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TITLE:

Tracking Superparamagnetic Iron Oxide-Labeled Mesenchymal Stem Cells Using MRI after Intranasal Delivery in a Traumatic Brain Injury Murine Model

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KEYWORDS:

intranasal delivery, cell tracking, SPIO, cell label, in vivo imaging, traumatic brain injury

SUMMARY:

Presented here is a protocol for non-invasive mesenchymal stem cell (MSC) delivery and tracking in a mouse model of traumatic brain injury. Superparamagnetic iron oxide nanoparticles are employed as a magnetic resonance imaging (MRI) probe for MSC labeling and non-invasive in vivo tracking following intranasal delivery using real-time MRI.

ABSTRACT:

Stem cell-based therapies for brain injuries, such as traumatic brain injury (TBI), are a promising approach for clinical trials. However, technical hurdles such as invasive cell delivery and tracking with low transplantation efficiency remain challenges in translational stem-based therapy. This article describes an emerging technique for stem cell labeling and tracking based on the labeling of the mesenchymal stem cells (MSCs) with superparamagnetic iron oxide (SPIO) nanoparticles, as well as intranasal delivery of the labeled MSCs. These nanoparticles are fluorescein isothiocyanate (FITC)-embedded and safe to label the MSCs, which are subsequently delivered to the brains of TBI-induced mice by the intranasal route. They are then tracked non-invasively in vivo by real-time magnetic resonance imaging (MRI). Important advantages of this technique that combines SPIO for cell labeling and intranasal delivery include (1) non-invasive, in vivo MSC tracking after delivery for long tracking periods, (2) the possibility of multiple dosing regimens

due to the non-invasive route of MSC delivery, and (3) possible applications to humans, owing to the safety of SPIO, non-invasive nature of the cell-tracking method by MRI, and route of administration.

INTRODUCTION:

Mesenchymal stem cells (MSC) are attractive candidates for stem cell-based therapies in treatments of central nervous system (CNS) disorders and injuries in humans. Moreover, MSCs have been used as a vehicle for the delivery of therapeutic proteins at injury sites[1,2]. In recent years, promising innovations have been developed to establish 1) novel routes of cell delivery and 2) cell tracking for stem cell-based therapies of CNS disorders. The intranasal delivery of stem cells into the brain depends on the ability of cells to bypass the cribriform plate and enter the olfactory bulb partially via a parenchymal route[3]. The combination of intranasal delivery and the labeling of MSCs with superparamagnetic iron oxide (SPIO) nanoparticles represents a promising approach for clinical applications of MSCs in treating CNS disorders, since SPIO nanoparticles are safe probes for magnetic resonance imaging (MRI) and allow non-invasive sensitive longitudinal tracking of MSCs post-delivery by MRI[3-5]. Furthermore, intranasal delivery is a safe and non-invasive route that allows repeated administration within a short period of time.

This article describes a highly sensitive and non-invasive technique for tracking MSCs in vivo post-intranasal delivery in a mouse model of traumatic brain injury (TBI), which employs SPIO-labeled cells and MRI. One important advantage of the SPIO labeling is the sensitive detection of SPIO in tissue by MRI, which makes it possible to track cells efficiently and non-invasively. The SPIO nanoparticles used here are commercially available and tagged with a fluorescein isothiocyanate (FITC) fluorophore, which allows for the detection of SPIO in tissue without immunostaining or additional processing. Furthermore, it is possible to perform longitudinal real-time tracking and investigate the biodistribution of the delivered MSCs.

PROTOCOL:

All procedures involving animals in this protocol were approved by the Institutional Animal Care and Use Committee, with the approval of the Ethics Committee for animal use in Taipei Medical University (approval no. LAC-2018-0574; 15.03.2019).

1. Labeling of MSCs with SPIO particles

1.1. To label MSCs with SPIO, add 6 mL of labeling media (25 µg/mL of SPIO in Dulbecco's modified Eagle medium [DMEM] with no fetal bovine serum [FBS]) to a T75 flask containing MSCs (80% confluency).

1.2. Incubate the cells with the labeling media in a CO_2 incubator (37 °C, 5% CO_2) without shaking. After 24 h, gently remove the labeling media using a sterile Pasteur pipette with a plastic tip attached to a vacuum. Wash the cells monolayer 2x with 6 mL of phosphate buffered saline (PBS) to remove any traces of uninternalized SPIO.

1.2.1. To determine whether the cells have been successfully labeled, check the labeled cells under a fluorescent microscope or confocal microscope if the SPIO are tagged with a fluorophore (i.e., like those used here; **Figure 1B,C**). Alternatively, perform Prussian blue staining for the cells (see steps 6.2–6.4 for the staining protocol).

1.3. Harvest adherent cells by treatment with 3 mL of trypsin and incubate at 37 °C. After 5 min of incubation, add 7 mL of pre-warmed DMEM media with 10% FBS (v/v) to inactivate the trypsin. Collect the cell suspension using a pipette into a 15 mL conical tube.

1.4. Centrifugate the cell suspension at 300 x *g* for 5 min. Discard the supernatant and resuspend the cell pellet in PBS. Count the viable cells using trypan blue dye and a hemocytometer.

NOTE: The cell pellet of the labeled cells will appear as a dark color due to iron loading (**Figure 1D**). This protocol is relevant for MSC labeling and MRI imaging. The procedure for MSC labeling[6] has been previously optimized, and only the steps to prepare labeled MSCs to track in vivo are included here, since in vivo tracking is the focus. The protocol for culturing and labeling of other cell types should be optimized by the researcher.

1.5. Adjust the cell concentration using PBS to 150,000 cells (or a number that results in a sufficient MRI signal) in 18 µL of PBS (or the total volume that will be used in the intranasal delivery procedure).

