Methods Molecular Biology (2013) 1052: 13–28
DOI 10.1007/7651_2013_21

Published online: 3 May 2013

Labeling and Tracking of Human Mesenchymal Stem Cells Using Near-Infrared Technology

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Abstract

The recently developed near-infrared (NIR) light imaging technology combines low background noise with deep tissue penetration and readily allows imaging and tracking of NIR-labeled cells, following transplantation in small animal model of diseases. The real-time and longitudinal detection of grafted cells in vivo, as well as their rapid ex vivo localization, may further clarify graft interactions with the surrounding, in target and nontarget organs throughout the body, over time. The present chapter describes a protocol for (1) the efficient labeling of human mesenchymal stem cells (hMSCs) using a membrane intercalating dye, emitting in the NIR 815 nm spectrum; (2) the stereotaxic transplantation of NIR815-hMSCs in rodent model of Parkinson's disease; and (3) the longitudinal in vivo detection of the grafted cells and the subsequent ex vivo imaging in selected tissues.

Keywords: Cell imaging, 6-Hydroxydopamine, Intra-striatal injection, Neurodegeneration, Stem cell transplantation, Cell therapy, Parkinson's disease

1 Introduction

Endogenous regeneration in the central nervous system (CNS) is very limited and allogeneic stem cells (SCs) have been increasingly recognized as a potential tool to replace or support lost cells injured by neurodegenerative processes. Several clinical studies, despite comparable setups, have reported contradictory results (1). Efficient SC therapy requires a still missing extensive comprehension of the progressive environmental cues and reciprocal interactions between the host and the donor cells. Facilitating and improving the number of cells engrafting in affected brain area are very important to achieve the best functional efficacy. This may be addressed by experimental work seeking to optimize cell delivery, improve characteristics of grafted cell, or modulate the CNS surroundings in the constant effort to better monitor cells and translate potential neurorescue mechanisms into novel targets for therapy (2).

The availability of experimental animal models to assess new therapeutic strategies in neurodegenerative disorders, including Parkinson's disease (PD), has been a fundamental breakthrough in the field of neuroscience research. Despite the increasing availability of transgenic and innovative models, the neurotoxin 6-hydroxydopamine (6-OHDA) remains one of the most widely used tools to induce a lesion in the rat (3). In particular, the intrastriatal (IS) injection of 6-OHDA in rats induces slow and retrograde degeneration of the nigro-striatal pathway that mimics, at least in part, the disease progression in humans. The characteristic progressive neuronal loss induced by this toxin in animals creates a therapeutic time-window wherein the neuroprotective potential of numerous pharmacological and non-pharmacological therapies has been successfully evaluated (3).

Near-infrared (NIR) light imaging offers new opportunities as a sensitive and noninvasive detection technique for diagnostic purposes. It represents an important advantage compared to other procedures, and avoids the use of retro- or lentiviruses, not transferable to clinical use. Particularly, it offers promising opportunities to develop noninvasive imaging protocols readily applicable to patients. This technology holds enormous potential for a wide variety of in vivo applications and is being increasingly used in small animal research. The use of NIR wavelengths for imaging allows deep penetration into tissues with minimal background and high optical contrast (4, 5). This simple, noninvasive technology consents live and real-time determination and imaging of biological targets without the need of exhaustive tissue sampling (6). In humans, the use of noninvasive NIR imaging has already been proposed as a routine diagnosis tool in stroke (7) and it is currently employed as a valuable bedside device for in vivo targeting of cancer and other tissue abnormalities (7, 8). Moreover, we have recently demonstrated that NIR technology allows longitudinal detection of fluorescent-tagged cells in living animals, giving immediate information on how different delivery routes affect cell permanence/persistence (9). NIR imaging allows investigations of transplanted cells from whole animals to the single-cell level over time, thus allowing cell tracking and assessment of integration/localization/migration in host surroundings and considerably reducing the number of animal experiments needed, as well as interindividual variability.

NIR technology can be readily accessible in any laboratory without the requirement for expensive clinical diagnostic equipment and specialized technical abilities, compared to those required for other in vivo imaging procedures (i.e., magnetic resonance). In this protocol we describe how human mesenchymal stem cells (hMSCs) can be rapidly and simply labeled with an NIR membrane intercalating dye, easily visualized in live animals after grafting in an animal model of PD and subsequently analyzed ex vivo using a suitable imaging platform.

