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TITLE:

Intracerebral Transplantation and *In Vivo* Bioluminescence Tracking of Human Neural Progenitor Cells in the Mouse Brain

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KEYWORDS:

Cell transplantation, luciferase, *in vivo* imaging, GFP, brain section, intraparenchymal transplantation

SUMMARY:

We describe the intraparenchymal transplantation of human neural progenitor cells transduced with a dual reporter vector expressing luciferase–green fluorescent protein (GFP) in the mouse brain. After transplantation, the luciferase signal is repeatedly measured using *in vivo* bioluminescence and GFP-expressing grafted cells identified in brain sections using fluorescence microscopy.

ABSTRACT:

Cell therapy has long been an emerging treatment paradigm in experimental neurobiology. However, cell transplantation studies often rely on end-point measurements and can therefore only evaluate longitudinal changes of cell migration and survival to a limited extent. This paper provides a reliable, minimally invasive protocol to transplant and longitudinally track neural progenitor cells (NPCs) in the adult mouse brain. Before transplantation, cells are transduced with a lentiviral vector comprising a bioluminescent (firefly-luciferase) and fluorescent (green fluorescent protein [GFP]) reporter. The NPCs are transplanted into the right cortical hemisphere

using stereotaxic injections in the sensorimotor cortex. Following transplantation, grafted cells were detected through the intact skull for up to five weeks (at days 0, 3, 14, 21, 35) with a resolution limit of 6,000 cells using *in vivo* bioluminescence imaging. Subsequently, the transplanted cells are identified in histological brain sections and further characterized with immunofluorescence. Thus, this protocol provides a valuable tool to transplant, track, quantify, and characterize cells in the mouse brain.

INTRODUCTION:

The mammalian brain has limited regenerative capacities following injury or disease, requiring innovative strategies to promote tissue and functional repair. Preclinical strategies focus on different aspects of brain regeneration, including neuroprotection, neurogenesis, angiogenesis[1,2], blood-brain-barrier repair[3,4], or cell therapy[5,6]. Cell therapy has the advantage of being able to promote many of these pro-repair processes simultaneously. In experiments with transplantation of cells, tissue repair has occurred through (1) direct cell replacement and (2) production of cytokines leading to angiogenesis and neurogenesis[7]. Recent advancements in stem cell technology have further facilitated the development of scalable, well-characterized neural cell sources that are now in the pipeline for clinical trials (reviewed in [7–9]). Although cell therapies have reached the clinical stage for a few neurological diseases (e.g., Parkinson's disease[10], stroke[11], and spinal cord injury[12]), their efficacy has been variable, and more preclinical research is needed to understand the mechanisms of graft–host interactions.

One major limitation of many preclinical studies is the continuous tracking of the transplanted cells inside the host. Often only end-point measurements are performed, omitting the dynamic migratory and survival processes in the host[6,13]. These limitations result in the poor characterization of the grafted cells and require high animal numbers to comprehend longitudinal changes. To overcome these limitations, in this study, we transduce induced pluripotent stem cell (iPSC)-derived neural progenitor cells with a commercially available dual-reporter lentiviral vector consisting of red firefly luciferase and enhanced green fluorescent protein (rFluc-eGFP). These cells are transplanted via stereotaxic intraparenchymal injection into the mouse brain and are longitudinally tracked using *in vivo* bioluminescence imaging over 5 weeks. After brain tissue collection, the GFP-expressing grafted cells are identified and further characterized in histological brain sections. This method can be smoothly adapted to alternative transducable cell sources and routes of transplantation for *in vivo* applications in the rodent brain. Overall, the procedure is valuable to obtain longitudinal information of graft survival and migration in the mouse brain and facilitates subsequent histological characterization.

PROTOCOL:

NOTE: All experiments involving mice were conducted in accordance with governmental, institutional, and ARRIVE guidelines and were approved by the Cantonal Veterinary Office of Zurich. Adult male and female non-obese diabetic SCID gamma (NSG) mice (10–14 weeks, 25–35 g) were used. Mice were housed in regular Type II/III cages in groups of at least two animals per cage in a humidity- and temperature-controlled room with a constant 12/12 h light/dark cycle. .).

1. Cell culture and viral transduction

1.1. Differentiate neural progenitor cells (NPCs) from iPSCs using small molecule inhibitors as previously described[14].