NOTE: It was noticed that a cell concentration higher than 150,000 cells/18 µL of PBS leads to cell aggregation, which may affect the efficiency of intranasal delivery. If a higher number of cells is needed for intranasal delivery, increase the total volume of cell suspension and increase the number of intranasal administrations, as intranasal administration is a non-invasive procedure, and multiple dosing is possible.

2. Controlled cortical impact (CCI) injury

NOTE: In this protocol, male C57 BL/6 mice (7–8 weeks old) were kept in a 12/12 h light/dark cycle with *ad libitum* access to food and water.

2.1. To prepare each mouse for CCI injury, administer the zolazepam (50 mg/kg) and xylazine (20 mg/kg) anesthetizing cocktail via intraperitoneal (i.p.) injection (1 mL/kg). Ensure that the depth of anesthesia is sufficient by a lack of toe-pinch response. Alternatively, place the mouse in a chamber supplied with 2%–4% isoflurane for 60 s**.**

2.2. Shave the fur of the dorsal surface of the skull between the ears using an electronic hair clipper. Clean the shaved area several times using a sterile cotton swab soaked in iodine. Use a cotton swab soaked in 70% ethanol to clean off the iodine.

2.3. Place the anesthetized mouse in the stereotactic frame and secure the mouse using ear bars and nose bars. Make a midsagittal incision (approximately 2.5 cm) in the shaved skin using sterile

scissors to access the surface of the skull.

2.4. Remove the tissue on the bone using a cotton pad to expose the skull. Clean the skull surface using a cotton swab soaked in a 3% H_2O_2 for 10 s, then clean it with a dry cotton pad.

NOTE: The skull sutures and both bregma and lambda can now be easily identified.

2.5. Identify the coordinates of choice on the skull surface for the CCI injury and draw a circle (4 mm diameter) around the coordinates using a pencil or proper marker.

NOTE: In this protocol, the coordinates at anteroposterior (AP) −2.0 mm and mediolateral (ML) +1.5 mm were used for CCI induction.

2.6. Use a microdrill and round burr (0.5 mm diameter) to thin the skull at the marked circle. Avoid applying pressure while drilling, as drilling through the bone may cause damage to the brain parenchyma. Clean bone dust away using a clean and dry cotton swab.

2.7. Gently remove the bone flap using sterile fine forceps to expose the dura mater while keeping it intact. Remove the mouse from the stereotactic frame that was used for pre-injury preparation and place it into the stereotactic frame of the CCI device.

2.8. Stabilize the head of the mouse using the ear bars and nose bars. Make sure the head of the mouse is level in the rostral-caudal direction and adjust the nose bars, if needed.

2.9. Follow the instructions on the control box to zero the impactor tip to the exposed cortical surface. Make sure that the impactor tip is aligned directly above the desired cortex coordinates to be impacted using the X and Y control wheels on the base of the impactor.

2.10. Set the experiment parameters using the control box with a velocity of 5 m/s, dwell time of 250 ms, and injury depth of 1 mm to induce mild injury in the mouse.

2.11. Induce injury by pressing the ''impact'' button on the control box. Swab any bleeding that occurs using a sterile cotton swab.

2.12. Remove the mouse from the stereotactic frame and close the incision using silk surgical sutures. Do not use metal clips to close the surgical site, since the mouse will be subjected to a magnetic field for MRI.

2.13. Apply topical antibiotics (bacitracin neomycin) to the surgical site to prevent infections. Keep the mouse on the heating pad and monitor it closely during the recovery phase.

2.14. Administer ketoprofen (2.5 mg/kg, IP) daily for 3 days after surgery, unless the ketoprofen administration contradicts with the study goals.

3. Intranasal delivery

3.1. At 1 day post-CCI induction, administer the zolazepam (50 mg/kg) and xylazine (20 mg/kg) anesthetizing cocktail via i.p. injection. Ensure that the mouse is deeply anesthetized by lack of toe-pinch response.

3.2. Prepare the mouse for intranasal delivery of MSCs by hyaluronidase treatment.

3.2.1. Grab the mouse's scruff and turn on its back firmly while immobilizing the skull. Place the tip of a pipette that contains hyaluronidase in sterile PBS (4 U/µL) near the nostril of the mouse at a 45° angle.

3.2.2. Administer 3 µL of hyaluronidase suspension in each nostril. Keep the mouse immobilized and facing upward on a clean pad for 5 min. Repeat hyaluronidase treatment 4x (total of 100 U hyaluronidase suspension).

3.3. After hyaluronidase treatment, keep the treated mouse on a clean pad facing up for 30 min.

3.4. To deliver MSCs into the brain, hold the mouse firmly, as described in step 3.2.1. Administer 3 µL/nostril of MSC suspension with a 3 s interval. Keep holding the mouse in the same position for 30 s until the sample drops have completely disappeared.

NOTE: Avoid forming air bubbles during administration.

3.5. Repeat the administration with a 2 min interval up to 3x.

NOTE: The total number of cells to be delivered is 150,000, such that 18 µL of the cell suspension can be delivered at a 3 µL dosage for each nostril, 3x each.

3.6. Return the mouse to its cage and monitor it closely until it fully recovers from anesthesia.

4. In vivo magnetic resonance imaging

NOTE: Histological staining of brain tissue has previously been used to confirm the successful delivery of stem cells after intranasal administration. However, this method can only be used as an endpoint of a study, not longitudinally. Using MRI probes to label therapeutic stem cells will allow for longitudinal, non-invasive, in vivo tracking of the cells using MRI. Importantly, this protocol efficiently reduces the number of animals required. In this protocol, MRI scanning was performed at days 1, 7, and 14 post-delivery of MSCs.