2 Materials

2.1 Cell Labeling

1. Fully equipped sterile hood.
2. Water bath reaching 37 °C temperature.
3. Disposable sterile pipettes, flasks, polypropylene test tubes, and tips.
4. Centrifuge.
5. Human commercial mesenchymal stem cells (Cambrex, Walkersville, MD, USA).
6. Mesenchymal stem cell growth medium (MSCGM) (Cambrex).
7. Trypan blue (Invitrogen, Carlsbad, CA, USA).
8. Phosphate buffered saline (PBS) (without Ca^{2+} and Mg^{2+}) pH 7.2–7.4 (Invitrogen).
9. Trypsin 0.05 %–EDTA 0.02 % (Sigma-Aldrich, Saint Louis, MO, USA).
10. Fetal bovine serum (FBS) (Sigma-Aldrich).
11. CellVue® NIR815 Midi Kit for membrane labeling containing diluent C and dye (Polyscience, Warrington, PA, USA) (see Note 1).
12. Cytocentrifuge equipped with appropriate cuvettes and filters (Shandon, Pittsburgh, PA, USA).
13. Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

2.2 Transplantation of Labeled hMSCs

1. Sprague–Dawley male rats (200 g at the time of surgery; Charles River, Calco, Italy).
2. Thiopental (50 mg/kg) (Rotexmedica GmbH, Trittau, Germany).
3. Sterile saline solution.
4. Gel foam (Harvard Apparatus Inc., Holliston, MA, USA).
5. Stereotaxic instrument for rat complete with ear bars (Stoelting, Kiel, WI, USA).
6. Optical fibers.
7. Disinfectant (any type is suitable).
8. Hamilton syringe 10 μl attached with a 26 gauge needle (7000 Series, Sigma-Aldrich).
9. Surgical tools: Scissors, sharp forceps, scalpels, sterile cotton pads, syringe and needles, metal clips, applying forceps.
10. Electric shaver.
11. Heating pad.
12. Odyssey® Infrared Imaging System (LI-COR).
13. MousePOD® in vivo Imaging Accessory (LI-COR).

2.3 Ex Vivo Analysis of Transplanted Labeled hMSCs

1. Cryostat.
2. Polylysine slides (Thermoscientific, Braunschweig, Germany).
3. Coverslides.
4. Pap pen (Sigma-Aldrich).
5. Neutral buffered formalin (NBF; Carlo Erba, Italy).
6. Primary antibody: Mouse anti-tyrosine hydroxylase, TH, (MAB 318, Millipore, Billerica, MA, USA).
7. Secondary antibody: IRDye® 700 goat anti-mouse antibody (LI-COR).
8. PBS (Invitrogen).
9. Triton X-100 (Sigma-Aldrich).
10. Tween 20 (Sigma-Aldrich).
11. Normal goat serum (NGS) (Sigma-Aldrich).
12. Blocking solution: PBS containing 10 % NGS and 0.3 % Triton X-100 (store fresh solution at 4 °C for up to 1 week).
13. Antibody solution: PBS containing 1 % NGS and 0.3 % Triton X-100 (store fresh solution at 4 °C for up to 1 week).
14. Odyssey® Infrared Imaging System (LI-COR).

3 Methods

3.1 Thawing and Culture of hMSCs

Commercial mesenchymal stem cells (Cambrex) are seeded following the manufacturer's instructions (see Note 2):

1. Quickly thaw cell-containing cryovials in a 37 °C water bath until the last sliver of ice melts.
2. Gently add thawed cells to a tube containing 5 ml of temperature-equilibrated MSCGM using a micropipette.
3. Centrifuge cell suspension at 200 × *g* for 10 min at room temperature.
4. Count the total number of viable cells with the trypan blue exclusion method and seed them at a density of 5,300 cells per cm^2 (around 400,000 cells in a T75 flask).
5. Grow cells in an incubator at 37 °C, 5 % CO_2 for at least two passages (see Note 3).

3.2 NIR Labeling of hMSCs

Use cells at low passages (up to the fourth passage) (see Note 4). All the manipulations should be done at room temperature and preferably avoiding direct light exposition.

1. Discard supernatant from cell cultures and wash once with PBS.
2. Aspirate PBS, add 2 ml of trypsin–EDTA, and incubate at 37 °C, 5 % CO_2 for 5–10 min.
3. Gently shake the flask to resuspend loosely attached cells.
4. Add 10 ml of MSCGM to inactivate the trypsin–EDTA.
5. Collect cells in a 15 ml conical polypropylene tube and centrifuge at 500 × *g* for 10 min at room temperature.
6. Wash cells with PBS: Resuspend cells in 10 ml PBS and centrifuge as above (step 5).
7. Resuspend cell pellet in a small volume of PBS (around 100 μl) and transfer to a small conical bottom 1.5 ml polypropylene tube.
8. Centrifuge as above (step 5) and wash once again with PBS (volume around 100 μl).
9. After centrifugation, carefully aspirate the supernatant with a micropipette or an insulin syringe, being careful not to remove any cells.
10. Resuspend cells in 50 μl of diluent C, contained in the CellVue 97® NIR815 Midi Kit (for 1×10^6 cells) with gentle mixing (see Note 5).
11. Immediately prior to staining prepare the staining solution by mixing 50 μl of diluent C and 1 μl of NIR815 dye in a sterile 200 μl polypropylene tube (see Note 6).
12. Add the staining solution to the cell suspension, immediately mix the sample by pipetting, and incubate for 5 min with periodic mixing (see Note 7).
13. Block staining by adding 200 μl of FBS, transfer cells in 15 ml conical bottom polypropylene tube, and wash cells twice with 10 ml of MSCGM to eliminate unbound dye.
14. Resuspend cells in MSCGM (for 1×10^6 cells use 500 μl) in a sterile 500 μl polypropylene tube. Keep a small aliquot (5 μl) to assess adequate labeling of cells (see Section 3.3).
15. Use 10 μl of the cell suspension to check viability of the cells by trypan blue exclusion test.
16. Plate cells in a T75 cm^2 flask in MSCGM overnight, if needed (see Note 8).