1.2. Culture NPCs from passage 2 onwards in Neural Stem cell Maintenance Medium (NSMM; **Table 1**) supplemented with small molecules (**Table 1**) in 6-well plates (2 mL of medium per well) coated with poly-ornithine/laminin521 (pLO/L521). Change the medium daily.

NOTE: To passage NPCs, add 1 mL of cell dissociation reagent per well (see the **Table of Materials**) and incubate at 37 °C for 1 min until most cells detach.

1.2.1. For coating, incubate 150 µL of pLO in 1 mL of 0.1 M phosphate-buffered saline (PBS) per well for 2h at room temperature (RT). After three washes with PBS, incubate 10 µg of L521 in 1 mL of PBS per well for 2 h at RT.

1.3. For viral transduction, plate 50,000 cells per well in a 24-well plate coated with pLO/L521 and add prepackaged viral vectors (pLL-EF1a-rFLuc-T2A-GFP-mPGK-Puro, LL410PA-1) to each well.

NOTE: Total infectious units (IFU) provided are >2 × 10^6 IFU and are enough to infect 100,000 cells at a multiplicity of infection (MOI) of 20. Cell counting has been performed with an automated cell counter. The transduction efficiency strongly depends on the used cell line. Work with lentivirus requires compliance with the local guidelines for biosafety level 2 (BSL-2) products.

1.4. Incubate the cells at 37 °C for 72 h, while continuing daily medium changes.

1.5. Confirm successful transduction by checking the cells for GFP expression in a fluorescence microscope. Set the microscope magnification to 10x or 20x and use the appropriate excitation (460–480 nm) and emission range (490–520 nm) to detect transduced GFP-expressing cells.

1.6. Quantify the ratio of GFP-transduced to total cells to estimate general transduction efficacy.

NOTE: Transduction rates of 65–95% were achieved with this protocol. A transduction efficacy of >50% is recommended as a go/no-go criterion before transplantation. If 50% transduction efficacy cannot be achieved, perform puromycin selection or sort the cells using flow cytometry to increase the yield of transduced cells.

1.7. Optional: Freezing of cells

1.7.1. Spin the cells down (300 × *g*, 5 min) and discard the supernatant. Resuspend the pellet in 1 mL of freezing medium (see the **Table of Materials**) and transfer the suspension into vials to

obtain 10^6 cells/vial. Transfer the cells to freezing boxes for 24 h at -80 °C and then to -150 °C for long-term storage.

2. Cell preparation for transplantation

2.1. Collect a vial of cells from -150 °C storage and transfer it to the laboratory. Count the cells using an automated cell counter.

NOTE: The vial contains 1.5–2 × 10^6 cells.

2.2. Quickly transfer the vial to a 37 °C water bath until no ice crystals remain (2–3 min).

NOTE: It is important to thaw rapidly to minimize any damage to cell membranes. Do not immerse the vial completely in the water bath as it can increase the risk of contamination.

2.3. Transfer the vial to the biosafety cabinet and pipette the whole content (~1 mL) into a sterile 15 mL conical tube.

NOTE: Work with lentivirally transduced cells requires BSL-2. However, washing and passaging cells remove viral particles from the medium. Information about when a transfer from BSL-2 to BSL-1 is permitted should be obtained from the local authorities.

2.4. Add 9 mL of sterile 1x PBS and centrifuge for 5 min at 300 × *g*, RT.

2.5. Remove the supernatant by aspiration using a pipette (1–10 mL); gently tilt the suspension towards the pipette tip and start aspirating. Be careful not to disturb the pellet.

2.6. Wash the cells by resuspending in 10 mL of sterile 1x PBS.

NOTE: Gently tab the tube to resuspend cells in the residual volume. Slowly triturate the cell suspension using a 1 mL pipette until it does not contain clumps or aggregates.

2.7. Count the cells before the final spin using an automated cell counter.

2.8. Centrifuge for 5 min at 300 × *g*, RT.

2.9. Remove the supernatant (step 2.5) and resuspend the cell pellet in the required volume of sterile PBS to a concentration of 8 × 10^4 cells/µL. Place the cells on ice and use them for transplantation within the next 5h.

NOTE: A volume of 1.6 × 10^5 cells/2 µL of PBS was used in this protocol.

3. Transplantation procedure

3.1. Preparation for surgery

3.1.1. Clean and sterilize the surgery equipment.

3.1.2. Prepare the stereotaxic device and the microinjection pump system.

NOTE: It is critical to test the Hamilton syringe and the 30 G, 2 inches needle before starting. Insert the needle into a tube containing sterile 0.9% NaCl and slowly draw the solution in and out.