4.1. To prepare the mouse for MRI scanning, anesthetize the mouse with isoflurane (5% isoflurane in 1 L/min of O_2 for induction, 1.5%–2% isoflurane for maintenance). Perform a toe-pinch to ensure that the mouse reaches the required anesthesia level.

4.2. Place the mouse on the imaging holder and secure its position using taps or any other proper method. Move the holder to the center of the MRI coil (7 T/40 cm magnet) and connect the monitoring connection.

4.3. To acquire T2*-weighted scans using a spin-echo sequence, set the repetition time (TR) to 1500 ms and echo time (TE) to 2.8 ms.

4.4. Use a 16 mm x 16 mm field of view (FOV), 128 x 128 acquisition matrix (MTX), and slice thickness of 0.75 x 0.8 mm with four signal averages and a 90° flip angle (FA).

4.5. Retract the mouse holder from the MRI coil center after completing the scans. Return the mouse to its cage and monitor it closely until it completely recovers from anesthesia.

4.6. To track and quantify the labeled MSCs on the T2*-weighted images, use ITK-SNAP software (version 3.8.0)[7].

4.6.1. Transfer the raw data of the MRI scans from the MRI machine's computer to the computer used for analysis in a DICOM (digital imaging and communications in medicine) format.

4.6.2. Run the ITK-SNAP software and load the MRI images by clicking on the **File** button. Then, click on **Open Main Image** in the menu. Press on the **Open Image** button in the display window, then locate and open the MRI images using the **Browse** button.

NOTE: The visualization of labeled cells in the MRI images will appear as hypointense areas. If the image contrast needs to be adjusted, select **Tools** | **Image Contrast**.

4.6.3. Create segmentations of the hypointense areas and lesion or other brain parts by selecting **Active Label** in the segmentation labels section. Use different label colors for different segments (if the segmentation of more than one part is needed).

4.6.4. Use the **Polygon** tool in the **Main Toolbar** to draw around the hypointense areas representing the SPIO-labeled MSCs. Select **Accept**, located below the MRI image. The segmented areas will appear as the same color of the active label assigned to that particular segment. Repeat this segmentation step for all MRI slices.

4.6.5. Develop a 3D map of the segmented areas to represent the MSC distribution in the whole brain by selecting the **Scalpel Tool** at the bottom of the **3D Toolbar**, located in the **Segmentation Labels** section at the bottom of TheITK-SNAP toolbox. Then, press **Accept** at the bottom of the created 3D map.

4.6.6. To perform a quantitative analysis (volume and intensity mean) of the segmented hypointense areas representing labeled cells, press the **Segmentation** button in the top panel and select **Volume and Statistics**.

5. Fixation of the mouse brain and cryosectioning

5.1. To fix the mouse brain, perform transcardiac perfusion with 4% paraformaldehyde (PFA) following the last MRI scan, as previously described[8].

5.1.1. Decapitate the head and extract the brain[8]. Fix the brain with 4% PFA for at least 48 h at 4 °C.

5.1.2. Dehydrate the brain by immersing it into a 30% sucrose solution at 4 °C until the brain sinks to the bottom of the solution.

5.2. Embed the brain in the optimal cutting temperature (OCT) solution and freeze at -20 °C. Section the brain with a cryostat microtome into slices with 14 µm thickness and mount them onto slides. Store the sections slides at -20 °C until further use.

6. Prussian blue staining

NOTE: Prussian blue staining is commonly used to detect the iron content in SPIO-labeled cells. Here, Prussian blue staining is used to confirm that the hypointense signals in the MRI images correspond to the SPIO-labeled MSCs and not to artifacts. Prussian blue staining is one of the most sensitive histochemical methods used to detect iron in tissues and can be used to identify even a single granule of iron in the cells.

6.1. Wash the slides of brain sections with distilled water for 5 min.

6.2. Perform Prussian blue staining by immersing the slides for 30 min in the staining solution, which contains equal parts hydrochloric acid (10%) and potassium ferrocyanide (10%) prepared immediately before use.

6.3. Wash 3x with distilled water, for 5 min each. Counterstain the sections with nuclear fast red for 5 min. Rinse the slides 2x with distilled water.

6.4. Dehydrate the sections gradually by immersing the slides in 95% and 100% alcohol for 2 min each. Add coverslip with a resinous mounting medium.

6.5. Use a light microscope to detect the stained cells in the brain sections.

NOTE: The iron in the labeled cells will appear as blue colored deposits.

REPRESENTATIVE RESULTS:

Twenty-four hours following intranasal delivery, the SPIO-labeled MSCs were detected as strong hypointense areas medial to the cortical injury on T2*-weighted images (**Figure 2B**), indicating the targeted migration of SPIO to the injury site. This migration remained visible up to 14 days post-delivery, as the hypointense signals were found to be visible without significant reduction

for this time period (**Figure 2B**). The injured animals treated with PBS showed no hypointense areas, indicating that the observed hypointense areas correspond to the SPIO labeled MSCs and not to signal artifacts (**Figure 2A**) The biodistribution of the labeled MSCs that were observed in vivo with MRI was visualized using 3D reconstruction (**Figure 2C,D**). The migration of MSCs to the injured cortex was confirmed histologically by Prussian blue staining and FITC channel detection of the FITC-tagged SPIO in the labeled MSCs (**Figure 3A,B**).