3.3 Assessment/ Control of Adequate NIR815 Cell Labeling

To check the adequate labeling of cells, a cytocentrifugation onto glass slides, immediately after staining procedures, may be performed.

1. Place slides and filters into appropriate slots in the cytospin and load not fewer than 100 μl of cell suspension (containing around 20,000–50,000 hMSCs) in each cuvette (see Note 9).
2. Spin the sample for 7 min at 400 rpm (see Note 10).

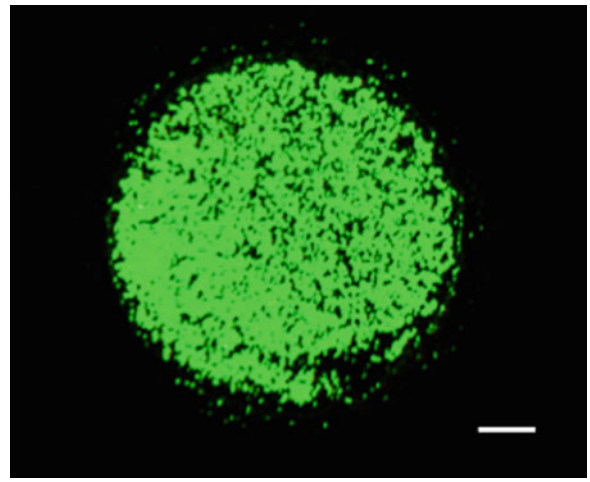

Fig. 1 Cytospin. Representative image showing a cytospin of NIR815-labeled hMSCs. Scale bar: 1 mm. (Modifed from Bossolasco et al., Int J Nanomed 2012)

3. Remove the filters from their slides and proceed to scan using the Odyssey® Infrared Imaging System.
4. Place the slide on the surface of the imager cells facing upwards and determine the scan area.
5. Using the software dedicated to the imager insert the coordinates of the scan area and set the scan parameters. Set the "offset focus" to 1 mm (see Note 11). At this point a rapid scan with low resolution (169 μm) and quality (lowest) is enough to assert adequate labeling of cells (Fig. 1). Scan intensity is usually set to 3 but needs to be adapted to the amount of cells (see Note 12).

3.4 Intra-cerebral Transplantation of NIR815-Labeled hMSCs

All animal procedures are carried out in accordance with the European Communities Council Directive of November 24th, 1986 (86/609/EEC), and are approved by the local Animal Care Committee.

The original transplantation protocol has been applied to PD-like rats bearing a unilateral 6-OHDA-induced lesion of the nigro-striatal pathway already described elsewhere (9). The transplantation procedure described below can however be applied to any animal model of neurodegeneration or brain injury (see Note 13).

1. Prepare the stereotaxic frame mounted with the Hamilton syringe.
2. Weigh and anesthetize male Sprague–Dawley rats using 50 mg/kg thiopental (intraperitoneal injection). Leave the animal in a cage with bedding until unconscious (see Note 14).
3. Accurately shave the animal's head with an electric shaver to uncover skin and wipe the area with a sterile cotton pad imbedded with disinfectant solution.

4. Place the anesthetized animal in the stereotaxic frame with the tooth bar set at −3.3 mm (see Note 15).
5. Perform a net 2 cm midline incision on the skin with a sharp scalpel starting between the eyes and fix the skin with two metal clamps to keep the skull uncovered.
6. Remove the connective tissue above the bone by scraping with the scalpel to allow visualization of bregma.
7. Find the bregma and point the needle exactly on it. Read the precise anterio-posterior (AP) and lateral (L) coordinates on the manipulator (*x*- and *y*-axis) of stereotaxic frame and retract the needle. Calculate the location for the injection site and then move and lower the needle to the specific coordinates (see Note 16).
8. Evidence the injection position on the skull with a fine marker and retract the needle.
9. Drill a small hole through the skull bone above the injection coordinate until you reach the dura mater (see Note 17).
10. Fill the syringe with the previously optimized cell dose (see Note 18).
11. Lower the needle until it reaches the dura mater without piercing it (see Note 19).
12. Determine the DV position on the stereotaxic frame. Calculate the injection coordinate and slowly lower the needle to the desired depth (see Note 20).
13. Slowly infuse the cells (see Note 21). Cells are injected at 0.5 μl/min and needle is left in place to allow diffusion of the injected volume.
14. After at least 5 min retract the needle VERY SLOWLY to avoid suction of the cells along the needle track due to pressure.
15. Fill burr hole with foam and clip wound.
16. Place the animal in a clean cage with heating pad until it regains consciousness.

