen (0.5 L/min) and nitrogen (1 L/min) mixture for 2 minutes. Sacrifice the mice by cervical dislocation.
2. Remove the mouse brain from the skull and fixate the brain tissue in 4% paraformaldehyde in PBS for 2 hours.
3. Dehydrate the brain tissue by placing the brain subsequently in different gradients of sucrose: 2 hr in 5% sucrose in PBS, 2 hr in 10% sucrose in PBS, overnight in 20% sucrose in PBS.
4. Freeze the brain tissue using fluid nitrogen and store the tissue at -80 °C until sectioning.
5. Section the brain tissue in 10 µm thick sections using a cryostat.
6. Screen unstained cryosections for blue fluorescence from the GB MPIO particles and green fluorescence from the eGFP expressing cells using a fluorescence microscope.
7. Screen unstained cryosections for green/red background fluorescence from inflammatory cells.
8. Perform further immunohistochemical and immunofluorescent stainings to identify endogenous cell populations (e.g. microglia, astrocytes, T cells,...) interacting with cellular grafts.

6. Representative Results

We here visually presented an optimized sequence of events for successful multimodal imaging of Luciferase/eGFP-expressing (stem) cell populations in the CNS of mice. At first, the described GB MPIO labeling procedure results in highly efficient labeling of BMSC-Luc/eGFP cells, which can easily be validated using fluorescence microscopy (**Figure 2A**). Next, upon grafting of GB MPIO labeled BMSC-Luc/eGFP in

the CNS of mice, survival of the cellular graft can be monitored by *in vivo* BLI based on luciferase activity (**Figure 2B**). Additionally, the exact localization of grafted cells can be monitored by *in vivo* MRI based on the iron content of the GB MPIO (**Figure 2C**). Finally, histological analysis allows validation of the results obtained by BLI and MRI. Stable survival was confirmed by a stable eGFP expression in time (**Figure 2D**). For this, colocalization of eGFP-expressing cells with blue fluorescent MPIO allows for exact determination of localization and survival of grafted cells. In addition, this dual fluorescent labeling of the stem cells reduces the possibility to observe false positive results based on background fluorescence created by inflammatory cells[5].