3.1.3. Set up the anesthesia machine. Test the machine before involving any animals. Clean the induction chamber with 70% ethanol.

3.2. Preparation of animals

3.2.1. Keep the mice for at least 7 days prior to the experiments in standard conditions to acclimate them.

NOTE: The following animals were used for this protocol: female NOD/SCID/IL2rγnull (30–35 g, also known as NSG) and female C57BL/6J (20–25 g, also known as B6). The procedure can also be performed with male mice.

3.2.2. Measure the mouse body weight and adjust the dose of the pain killer to be injected. Administer carprofen (5 mg/kg body weight) intraperitoneally to reduce pain and/or prevent an inflammatory response.

3.2.3. Anesthetize the animals using isoflurane (3% in the induction phase and 1.5–2% in the maintenance phase during surgery) vaporized in oxygen.

NOTE: Gaseous anesthesia is preferred due to a quick wake-up after the surgical procedure and because the levels of anesthetic gas can be easily adjusted.

3.2.4. Use nociceptive reflexes to ensure the animal is deeply anesthetized (e.g., toe pinches). When deep anesthesia is reached, transport the animal from the induction chamber to the stereotaxic frame. Maintain anesthesia using a face mask.

NOTE: The breath rate needs to be monitored visually throughout the procedure (40–60 breaths per minute). Use a warming pad to avoid hypothermia during the procedure.

3.2.5. Apply ophthalmic lubricant to prevent the eyes from drying out.

3.2.6. Shave the mouse scalp with an electric razor and disinfect the skin with 5% betadine solution using cotton swabs.

3.2.7. Secure the mouse head and insert the ear bars into the external meatus.

NOTE: Be careful not to damage the eardrums. Apply lidocaine ointment to both ear canals before inserting the ear bars. To check if the animal's head is in a stable position, carefully push down on the head to see if there is movement. If movement is noted, either the ear bar, nosepiece placement, or both are incorrect and need to be readjusted.

3.3. Craniotomy

3.3.1. Use a surgical blade to make a cut along the midline big enough to reveal the lambda and bregma landmarks.

NOTE: Skin retractors can be applied to keep the skull exposed.

3.3.2. Retract the periosteum and fascia with a scalpel and use sterile cotton swabs to dry the skull surface.

3.3.3. Adjust the ear and mouth bars to standardize the head position.

NOTE: The vertical coordinates for bregma and lambda need to be identical for anteroposterior positioning.

3.3.4. Place the needle at the bregma and calculate the coordinates of the desired injection points (the coordinates of interest chosen for this protocol: anterior–posterior (AP): + 0.5 mm, medial–lateral (ML): + 1.5 mm). Move the needle to that point and mark it with ink.

NOTE: The coordinates were chosen based on the Franklin and Paxinos Mouse Brain Atlas [15]. Distances are mm from the bregma.

3.3.5. Drill a hole with a diameter of 2–3 mm through the skull with a surgical, dental drill.

3.3.6. Move the needle to the surface of the dura and calculate the depth coordinates.

3.4. Transplantation procedure

3.4.1. Resuspend the cells in the tube (step 2.9) and draw 2 µL of cell suspension into a syringe (5 µL or 10 µL).

NOTE: Make sure that no air bubbles are present in the cell suspension. The syringe needs to be kept in a horizontal position until mounted into the stereotactic device to avoid cell sedimentation.

3.4.2. Place the syringe above the target site (calculated coordinates: AP: + 0.5 mm, ML: + 1.5 mm) and slowly move the needle to the surface of the dura.

NOTE: If unsure about the correct coordinates, perform injections with a dye and histological evaluation of the injection site before transplanting cells (for more details, see [16]).

3.4.3. Guide the needle at a rate of 0.02 mm/s into the brain up to the proper depth (the coordinate chosen for this protocol is dorsal–ventral (DV) – 0.8 mm). Overshoot the depth by 0.1 mm and withdraw the needle over the same distance to create a pocket for the injected cells.

3.4.4. Apply tissue adhesive around the needle using forceps to prevent leakage of cells.

3.4.5. Inject 2 µL of the prepared cell suspension at a constant rate of 3–5 nL/s.

NOTE: The injection procedure will last between 7 and 12 min.