3.5 In Vivo Tracking of NIR-Labeled hMSCs

1. In vivo tracking of NIR815-labeled cells can be performed at any time point following intracerebral transplantation. Before starting with the animal procedure turn on the Odyssey® Imager (LI-COR) and MousePOD® in vivo Imaging Accessory (Fig. 2a) so that the chamber can reach the desired temperature (37 °C). Constant temperature is maintained in the chamber throughout the in vivo imaging procedure.
2. Weigh and anesthetize transplanted rats using thiopental (50 mg/kg, intraperitoneal injection). Animals need to be

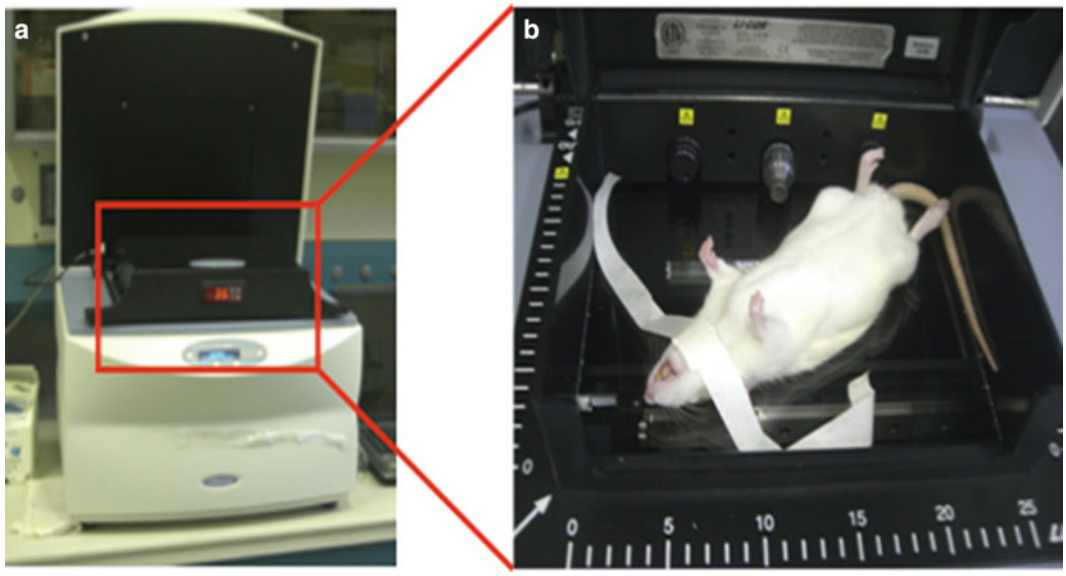

Fig. 2 NIR imaging system. (**a**) Photograph of the Odyssey® Infrared Imaging System mounted with MousePOD® in vivo Imaging Accessory. (**b**) Photograph of anesthetized animal adequately positioned on the glass surface of the imager to allow correct imaging procedure

maintained under sedation throughout the entire in vivo imaging procedure (see Note 22).

3. Accurately shave the fur of the animal in the area that will be exposed to the imager (see Note 23).
4. Place the animal on the instrument in a supine position so that the area to be scanned is in direct contact and lies flat with the imager surface, determine the scan area, and close the chamber (Fig. 2b) (see Note 24).
5. Insert the coordinates of the scan area, set the scan parameters in the dedicated software, and initially perform a rapid scan at low resolution (169 μm) and quality (lowest) to assert the precise localization of the transplanted cells and determine the best "focus offset." Scan intensity of the 800 nm laser is typically set between 7 and 10 (see Note 25).
6. Perform a second scan to obtain a high-resolution image (Fig. 3a) (see Note 26).
7. Once the scan is completed a semiquantitative evaluation of the NIR815 can be performed (Fig. 3b) (see Note 27).
8. Images can be transformed in pseudo-colors to obtain a visual representation of NIR815 intensity levels (Fig. 3c).