FIGURE LEGENDS:

Figure 1: Schematic flowchart of the protocol and in vitro confirmation of SPIO uptake by MSCs. (**A**) MSCs were incubated with SPIO for 24 h for labeling. Then, the labeled MSCs were delivered into a TBI mouse model via an intranasal (IN) route. MRI at different timepoints was performed to track the labeled MSCs. Confirmation of sufficient labeling of MSCs by SPIO was achieved by (**B**) fluorescence microscopy and (**C**) confocal microscopy using the FITC channel, since SPIO nanoparticles were tagged with FITC. (D) The cell pellet of the labeled MSCs appeared dark in color due to iron loading. FITC: fluorescein isothiocyanate; SPIO: superparamagnetic particles of iron oxide; MSCs: mesenchymal stem cells; MRI: magnetic resonance imaging; IN: intranasal; TBI; traumatic brain injury.

Figure 2: Real-time MRI enables the detection and tracking of SPIO-labeled MSC migration toward injury sites in the brains of TBI-induced mice. (**A**) Mice were subjected to TBI, followed by treatment with PBS or SPIO-labelled MSCs, administered via an intranasal route 24 h after injury. Coronal sections of T2*-weighted images showed the labeled MSCs as a hypointense area (arrowhead) on the edge of the injury site (outlined area) at 1, 7, and 14 days post-delivery. The PBS-treated mice show no hypointense area. (**B**) Segmentation process of the injury site area (green) and labeled MSCs (red) based on coronal T2*-MRI images. (**C**) 3D reconstruction of the mouse brain treatment based on T2*-weighted images illustrating the biodistribution of SPIO-labeled MSCs in the brain 14 days post-delivery.

Figure 3: Histological analysis confirms the presence of SPIO-labeled MSCs in the brains of the treated animals. Prussian blue staining of brain sections of a (**A**) mouse treated with PBS (control) and (**C**) mouse treated with SPIO-labeled MSCs. SPIO-positive cells were detected in MSC-treated mouse (boxed cells, blue), while the control mouse showed no positive cells at the injury site in the cortex at 14 days post-delivery, confirming MRI observations. Fluorescence microscopy analysis of the cortex of a (**B**) control mouse treated with PBS and (**D**) mouse treated with SPIO-labeled MSCs was conducted 14 days post-delivery. The analysis revealed the presence of FITC-tagged SPIO-positive cells (boxed cells, green) at the injured cortex in the MSC-treated mouse, but no FITC signals were observed in the cortex of the PBS-treated mouse. Scale bars = 50 µm, unless stated otherwise.

DISCUSSION:

The protocol described here represents general procedures for the SPIO labeling of MSCs and MRI tracking of SPIO-labeled MSCs post-intranasal delivery. The protocol allows the opportunity to study the migration and biodistribution of MSCs post-delivery in vivo in the brain, using a non-

invasive method.

MSCs are attractive candidates for stem cell-based therapies for CNS disorders and injuries due to their ability to secrete trophic factors that 1) trigger neurorestorative processes and 2) provide neuroprotection, owing to their anti-inflammatory effects within the injury area[9-12]. Although long-term MRI tracking and detection of SPIO-labeled MSCs may be limited due to the dilution of intercellular SPIO with cell division, labeled cells can be detected for up to several weeks post-transplantation in the brains of animal models[13].

Also described here is the labeling protocol of MSCs with SPIO nanoparticles coated with dextran without transfection agents. Other protocols have been used in the literature[14-16]. However, in all cases, these protocols should be adjusted for cell type, SPIO size, incubation time, and SPIO concentration. MSCs have been shown to have impaired chondrogenic differentiation potential but not adipogenic differentiation upon SPIO labeling[17]. Therefore, it is highly recommended that differentiation assays be performed prior to stem cell delivery to evaluate the influence of SPIO on the differentiation potency of stem cells. In a previous study, it was demonstrated that MSC labeling with the same SPIO type and concentration used in the here did not affect the osteogenic or adipogenic differentiation potency of MSCs[6].

The intranasal route of therapeutic stem cell delivery for brain disorders and injuries is a promising approach for the clinical application of stem cells. However, the intrinsic and molecular mechanisms that dictate the behaviors of stem cells in the nasal cavity remain unclear. Although the intranasal route is widely explored for the delivery of small molecules, the size and biodistribution behavior of the therapeutic stem differ from small molecules. The current protocol demonstrates that MSCs tend to migrate toward the injury site after intranasal delivery.

Here, T2*-weighted images were used to track the SPIO-labeled MSCs. Other reports have used gradient echo imaging. However, susceptibility artefacts are often observed in gradient echo imaging due to intercellular SPIO. In the current protocol, the location of the hypointense areas representing the SPIO-labeled MSCs on T2*-weighted images was the same as the location of the SPIO in brain sections as detected by histological examination (**Figure 3**). This indicates the adequate sensitivity of T2*-weighted spin echo imaging for SPIO-labeled MSC tracking in the brain.

In summary, the described protocol is beneficial for in vivo stem cell tracking studies of brain injuries and disorders. The longitudinal tracking of stem cells in vivo has traditionally been performed by sacrificing animals at multiple timepoints. The current protocol provides a non-invasive and efficient approach for MSCs delivery and tracking, which represents a potential procedure for stem cell-based therapy for brain injuries and disorders in clinical settings.

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DISCLOSURES:

The authors have nothing to disclose.