3.4.6. Following injection, leave the needle in place for at least 5 min before slowly withdrawing it. Apply tissue adhesive to seal the hole in the skull and wait for another 2 min.

3.5. **Sutures and post-care**

3.5.1. Apply sterile 0.9% NaCl solution to the exposed skull to avoid dehydration.

3.5.2. Close up the wound with a 5/0 silk suture thread.

3.5.3. Hydrate the animal with 0.5 mL of ringer lactate solution subcutaneously injected in the lower back.

3.5.4. Interrupt anesthesia delivery and carefully remove the mouse from the stereotaxic apparatus and place it back in a cage kept on a heating pad.

3.5.5. Monitor the animals during the acute phase postinjury. Check the suture, the animal weight, and overall health at least twice a day.

4. *In vivo* imaging

4.1. Preparation of luciferin

4.1.1. Thaw D-luciferin potassium salt at RT and prepare a fresh stock solution of D-luciferin at 30 mg/mL in PBS.

4.1.2. Sterilize the stock solution through a 0.22 µm syringe filter.

NOTE: Immediate use of the working solution is recommended. If necessary, dissolved luciferin can be stored at -20 °C. However, prolonged storage may result in the degradation of signal. Luciferin is a light-sensitive reagent; keep it out of direct light whenever possible. Alternative

substrates may also be considered, e.g., cycluc, to improve the resolution limit[17].

4.2. Imaging

4.2.1. Initial setup

NOTE: Bioluminescence imaging was performed using an *in vivo* imaging system (see the **Table of Materials**) consisting of a dark chamber and a cooled charge-coupled device (CCD) camera.

4.2.1.1. Double-click the **Living Image software** icon and select a **user ID** from the drop-down list.

4.2.1.2. Click **Initialize** in the **control** panel that appears. Once the initialization process is completed, the **temperature box** in the control panel will turn green.

4.2.1.3. In the **control** panel, check the **Luminescent** and **Photograph** boxes and select **Auto exposure** (~60 s). Select a **field of view** (**D**/12.5cm was chosen for this protocol). Enter the subject height (1.5 cm) and select the **use subject height** focus option. Manually set the following parameters: **large binning, f/2, blocked excitation filter,** and **open emission filter**.

4.2.2. Determine the injection amount of D-luciferin at 300 mg/kg body weight.

NOTE: The standard recommended dose is 150 mg/kg of D-luciferin. This procedure was adjusted according to a protocol reporting higher sensitivity using 300 mg/kg[18].

4.2.3. Inject the luciferin intraperitoneally (i.p.).

NOTE: If the animal needs to be sedated before injection, be aware that it may extend the peak luciferase expression time.

4.2.4. Wait for 5 min, then anesthetize animals with a continuous supply of isoflurane (4.5% in the induction phase and 1.5–2% in the maintenance phase during imaging procedure).

4.2.5. Shave the sedated animals on the head region using a conventional hair shaver. Place the animals in the imaging chamber and start imaging 15 min after the luciferin injection by clicking **Acquire** in the **control** panel.

5. Perfusion

5.1. Anesthetize the animals by an i.p. injection of sodium pentobarbital (150 mg/kg body weight). Wait until the mouse no longer responds to painful stimuli, such as toe pinches.

5.2. Lay the mouse on its back and use tweezers and scissors to open the chest cavity.

5.3. Use standard scissors to open the diaphragm.

5.4. Expose the heart and insert a needle (from the tubing with ringer/4% paraformaldehyde solution (PFA)) into the apex of the left ventricle.

NOTE: Be careful to keep the tip of the needle in the lumen of the ventricle.

5.5. Cut the right ventricle using scissors.

5.6. Perfuse with ringer solution (can be kept at RT) for 3–4 min (flow rate: 17 mL/min). Continue until the heart is clean.

5.7. Switch the stopcock to allow for the flow of PFA (store at 4 °C; keep on ice during the procedure) and perfuse for another 5 min (~100 mL).

5.8. Stop the pump and remove the needle from the left ventricle.

NOTE: The perfusion with PFA preserves tissue integrity uniformly. It also facilitates the preservation of GFP signal in the transplants that otherwise may be lost due to diffusion.