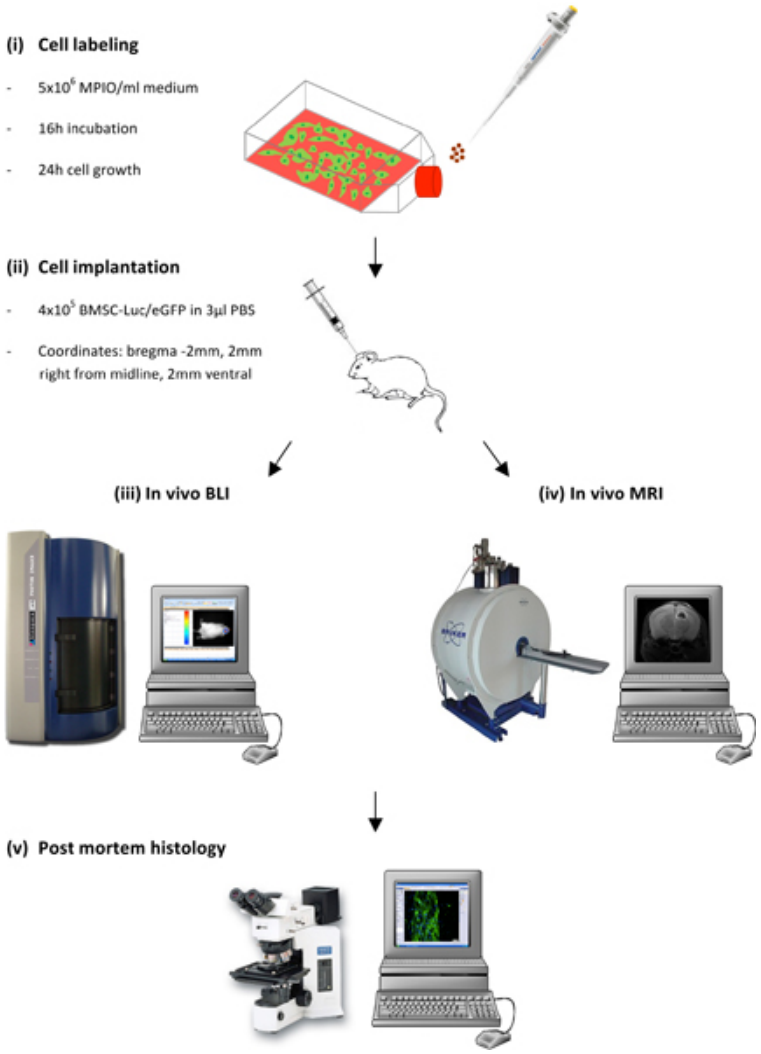

Figure 1. Overview of the handling sequence

i. Cell labeling: BMSC-Luc/eGFP are labeled with 5 x 10^6 GB MPIO particles per ml culture medium. After 16 hr of incubation and a following rest period of 24 hr, cells are harvested for intracerebral implantation.
ii. Cell implantation: GB MPIO labeled BMSC-Luc/eGFP are grafted in mouse brain at the following coordinates: bregma -2 mm, 2 mm right from the midline and 2 mm under dura.
iii. *In vivo* BLI: Following intravenous administration of 150 mg D-luciferin/kg body weight, the generated light was acquired during 5 minutes using the Photon Imager.
iv. *In vivo* MRI: A set of 10 coronal T2-weighted spin echo (SE) images and T2*-weighted gradient echo (GE) images with an in-plane resolution of 70 µm^2 were acquired. Sequence parameters were as follows: repetition time (TR): 500 ms, echo time (TE): 8 ms (GE sequence) and repetition time (TR): 4200 ms, echo time (TE): 12.16 ms (SE sequence); field of view (FOV): 18x18 mm^2, matrix: 256x256, 1 mm slice thickness and 1 mm slice separation.
v. Post-mortem histology: 10µm thick cryosection were screened for eGFP expressing and blue fluorescently labeled BMSC using a fluorescence microscope.