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449

Figure 1

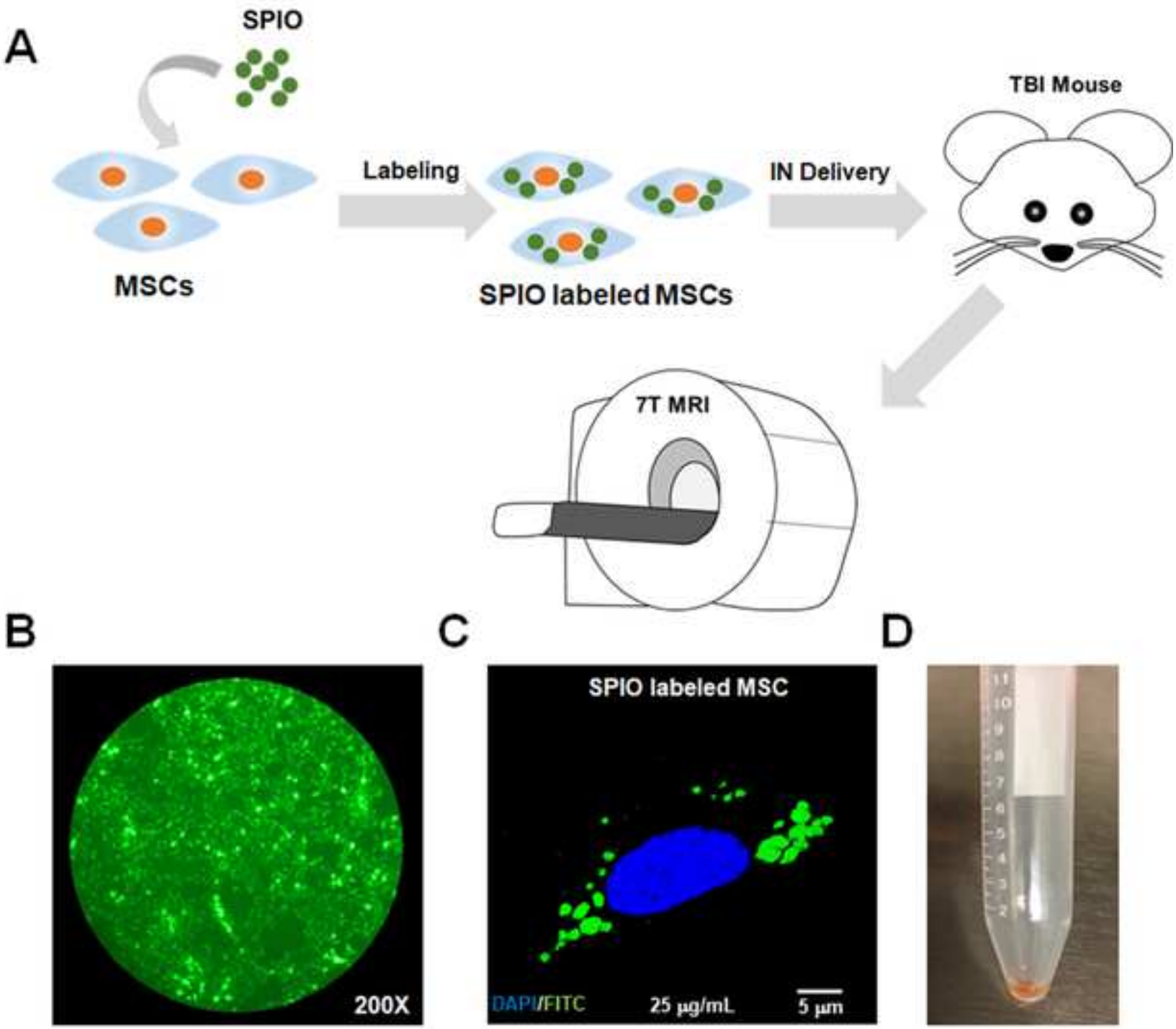

Figure 2