6. Processing

6.1. Tissue collection

6.1.1. Remove the head using standard scissors and make a midline incision in the skin.

6.1.2. Turn the skin over the eyes to expose the skull.

6.1.3. Start from the caudal part at the point of the parietal bone and make a small incision using spring scissors. Advance the scissors rostrally along the midsagittal suture up to a point between the eyes. Start again from the caudal part and make two cuts parallel and ~4 mm apart in the sagittal plane.

NOTE: Be careful not to damage the brain by pressing the scissors against the interior surface of the skull.

6.1.4. Use forceps to carefully tilt one side of the parietal bone and break it off. Do the same with the other side.

NOTE: Use a microspatula to free the bone from the meninges; otherwise, they may damage the brain while breaking off the skull. If parts of the frontal bone remain, make a small cut to tilt and break off the bone plate.

6.1.5. To release the brain, carefully slide the microspatula under the brain (olfactory bulbs) and

tilt it gently upward.

6.1.6. After collecting, keep the brain in 4% PFA solution for 4–6 h at 4 °C. Transfer it to sterile 1x PBS afterward.

6.2. Immunohistochemistry

6.2.1. Transfer the brain to a 30% sucrose solution for at least 48 h at 4 °C to prevent the formation of crystals during freezing.

6.2.2. Use a sliding microtome to cut coronal sections with a thickness of 40 µm. Collect and store the sections as free-floating sections (in a 24-well-plate) in a cryoprotectant solution (**Table 1**) at -20 °C until further processing.

6.2.3. Rinse the sections with 450 µL of 1x PBS for each well (3 times, 5 min each, RT).

6.2.4. Block non-specific sites with 450 µL of blocking solution for each well (**Table 1**) for 1 h at RT.

6.2.5. Incubate each well with 450 µL of primary antibodies at 4 °C overnight. Dilute the antibodies 1:200 in 3% donkey serum; 0.1% Triton-X-100 in PBS. To identify donor material in the host environment, use an antibody to human-specific nuclei (Anti-Human Nuclei Antibody, clone 235-1).

6.2.6. Wash the sections with 450 µL of 1x PBS for each well (3 times, 5 min each, RT).

6.2.7. Incubate each well with 450 µL of corresponding fluorescent secondary antibodies for 2–3 h (RT). Dilute the antibodies in 3% donkey serum; 0.1% Triton-X-100 in 1x PBS.

6.2.8. Wash the sections with 450 µL of 1x PBS for each well (3 times, 5 min each, RT).

6.2.9. Stain the nuclei with 450 µL of 0.1 µg/mL 4',6-diamidino-2-phenylindole (DAPI).

REPRESENTATIVE RESULTS:

We aim to longitudinally track transplanted neural progenitor cells in the mouse brain using *in vivo* bioluminescence imaging and identify the transplanted cells in subsequent histological analysis (**Figure 1A**). Therefore, neural progenitor cells are transduced with a lentiviral vector consisting of EF1α-rFluc-eGFP. Before transplantation, cells were tested for successful transduction by expression of eGFP *in vitro* (**Figure 1B**). The successfully transduced cells were stereotactically transplanted in the mouse brain at the desired coordinates (e.g., in the sensorimotor cortex). Following transplantation, the mice were systemically injected with D-luciferin, the substrate for rFluc, and signal intensities of the transplanted cells were measured to confirm successful transplantation (**Figure 1C**).

To evaluate the detection limit of the *in vivo* bioluminescence imaging, a range of 6,000–180,000 cells was transplanted in the right sensorimotor cortex of the mouse (**Figure 2A**). We detected <6,000 cells and a bioluminescence signal proportional to the transplanted cell count directly after transplantation (**Figure 2B**). As human cell sources are immunogenic to immunocompetent mice, NOD scid gamma (NSG) immunodeficient mice were used to observe the long-term survival of the cell grafts. Long-term survival and detection of a bioluminescence signal for up to 5 weeks were confirmed after cell transplantation (**Figure 2C,D**). The transplanted cells were successfully detected *ex vivo* in a subsequent histological analysis through the eGFP reporter and immunostaining with anti-human nuclei and anti-human mitochondrial antibodies (**Figure 2E**).

FIGURE AND TABLE LEGENDS:

Figure 1: Transplantation of neural progenitor cells. (**A**) Schematic overview of generation and transplantation of rFluc-eGFP NPCs. (**B**) Representative immunofluorescence image of transduced NPCs (GFP reporter, green) counterstained with DAPI (blue); scale bars = 5 µm. (**C**) *In vivo* detection of bioluminescence signal in transplanted cells; color bar = blue (0, min, no signal), red (4 flux, p/s × 10^5, max signal) Abbreviations: NPCs = neural progenitor cells; GFP = green fluorescent protein; rFluc-eGFP = red firefly luciferase and enhanced green fluorescent protein; DAPI = 4',6-diamidino-2-phenylindole; p/s = photons/s.