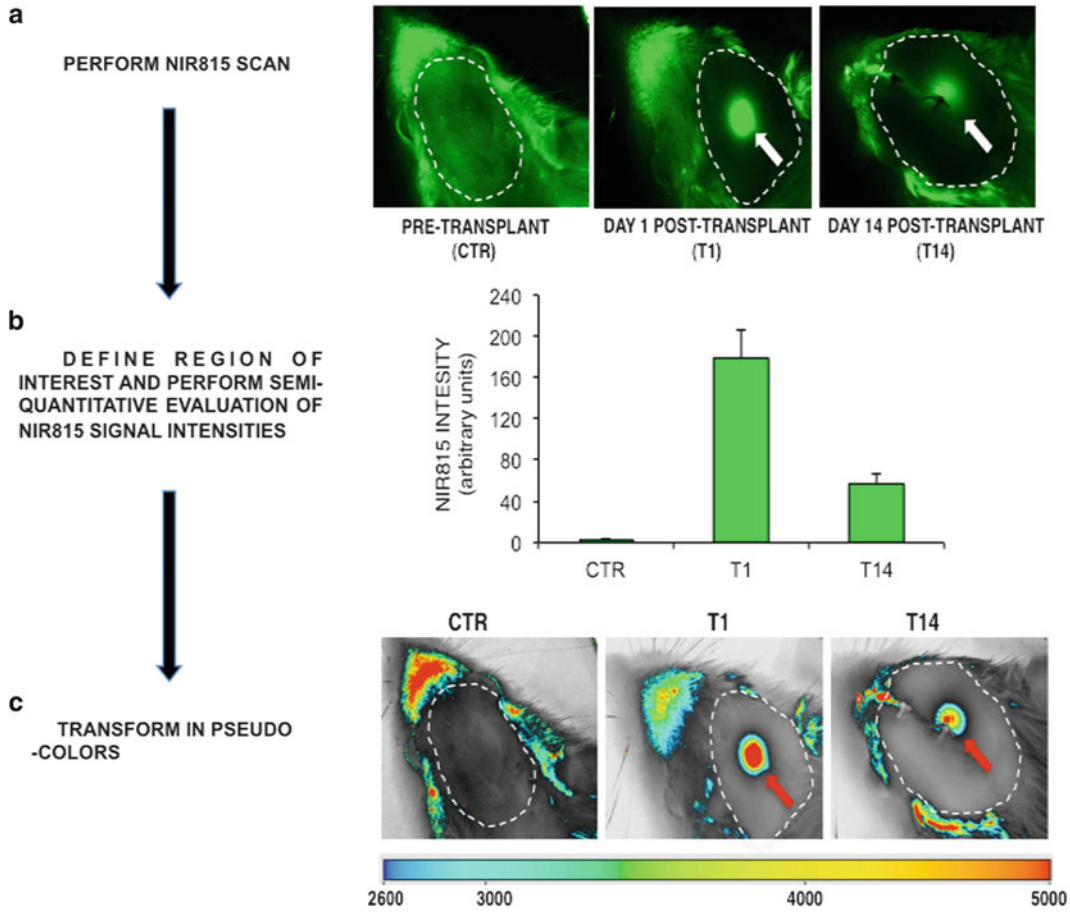

Fig. 3 In vivo and ex vivo NIR imaging of grafted NIR815 hMSCs. (**a**) Representative photograph of longitudinal NIR imaging in a live animal. *Dotted circles* represent the area of interest selected for the analysis. (**b**) Semiquantitative analysis of the scan. (**c**) Transformation of NIR815 intensities in pseudo-colors. (Modified from Bossolasco et al., Int J Nanomed 2012)

3.6 Ex Vivo Imaging of NIR-Labeled hMSCs

3.6.1 Whole Organ

1. At the end of the experimental paradigm anesthetize the animal (100 mg/kg thiopental) and sacrifice it by decapitation (see Note 28).
2. Immediately remove brain, place it on a glass slide, and scan rapidly. (Note that to avoid degradation of the tissue only a low-resolution (169 μm) and low-quality scan (lowest) is performed. The green signal indicates the presence of the transplanted cells (Fig. 4).) Moreover, at the time of sacrifice any organ may be removed and scanned to evaluate potential migration of cells to peripheral tissues/organs (see Note 29).
3. Freeze organs on dry ice immediately after scanning and keep at −80 °C for future evaluation.

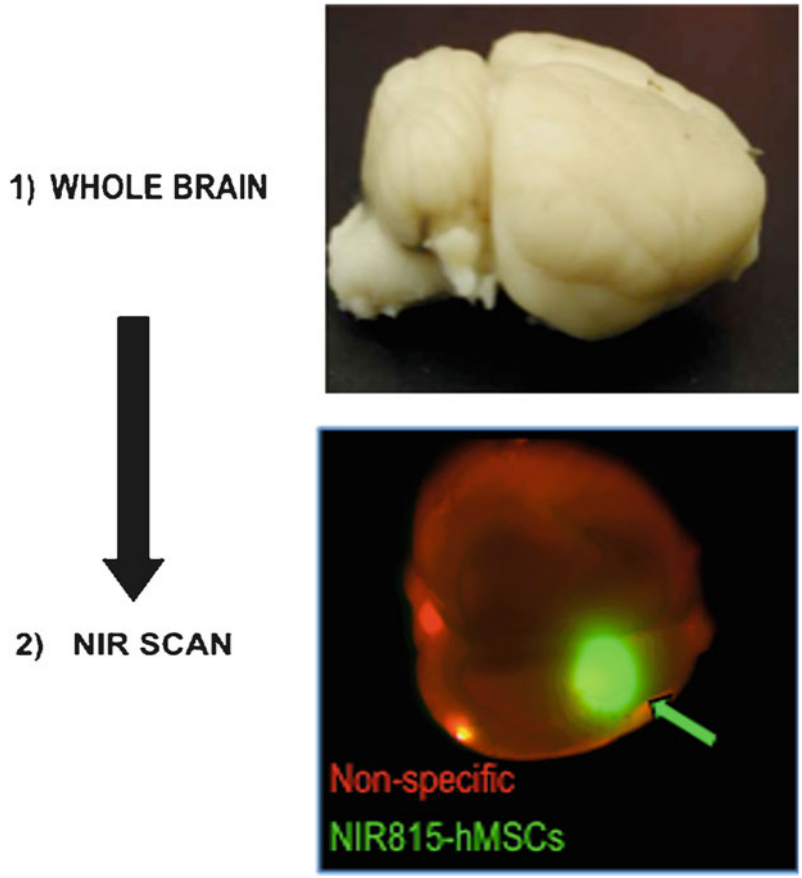

Fig. 4 Ex vivo NIR imaging of whole brain. The presence of the grafted cells is clearly indicated by the intense NIR815 signal (*green arrow*) visible at the site of transplantation. The *red* signal corresponds to a nonspecific background of the tissue in the *red* spectrum. (Modified from Bossolasco et al., Int J Nanomed 2012)