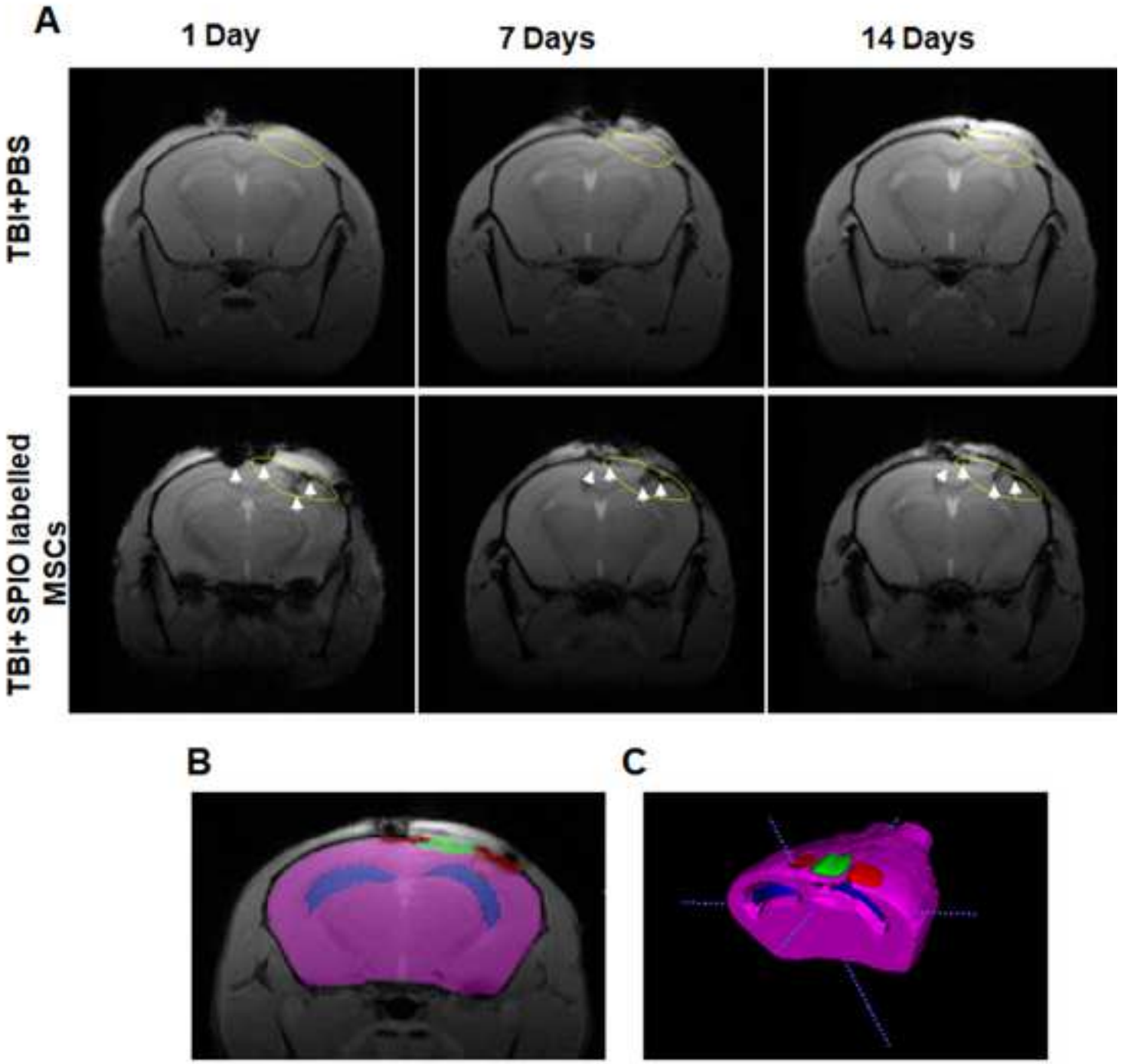

Figure 3

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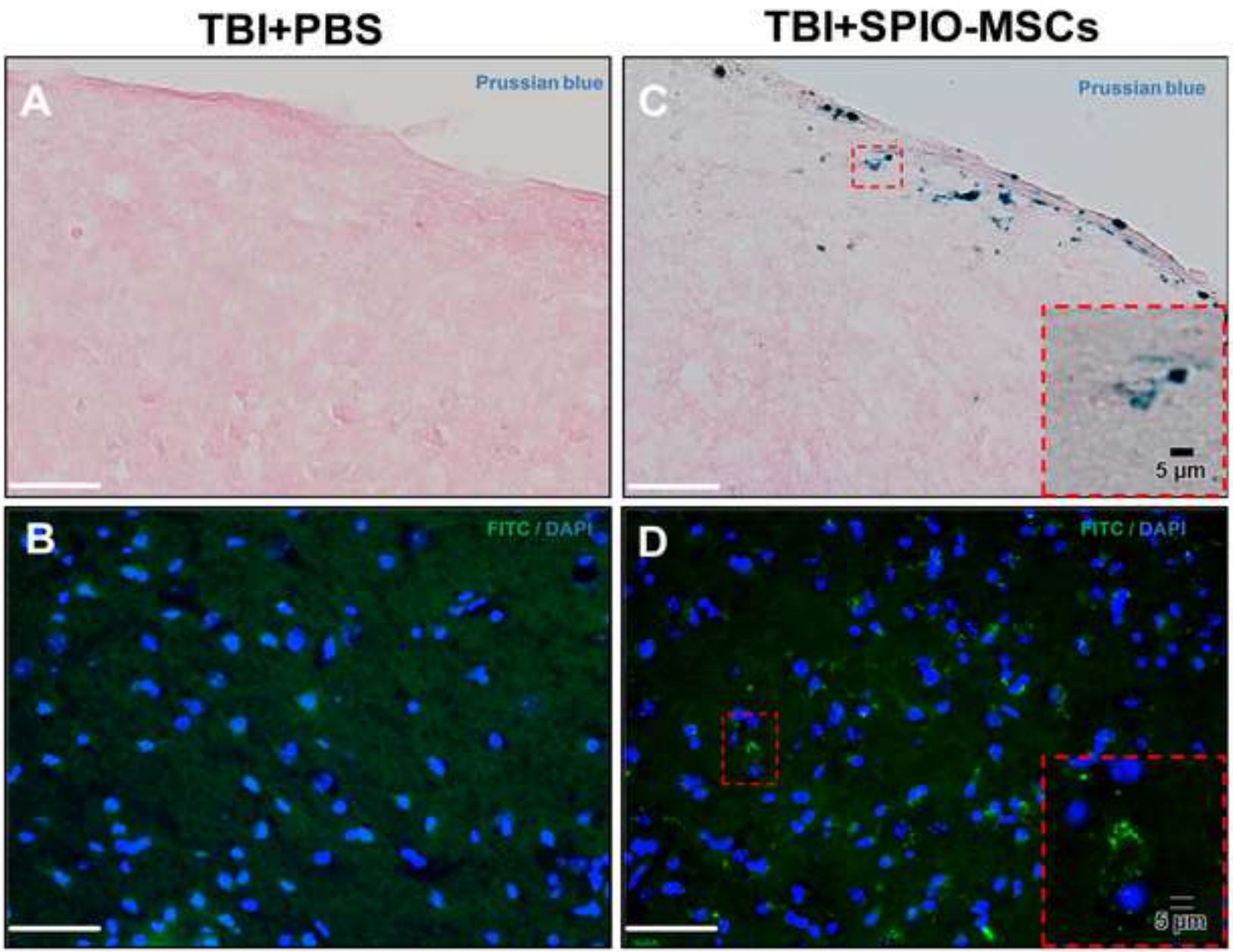