Figure 2: Time course of transplanted cells. (**A**) Schematic view of cell numbers for transplantation. (**B**) Detection limit of transplanted cells 1 h after transplantation. (**C, D**) Time course of transplantation (180,000 cells) for up to 35 days in NSG mice ; color bar = blue (0, min, no signal), red (4 flux, p/s × 10^5, max signal) Data are mean ± SEM (*n = 5*). (**E**) Representative fluorescence images of histological sections and transplanted cells 5 weeks following transplantation. Scale bar = 10 µm. Abbreviations: D = day after transplantation; NSG = immunodeficient NOD scid gamma; DAPI = 4',6-diamidino-2-phenylindole; GFP = green fluorescent protein; HuNu = Anti-Human Nuclei Antibody, clone 235-1; p/s = photons/s.

DISCUSSION:

Regenerating the injured brain to allow for functional recovery remains an unmet challenge. Many innovative preclinical approaches have evolved targeting, for example, immune modulation[18,19], angiogenesis[1,20–22], blood-brain-barrier integrity[2,3,23,24], and cell replacement[5,25]. Especially in recent years, cell-based therapies have emerged as a promising treatment strategy for the brain due to major advancements in stem cell technology and efficient differentiation protocols. This paper provides a valuable protocol for transplanting and tracking neural cells in the mouse brain. The method is applicable for all transducable cell lines for *in vivo* applications in the mouse brain.

The presented setup uses transplants of human origin in a mouse. These transplants are not viable in the long-term in immunocompetent wild-type mice due to immunogenicity. Hence, immunodeficient NSG mice were used to overcome this limitation. Alternatively, the use of mouse transplants may be preferred to overcome the immunogenic aspects. If transplantation of human cells is required, humanized mouse models represent an emerging alternative to reduce the probability of graft rejection[27].

A commercial dual-reporter viral vector consisting of firefly luciferase and eGFP under the EF1α promotor was used to visualize the transplants. This promotor was selected to achieve a high signal intensity. However, apart from NPCs, other cell types have been shown to promote brain function after injury, including pericytes[28] and astrocytes[29]; hence, depending on the cell line used, other promotors might be more suitable to achieve high expression levels. Additionally, the use of transgene promoters, such as CMV, may lead to downregulation, especially in long-term experiments[30]. The transduction efficiency of the lentiviral vector strongly depends on the used cell line and may vary between single experiments. Therefore, transduction efficiency must be evaluated before starting the *in vivo* experiments and to correct variations in transduction efficacy between experiments. The brain region of transplantation also influences the signal strength. Although a detection limit of <6,000 cells was achieved for cortical transplantations, it may require more cells to detect a signal in deeper brain regions, for example, striatum or hippocampus.

Transplantation volumes in the mouse brain are limited to 1–2 µL. Therefore, it is important to identify a suitable cell number for the experiments. It has been previously observed that increasing cell numbers leads to decreased survival rate, most likely due to limited availability of nutrients and oxygen in the region of transplantation[31]. *In vivo* bioluminescence imaging provides a relatively low spatial resolution compared to other *in vivo* imaging methods such as MRI or CT. Therefore, short migratory paths of grafted cells can only reliably be assessed in the subsequent *post-hoc* analysis.

The absolute signal strength of the bioluminescence is generally proportional to the transplanted cell number. However, the signal strength might be reduced if grafts are transplanted in deeper brain structures or if the signal strength is outside the linear detection spectrum of the *in vivo* imaging system. Currently, novel substrates are developed to ensure more efficient penetration across the blood-brain barrier than D-luciferin, including cycluc1. These substrates may further improve the detection limit of the grafted cells in the future[17]. Overall, this protocol allows a straightforward, minimally invasive procedure to transplant and observe grafts in the mouse brain.

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DISCLOSURES:
The authors have no potential conflicts of interest to declare.