3.6.2 Tissue Sections

1. Cut brain coronal sections (25 μm) using a cryostat and mount them on polylysine slides. Up to four sections containing the striatum can be mounted on each slide.
2. Let the sections dry, protected from light, for at least 30 min.
3. Place all slides side by side on imager surface, sections facing upwards.
4. Determine and insert the coordinates of the selected area and set the scan parameters to low resolution (169 μm) and low quality (lowest) to perform a high-throughput evaluation of all the brain sections (Fig. 5) (see Note 30).
5. Select slides and perform high-resolution scan, if necessary.
6. Selected slides can be processed for immunohistochemistry (Section 3.4, step 3).

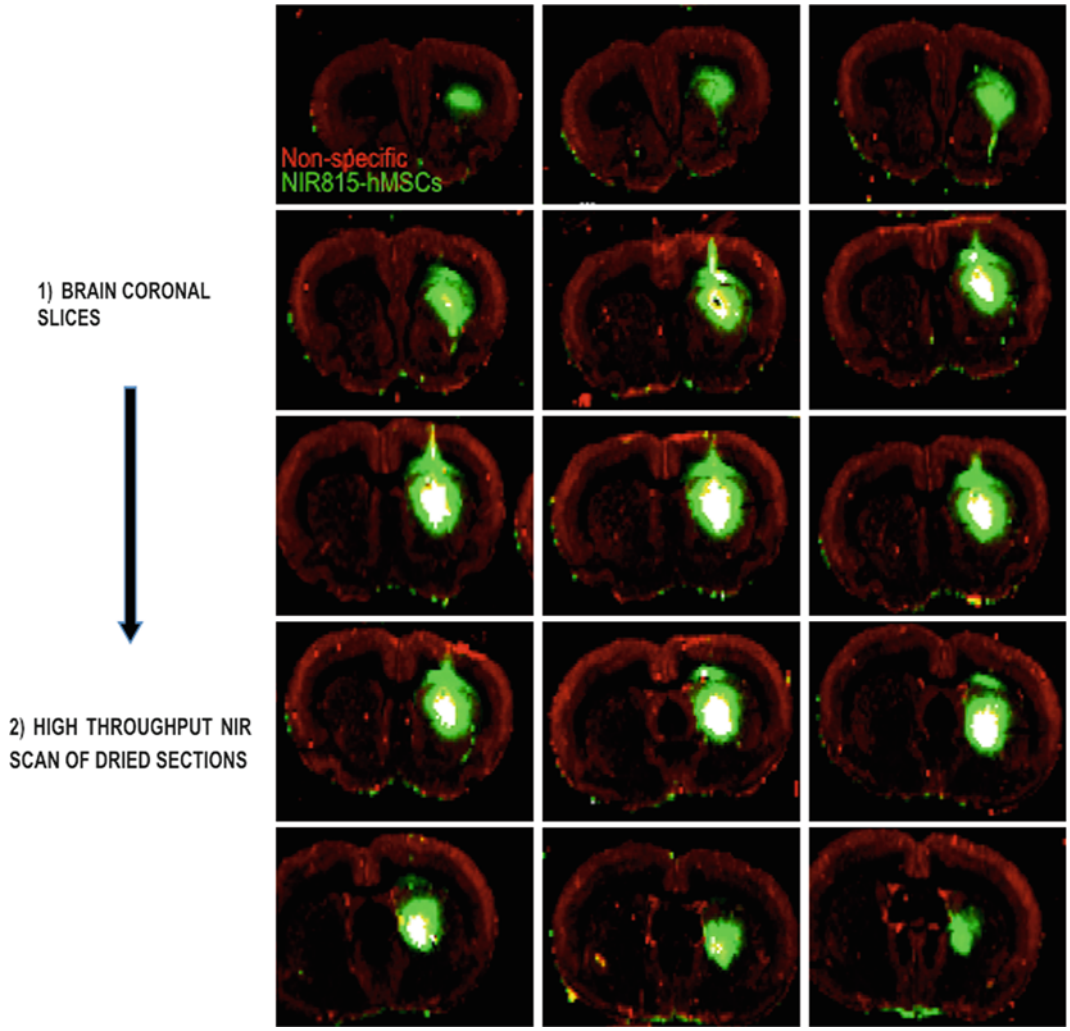

Fig. 5 High-throughput NIR imaging. Coronal brain sections containing the entire striatum are mounted on glass-slides and a high-throughput analysis can be performed at low resolution. Presence of the grafted hMSCs is clearly indicated by the intense *green* signal. The *red* signal corresponds to a nonspecific background of the tissue in the *red* spectrum

3.6.3 NIR Immunohistochemistry for Tyrosine Hydroxylase (TH)

Localization of transplanted NIR815-labeled cells within the 6-OHDA-lesioned striatum can be performed by conventional immunohistochemical procedure using secondary antibodies linked to an NIR fluorochrome (IRDye®).