Table of Materials

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Name of Material/Equipment	Company	Catalog Number
Cell culture supplies (Plastics)	ThermoFisher Scientific	Varies
Disposable Microtome Blade	VWR	95057-832
D-MEM/F-12 (1X) with GlutaMAX	GIBCO	10565-018
Embedding medium for frozen tissue specimens (O. C. T.)	Sakura Finetek	4583
Fetal Bovine Serum (FBS)	GIBCO	12662-029
Fluorescence Wild Field Microscope	Olympus	Olympus BX43
Forcept	Fine Science Tools	11293-00
Gentamicin (10 mg/mL)	GIBCO	15710-064
Hair clipper	Pet Club	PC-400
Head Trauma Contusion device	Precision Systems and Instrumentation	Model TBI-0310
Hyaluronidase from bovine testes	MilliporeSigma	H3506
ITK-SNAP Software	Penn Image Computing and Science Laboratory (PICSL) at the University of Pennsylvania, and the Scientific Computing and Imaging Institute (SCI) at University of Utah	ITK-SNAP 3.8.0
Ketamine (Ketavet)	Pfizer	778-551
Mice	National Laboratory Animal Center, Taiwan	C57BL6
Microdrill	Nakanishi	NE50
Microtome	Leica	RM2265
Mouse (C57BL/6) Mesenchymal Stem Cells	GIBCO	S1502-100
MRI scanner	Bruker Biospec	
Phosphate Buffer Saline (PBS)	Corning Cellgro/ThermoFisher	21-031-CV
Povidone-iodine 7.5%	Purdue product L.P.	
Prussian Blue Stain	Abcam	ab150674
Scissor	Fine Science Tools	14084-08
Stereotaxic frame	Kopf Instruments	Model 900

Superparamagnetic iron oxide (SPIO) nanoparticles	BioPAL	Molday ION EverGreen, CL-50Q02-6A-51
Suture monofilament	Ethicon	G697
Timer	Wisewind	
TrypLE	GIBCO	12604-013
Xylazine (Rompun)	Bayer	QN05 cm92

Comments/Description
Replaceable with any source
Surgery
Wild type mice strain used in the study
Combine with Burrs for generating the bone window
Surgical scrub
Surgery

stem cells labeling for in vivo tracking using MRI
Suture
Replaceable with any source

We greatly appreciate the efforts for handling and reviewing our manuscript as well as the valuable comments by the editor and reviewers. The editor and reviewers comments are constructive indeed and have been used as a guideline to revise our manuscript. Please find below the editor and reviewers comments repeated in italics and our responses inserted after each comment.

The language in the manuscript is not publication grade. Please employ professional copy-editing services.

Response: We revised the manuscript and corrected any detected syntax, wording and grammatical errors. We also had an experience writer to check the manuscript and improve the manuscript language.

Editorial comments:

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.*

Response: We apologize for the grammar and spelling issues. We revised the manuscript and corrected any detected syntax, wording, grammar and spelling issues.

2. Please revise lines 32-33 to avoid textual overlap with previously published work.

Response: We revised the above mentioned lines. Please check the revised manuscript

3. Please add a Summary section before the Abstract section to clearly describe the protocol and its applications in complete sentences between 10−50 words: "Here, we present a protocol to …"

Response: We added a Summery section, please check the revised manuscript (cover page).

4. Please define acronyms/abbreviations upon first use in the main text.

Response: We revised the main text and defined the undefined abbreviations.We also added a List of abbreviation after the references list

5. Introduction: Please describe the technique in the context of the literature (with citations).

Response: We revised the Introduction section, please check the revised manuscript.

6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. Step 1 followed by 1.1, followed by 1.1.1, etc. Each step should include 1−2 actions and contain 2−3 sentences. Use subheadings and substeps for clarity if there are discrete stages in the protocol. Please refrain from using bullets, dashes, or indentations.

Response: We revised the protocol section. Please check the revised manuscript

7. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

Response: We revised the protocol section. Please check the revised manuscript

8. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE."

Response: We revised the protocol section. Please check the revised manuscript

9. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

Response: We revised the protocol section. Please check the revised manuscript

10. Line 75: What volume of PBS is used to wash and how many times?

Response: We revised the protocol section. Please check the revised manuscript

11. Line 78: Is the supernatant discarded after centrifugation? Resuspend the cells in what?

Response: We revised the protocol section. Please check the revised manuscript

12. *Line 93: Please specify the age, gender and strain of mouse. Please mention how proper*
Response: We revised the protocol section. Please check the revised manuscript

13. Line 101: Apply H2O2 with what and what volume of H2O2 is used?

Response: We revised the protocol section. Please check the revised manuscript

14. Line 103: Draw with what?

Response: We revised the protocol section. Please check the revised manuscript

15. Line 128: When is intranasal delivery is done (i.e., how many days after surgery)?

Response: We revised the protocol section. Please check the revised manuscript

16. Lines 131 and 132: Please describe what is done here using the imperative tense.

Response: We revised the protocol section. Please check the revised manuscript.

17. Line 145: When is MRI done?

Response: We revised the protocol section. Please check the revised manuscript.

18. Lines 170-171: Please describe how perfusion and brain extraction are done or provide a relevant reference.

Response: We revised the protocol section. Please check the revised manuscript.

19. Line 176: Section the brain with what?

Response: We revised the protocol section. Please check the revised manuscript

20. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

We revised the Protocol section to combine the short steps

21. Please include single line spacing between each numbered step or note in the protocol.

We revised the protocol section

22. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

Response: We highlighted the protocol text that would be featured in the video. Please check the revised manuscript

23. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

Response: We highlighted the protocol text according to the suggested guidelines. Please check the revised manuscript.

24. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Response: We highlighted the protocol text according to the suggested guidelines. Please check the revised manuscript.

25. Line 244: Is the number 10 a reference? If so, it should be a superscript.

Response: We apologize for this typo. This has been corrected in the revised manuscript.

26. Where are references 1-3 cited? Each reference cited in text must appear in the reference list, and each entry in the reference list must be cited in text.