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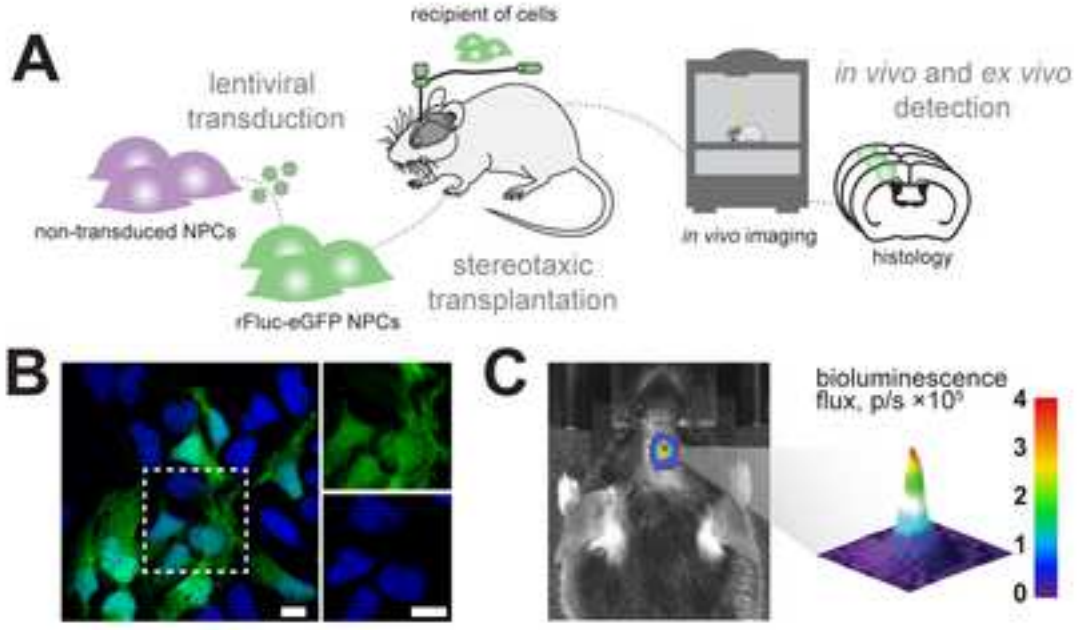