1. Circle the sections with a pap pen.
2. Fix section for 15 min with NBF.
3. Wash with PBS for 10 min (see Note 31); repeat wash four times.
4. Block sections with the blocking solution for 1 h at room temperature.

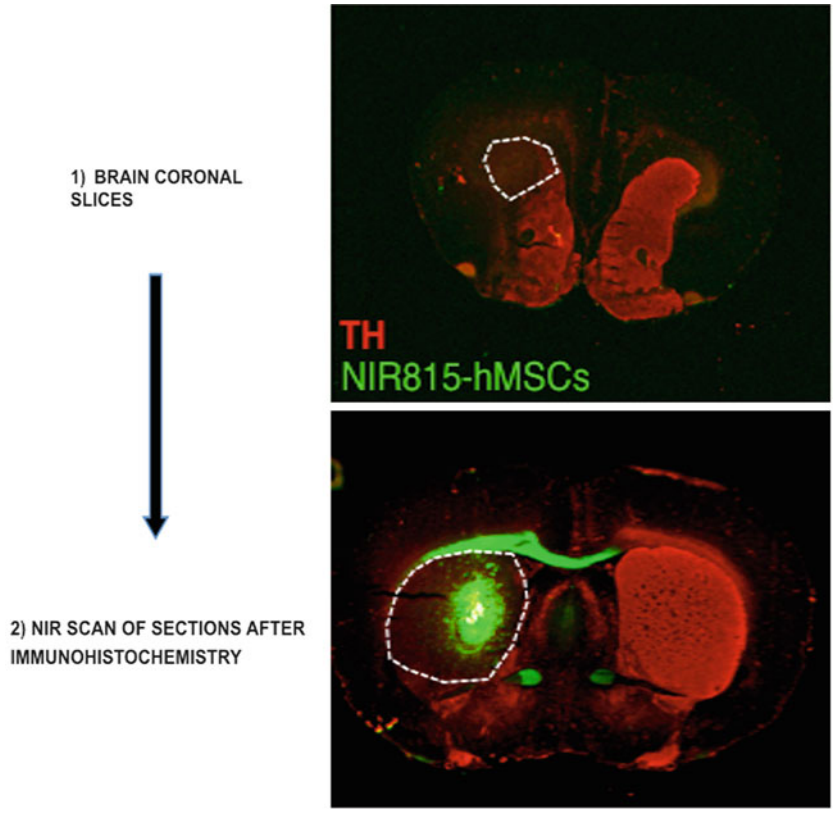

Fig. 6 NIR immunohistochemistry. A classical immunohistochemistry procedure is performed using a secondary antibody emitting in the *red* NIR spectrum (700 nm). At the end of the procedure sections are dried and can be scanned immediately. The *red* signal indicates the presence of tyrosine hydroxylase (TH)-positive dopaminergic terminals in the intact striatum. Localization of the grafted cells in the lesioned striatum (absence of *red* signal) is indicated by the intense *green* signal (800 nm). (Modified from Bossolasco et al., Int J Nanomed 2012)

5. Incubate with the primary anti-TH antibody in the antibody solution overnight at 4 °C (see Note 32).
6. Wash sections with PBS containing 0.1 % Tween 20 for 5 min; repeat wash four times.
7. Dilute secondary antibody (1:5,000) in the antibody solution and incubate for 1 h at room temperature.
8. Wash with PBS–0.1 % Tween 20 for 10 min; repeat wash six times.
9. Wash twice with PBS.
10. Wash once with distilled H_2O.
11. Let slides dry overnight protected from light (see Note 33). Perform high-resolution scan (see Note 28: sections do not need to be mounted to obtain high-quality NIR images) (Fig. 6).