Response: reference 1 was cited in page 2 and 10, reference 2 in page 5, and reference 3in page 10in the revised manuscript

27. References: Please do not abbreviate journal titles; use full journal name.

Response: We revised the References list

28. Figure 1C: Please use the micro symbol µ instead of u and abbreviate liters to L (L, mL, µL) to avoid confusion (i.e., µg/mL, 5 µm). Please include a space between all numbers and the corresponding unit (25 µg/mL).

Response: We apologize for these typos. We revised the figure accordingly.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In the manuscript "Non-Invasive and Real-Time Tracking of SPIO Labeled Mesenchymal Stem Cells Using MRI after Intranasal Delivery in Mice Model of Traumatic Brain Injury", the authors describe how to label MSCs, deliver them intranasally in a murine model of brain injury, and non-invasively detect MSC delivery to the brain using MRI. This protocol should be of interest to a wide range of neuroscience researchers.

Minor Concerns:

Figure 3 should show control images of PBS-treated animals next to the images of mice treated with MSCs.

Response: We revised the figure and included micrographs of Prussian blue staining and fluorescence microscope of brain section of PBS treated mouse. Kindly check the revised manuscript.

Reviewer #2:

Manuscript Summary:

About the Manuscript JoVE60450, entitled "Non-Invasive and Real-Time Tracking of SPIO Labeled Mesenchymal Stem Cells Using MRI after Intranasal Delivery in Mice Model of Traumatic Brain Injury", by Shahror et al, submitted as a paper to JoVE.

I have read the referred manuscript, and my opinion is the following:

I understand that the originality of the paper should not be evaluated, which is not, but the clarity of the description, which is not very clear, and it should be further evaluated by experts.

A list of abbreviations should be supplied, clearly describing all experimental procedures, including TBI and CCI. The demonstration claimed in P6 that MSCS migrate toward the injury site should be fully justified.

The manuscript can be of interest for specialists as long as the experiments are clearly described.

Response: We apologize for not carefully presenting more clear description current protocol in particular CCI procedure. This has been corrected by revising the entire protocol and better description of CCI procedure. We revised Figure 3 to support our primary finding that MSCs are detectable at the injury site in histological examination. We also added a List of abbreviation. Kindly check the revised manuscript.

Reviewer #3:

Manuscript Summary:

This manuscript describes 4 techniques concerning the real-time MRI tracking of the labelled mesenchymal stem cells (MSCs) in the mice model of traumatic brain injury:

(1) Procedure of MSCs labeling with superparamagnetic iron oxide nanoparticles.

(2) Technique of intranasal delivery of the labelled MSCs.

(3) Series of actions aiming to deliver the controlled cortical impact modelling the traumatic brain injury.

(4) Procedure of real-time magnetic resonance imaging.

In addition, the authors describe histological procedures aiming to confirm the MRI results excluding artifacts: (5) Fixation of mouse brains and cryosectioning, and (6) Prussian Blue Staining.

Demonstrating the efficacy of the method, authors present the results of MRI scanning and of histological analysis.

At the end of the manuscript, authors discuss the advantages and restrictions of the presented methodology.

In general, the manuscript contains rather detailed descriptions of the protocols. However, to make it reproducible by the others, the authors need to correct or even re-write some of its parts.

Response; We revised the manuscript to clarify the protocol. We also re-wrote some parts of the protocol to make it reproducible by the others

Major Concerns:

Some parts of the text are obscure. Meanwhile, the clarity of layout is especially critical for such manuscript.

For example, in the description of the Intranasal Delivery procedure (lines 127-144) there are repetitions and unclear parts:

1. Authors have to unify the descriptions for Hyaluronidase and MSCs-suspension application (see line 131 versus line 138).

2. Items 3-5 (lines 131-137) belong to Hyaluronidase pre-treatment. Therefore, it is better making the hierarchical outline - items 4 (lines 133-134), and 5 (lines 135-137) are sub-items of the item 3 (lines 131-132)

Response: We revised the manuscript and corrected any detected syntax and wording. We also clarify some parts and re-write other parts of the manuscript in attempt to clarify the current protocol. In the revised manuscript, we have elaborated our protocol hierarchy regarding the Hyaluronidase and MSCs-suspension application to address the point raised by the reviewer.

Minor Concerns:

1. Lines 86-126: Description of the CCI injury suggest that authors used two different stereotactic devices. One for preparatory procedure (line 98) and the other one equipped by TBI-0310 - for the Controlled Cortical Impact (line 111). If so, this should be stated more clear.

Response: We used two separate stereotactic devices, one for the pre-injury procedure and the other combined with TBI-0310. We revised the Protocol section to clarify this point.

2. Some abbreviations are not expanded.

For example, the nanoparticles were embedded with fluorescein isothiocyanate, which mentioned only as FITC (starting from the line 37), and never explained.

Controlled Cortical Impact (CCI) - also mentioned only in abbreviated form (first appearance - line 86).

Response: We apologize for not carefully explained the abbreviation. We add List of abbreviations list and revised the manuscript to expand the un-explained abbreviations.

3. As an additional note, the ultrasmall SPIOs might be more perspective that SPIOs (for review see Daldrup-Link HE. Ten Things You Might Not Know about Iron Oxide Nanoparticles. Radiology 2017)

Response: We are grateful for the reviewer's interesting literature suggestion. In future, we plan to examine the ultrasmal SPIO feasibility for MSCs labelling. However, the current protocol is already established in our lab and we interested to have it published in the current manuscript.

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Title of Article:	Non-Invasive and Real-Time Tracking of Superparamagnetic Iron Oxide Labeled Mesenchymal Stem Cells Using MRI after Intranasal Delivery in Mice Model of Traumatic Brain Injury
Author(s):	Rami Ahmad Shahror, Chung-Che Wu, Yung-Hsiao Chiang and Kai-Yun Chen

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