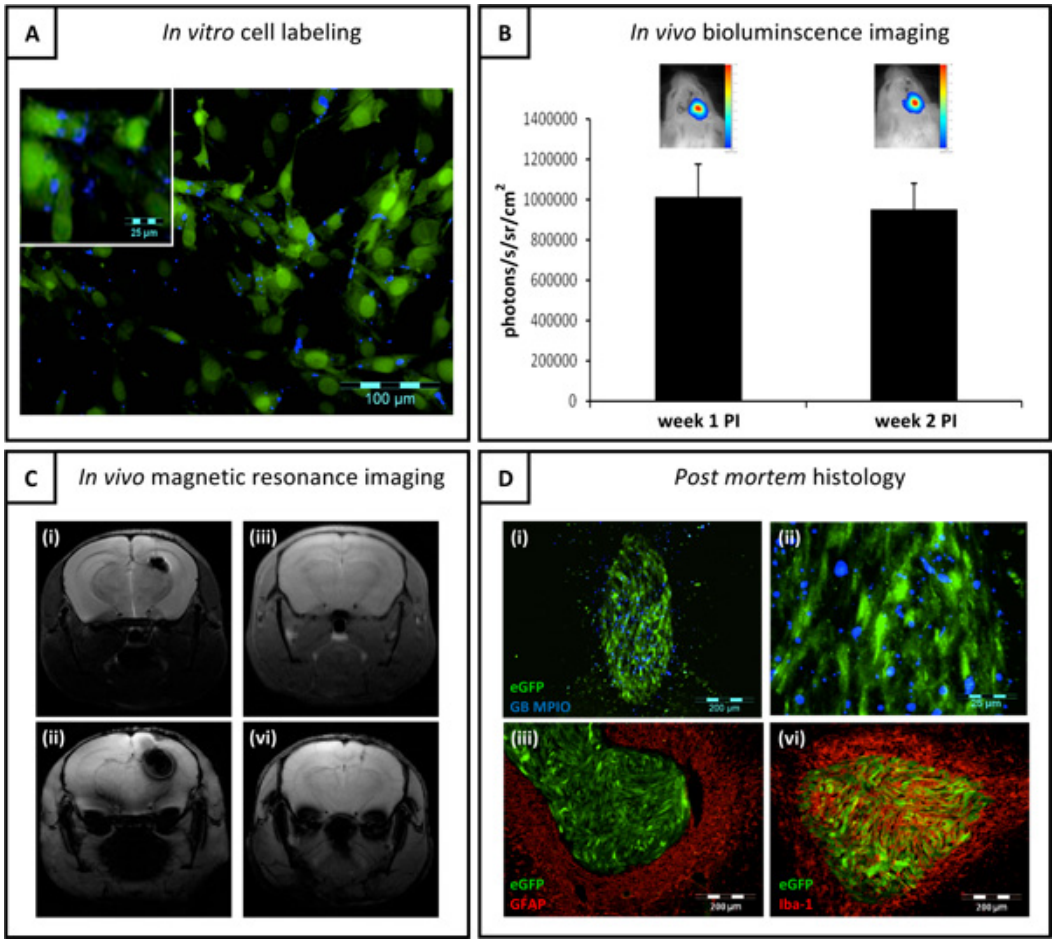

Figure 2. Multimodal imaging of stem cell grafts

A. Direct immunofluorescence microscopy of Glacial blue (GB) MPIO labeled BMSC-Luc/eGFP.
B. *In vivo* BLI of mice injected with $4x10^5$ GB MPIO labeled BMSC-Luc/eGFP cells at week 1 and week 2 after implantation. Statistical analysis of detected BLI signals at week 1 and week 2 post-implantation. Data are expressed as average number of photons per second per steradian per square centimeter (ph/s/sr/cm^2) from a 5 minute time period +/- standard error calculated from a fixed region of interest in mice showing a clear BLI signal at the injection site.
C. Coronal two-dimensional MRI of GB MPIO labeled BMSC-Luc/eGFP (left panel: (i) T2-weighted spin echo image and (ii) T2*-weighted gradient echo image) and unlabeled BMSC-Luc/eGFP (right panel: (iii) T2-weighted spin echo image and (vi) T2*-weighted gradient echo image) grafts in mouse brain.
D. Histological analysis of GB MPIO labeled BMSC-Luc/eGFP implants at week 2 post-implantation. (i) Direct immunofluorescence analysis for localization of eGFP-expressing and GB MPIO labeled BMSC-Luc/eGFP grafts. (ii) High magnification detail of (i). (iii) Immunofluorescent staining for GFAP antigen, indicating the development of astrocytic scar tissue in the surrounding of GB MPIO labeled BMSC-Luc/eGFP. (vi) Immunofluorescent staining for Iba-1 antigen, indicating the invasion and surrounding of GB MPIO labeled BMSC-Luc/eGFP by microglia.

Discussion

In this report, we describe an optimized protocol for the combination of three complementary imaging modalities (BLI, MRI and histology) for detailed characterization of cellular implants in the CNS of immune competent mice. A combination of reporter gene labeling of cells, based on genetic modification with the reporter genes firefly Luciferase and eGFP, and a direct cell labeling with GB MPIO, leads to an accurate assessment of stem cell grafts *in vivo*.

For particle labeling of BMSC, the GB MPIO particle size (1.63µm) in combination with the anionic surface (carboxyl substitutes) is suggested to facilitate endocytotic uptake of these particles by non-phagocytic cells[16-18]. However, it needs to be mentioned that the incubation time to obtain high labeling efficiency is cell type dependent. For example, phagocytic cells (like macrophages and dendritic cells) are able to internalize these particles more rapidly (incubation time of 0.5 - 1 hr) as compared to non-phaghocytic cell types (like BMSC or neural stem cells), which need an incubation time of minimum 16 hr for efficient labeling. This needs to be taken into account while performing cell labeling experiments and efficiency of labeling should always be determined in advance using fluorescence microscopy and/or flow cytometric analysis. As labeling of cell populations with GB MPIOs does not influence cell viability, cell proliferation or cell phenotype[5], the combination of iron oxide and a fluorophore in these particles therefore creates an ideal opportunity to visualize them both by MRI and optical imaging. Moreover, due to the high iron content and large particle size, MPIO particles can be used for single cell[12,19,20] and single particle imaging[21] by MRI. This is extremely interesting when it comes to study cell migration as single cells can be observed. However, the high iron content of the used GB MPIO particles