4 Notes

1. The NIR815 dye emits in the red spectrum and results in higher signal specificity and reduced background noise compared to NIR700 dyes.
2. Any other adherent or non-adherent cell types may be used, but will require previous optimization of the protocol.
3. Divide cells when they reach confluence; this will depend on their specific growth rate.
4. A karyotyping of cells may be performed to assess the absence of cell culture-induced aberrations.
5. A minimum of 1×10^6 cells can be labeled with this procedure. The volume indicated is optimized for 1×10^6 cells. If the number of cells to be labeled is larger, the volume needs to be proportionally adapted (i.e., 250 μl for 5×10^6 cells, 500 μl for 1×10^7 cells, etc.).
6. The volume of staining solution needs to be equal to the volume of the cell suspension. Note that concentration of the NIR815 dye should not exceed 1 % of the final volume. Higher percentage may affect cell viability. Dye concentration should be optimized for each cell type.
7. Labeling times exceeding 5 min may affect cell viability. Incubation time should be optimized for each cell type.
8. Cells may be transplanted immediately after staining or, if needed, may be grown in culture overnight or more (be careful that, although cell labeling is stable over time, the dye will be diluted by cell division over passaging).
9. Dilute the aliquot with PBS to obtain a final volume of at least 100 μl.
10. Alternatively, a drop of cell suspension may be placed onto a glass slide, sealed with a coverslip, and directly observed on the Odyssey® Infrared Imaging System.
11. Focus will depend on the thickness of the slide. A rapid scan can be previously performed to determine the optimal offset focus.
12. If necessary high resolution (21 μm) and quality (highest) can be performed. Highest resolution and quality of the image will take longer time to complete the scanning procedure.
13. Protocol for 6-OHDA injection has been described in details elsewhere (10).
14. Any other anesthetic may be used.
15. Before fixing the teeth of the animal assure that the animal is correctly placed; no left–right movement of the head should be possible. Correcting placing of the animal is fundamental

to properly calculate the coordinates and find the correct injection site.

16. The site of injection is determined using a brain atlas specific for the species of interest. For rats we use the Paxinos and Watson brain atlas (11). Find the brain area of interest in the atlas and determine the coordinates. Injection coordinates are expressed in millimeters relative to bregma. For example for unilateral injection of hMSCs in the right striatum the needle has to be moved to the following position: AP = +1 mm and L = −3 mm from bregma.
17. Be very careful not to damage the membrane; it will be used to calculate the dorsoventral (DV) coordinates.
18. Cells need to be resuspended in physiological solution or PBS; maximal volume that may be injected without damaging neuronal structure has to be determined a priori and will depend on the injection site. In addition optimum cell concentration will vary with each cell type; perform preliminary trials to determine the maximum cell concentration that can be used and that will not cause tapping of the needle.
19. To precisely assess the point at which the needle touches the dura, place yourself at an angle with respect to the surface of the membrane so that you may observe the reflection of a light beam. Carefully and slowly lower the needle; as the needle touches the membrane, the latter will be displaced, thereby modifying reflection of the light beam that will no longer be visible from your viewpoint.
20. For a single IS injection DV = −5 mm with respect to dura: Global coordinates with respect to bregma and dura for single unilateral injection in the right striatum are AP = +1 mm, L = −3 mm, and DV = −5 mm.
21. Manually and slowly rotate the tip of the Hamilton plunger with a light downward pressure until the desired volume has been injected.
22. The MousePOD® in vivo Imaging Accessory can be connected to an anesthetic system. Refer to the Li-Cor Web site for major information (http://www.licor.com/bio/products/accessories/odyssey_accessories/mousepod/mousepod.jsp).
23. Complete removal of the fur is fundamental for NIR analysis; the animal's hair may act as an optic fiber, thus interfering with the imaging process.
24. To obtain a distinct/clear signal any possible movements of the animal's head should be avoided.
25. Best detection of cells transplanted in the striatum is usually obtained with focus set at 4 mm.

26. Resolution of 84 μm reduces time of scan and allows the acquisition of high-quality images. Set scan intensity between 7 and 10 for the 800 nm laser (green); if necessary a high, 21 μm resolution can be performed but time to complete the scan will be considerably extended; the 700 nm channel (red) is not necessary for detection of NIR815-labeled cells and is usually excluded.
27. Although NIR values can be precisely determined, measurements should be considered semiquantitative because they may be slightly influenced by the position of the animal (head) on the surface of the imager. For repeated evaluations over time, users are advised to document the original position and scan area of the animal during the first imaging procedure and conform to them as closely as possible during successive measures.
28. Transcardiac perfusion of the animal is not necessary for ex vivo analyses. If necessary for other purposes, it can be performed and will not interfere with subsequent imaging procedures.
29. Alternatively, brain can be frozen on dry ice and scanned afterward.
30. Scan intensity will depend on the amount of NIR815-labeled cells present in the sections and is usually set between 3 and 5. Focus is normally set to 1 mm (thickness of the slide). Complete scanning of 30 sections typically takes 10 min. This first scan will determine the specific localization of the transplanted cells in sections and allow selection of more accurate areas for the subsequent analysis.
31. Add enough PBS so that sections are completely covered; 200–300 μl are typically required for four sections; this holds true for all subsequent washes and incubations with primary and secondary antibodies.
32. Best dilution of the primary antibody has to be previously determined for each lot; we found that a 1:1,000 dilution usually gives optimal results.
33. For long-term storage slides can be mounted with Fluorosave® and covered with coverslips. The mounting solution does not interfere with the imaging procedure.

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http://www.springer.com/978-1-62703-558-3

Imaging and Tracking Stem Cells
Methods and Protocols
Turksen, K. (Ed.)
2013, XI, 218 p. 40 illus. in color. With online files/update., Hardcover
ISBN: 978-1-62703-558-3
A product of Humana Press