has also certain disadvantages when aiming to delineate the exact size of the graft site by MRI. The very high iron content of MPIOs not only creates magnetic inhomogeneities at the implant site, but also in the surrounding of the cellular implant, resulting in an overestimation of the implant site volume and a less accurate discrimination between implanted cells and the surrounding tissue. Consequently, the type of particle, needed for cell labeling, will depend on the study design. When aiming to determine stem cell migration, a very high sensitivity and thus high iron containing MPIOs will be needed. On the other hand, when aiming for the accurate localization of stem cell grafts, smaller SPIOs with a lower iron content might be a preferable alternative[22]. Moreover, iron particles generate negative contrast introducing another problem concerning the discrimination between implanted cells and bleeding following cell grafting. Therefore a lot of research is going on towards the use of positive contrast agents for cell labeling. Next to the particle type, the type of sequence used for the MRI acquisition also depends on the aim of the study. When MRI is used to delineate the implant to obtain accurate information on the location of the implant and the implant volume, the T2 sequence (spin echo) is favourable as it provides anatomical information with high accuracy. On the other hand, the T2* sequence (gradient echo) is a sequence used to study possible migration of grafted cells as this sequence takes into account all inhomogenities of the magnetic field rendering a higher sensitivity towards compounds influencing the magnetic field. This sequence is absolutely necessary when it comes to the detection of a small amount of cells that might have migrated out of the implanted region. Using this sequence we were able to conclude that BMSC-Luc/eGFP cells do not migrate after striatal implantation in the CNS of mice.

While MRI provides data regarding cell implant localization, additional BLI analysis provides data regarding cell implant survival[2,4,11]. As the enzymatic reaction between the luciferase enzyme and the substrate luciferin needs the presence of O_2 and ATP, it is a reliable technique to study cell survival. Necrotic cells will not express the luciferase enzyme and no O_2 and ATP will be present. Moreover, only cells expressing the luciferase enzyme are able to catalyze the reaction, resulting in the production of light, which resembles the high sensitivity of the technique. BLI can be performed in a quantitative manner because the amount of produced light is proportional with the amount of luciferase-expressing cells. However, important considerations need to be taken into account while performing BLI quantitatively as several factors other than cell amounts can influence the expression level of luciferase or the amount of detected light. For example, anesthesia level can influence the luciferase enzyme and/or the luciferin availability[23], different factors and compounds can influence the substrate availability and/or uptake[24-26] or promoter activity[27-30], resulting in differences in signal output. Moreover the use of BLI is limited to small animals as the technique is restricted to low tissue penetration of light. Consequently, when aiming to perform quantitative BLI to follow up cell survival after transplantation, all parameters possibly influencing signal variations need to be kept as standard as possible. For example, implantation depth of cellular grafts, anaesthesia level and administration route / amount of the substrate administered before BLI acquisition, proper shaving of the animal's skin, etc.

Taking into account the above described advantages and disadvantages of molecular imaging, the obtained results always need to be validated by histological analysis. Hereby various cellular phenotypes and cellular interactions between different cell types can be visualized with highest sensitivity post mortem. Despite the fact that this technique is the validation tool of choice, the presence of background fluorescence created by inflammatory cells surrounding and/or invading the graft site needs to be considered, as this might lead to false positive results. However, the use of a dual labeling with a fluorescent reporter gene, such as eGFP, and a fluorescent probe, such as GB MPIO, reduces the possibility of misidentification (i.e. grafted cells should display both specific fluorescences, without displaying background fluorescence in irrelevant light channels).

Summarizing, while BLI is a unique technique to monitor cell survival in time in a quantitative manner with very high sensitivity but low resolution, MRI is the technique of choice to overcome the low resolution obtained with BLI. Combination of these *in vivo* techniques renders sufficient information regarding survival and localization of grafted cells *in vivo* in a non-invasive manner. Finally, cellular graft interactions with surrounding tissue and/or endogenous inflammatory cells can easily be monitored post mortem using histology. Although, at the moment, the latter still needs to be done by histological analysis, the main challenges for the future will be to improve existing *in vivo* molecular imaging modalities to allow real-time assessment of the parameters with similar resolution and sensitivity as obtained using histological analysis.

Disclosures

The authors declare no conflict of interest.

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