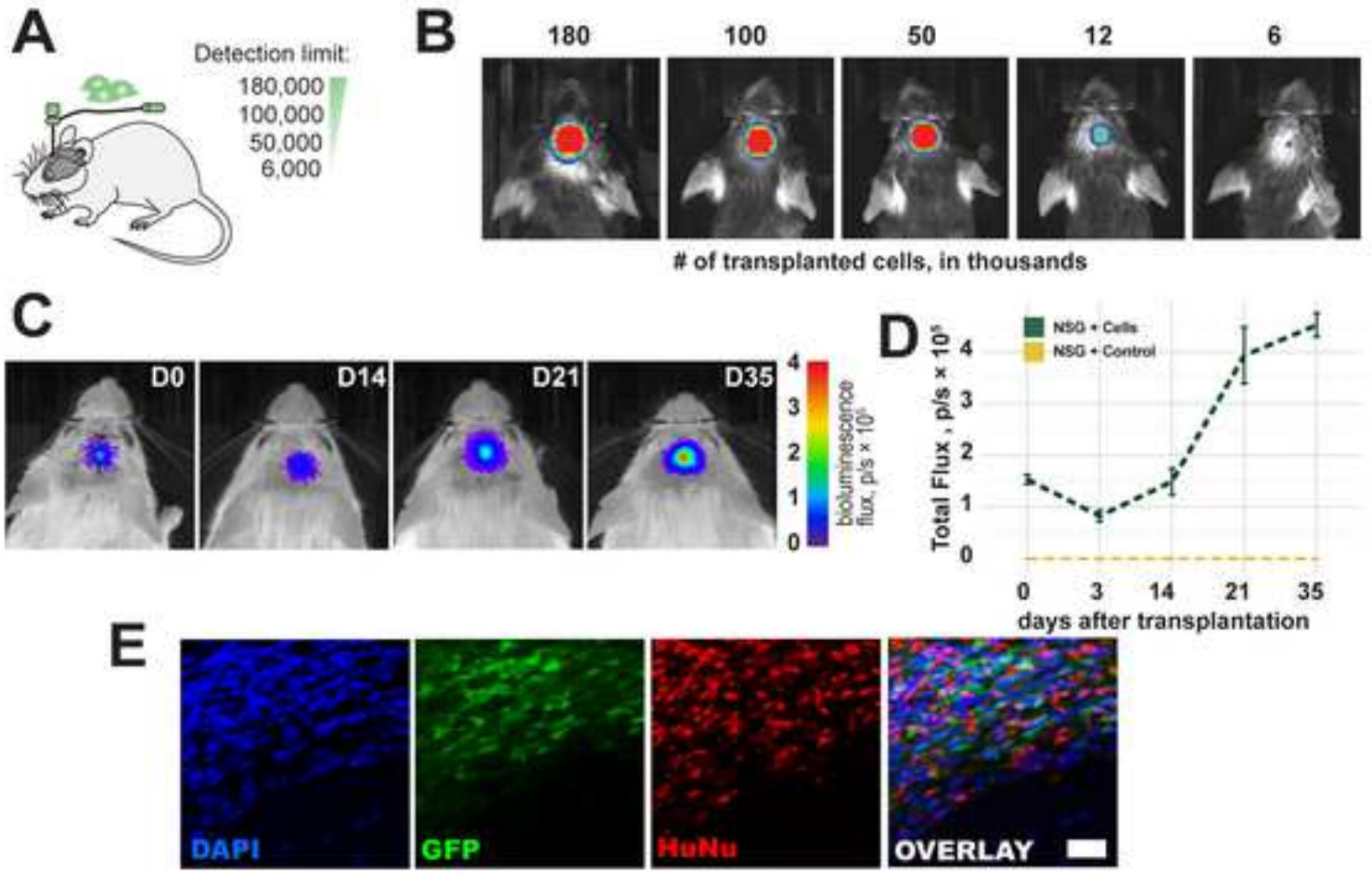

Neural Stem cell medium, 500 mL	
DMEM/F12	240 mL
Neurobasal	240 mL
N2-Supplement (100x)	5 mL
B27 Supplement (50x)	10 mL
Glutamax (100x)	5 mL

Growth factor supplementation for 50 mL of medium	
hLif (10 µg/mL, 1,000x)	50 µL
CHIR99021 (10 mM, 2,500x)	15 µL
SB431542 (10 mM, 3,333x)	10 µL
Fibroblast growth factor (FGF)	5 ng/mL

Cryoprotectant solution , 500 mL	
Sucrose	150 g
Ethylene glycol	150 mL
0.1 M PBS	make up volume to 500 mL

Blocking solution	
Donkey serum	5%
Triton X-100	0.10%
1x PBS	

Editorial comments:
Editorial Changes
Changes to be made by the Author(s):

We would like to thank the Editors for their valuable input and careful evaluation of the manuscript. We addressed the suggestions (see below) and changed/expanded the manuscript accordingly. Changes to the manuscript are highlighted in cyan.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you for the input. We thoroughly proofread and adapted the manuscript.

2. Please provide an abstract between 150-300 words, more clearly describing the goal of the protocol.

Thank you for the input. We adapted the abstract accordingly

3. Please provide references for lines: 60-72.

We added the references 6 and 13 for line 60-72

4. Please revise the following lines to avoid previously published work: 147-150, 183-184, 386-387.

We updated information to avoid previously published work throughout the manuscript wherever possible.

5. Please include the following details in the introduction as well:
a) A clear statement of the overall goal of this method
b) Information to help readers to determine whether the method is appropriate for their application

We added a statement of the overall goal and information about the scope of the application in line 71-75

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. For example: SystemBio, Histoacryl, etc.

All commercial language was removed from the protocol.

7. Please use SI unit denotation for all units throughout the manuscript: L, mL, µL, cm, kg, etc. Hours, minute, and seconds can be written as h, min, s, respectively.

We changed all units to SI units.

8. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

We included an ethics statement: lines: 78 - 84

9. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol.

We replaced any personal pronouns with appropriate third person pronouns and rewrote all sections containing phrases with “could be”, “should be” or “would be”.

10. Please add more details to your protocol steps. 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.

We adapted these changes across the entire manuscript.

Step 1.2: What was the volume of medium used to culture the cells? How was the pLO/L521 coating done? Please mention

We have added the information that 2ml medium were used per well and now provide the details for coating as follows: “For coating, 150µl pLO in 1ml PBS per well were incubated for 2h at room temperature. After 3x washing with PBS, 10µg L521 in 1ml PBS per well were incubated for 2h at room temperature.” (see line 95-97)

Step 1.3: How were the cells counted? What was the quantity of vector added?

The cells were counted using an automated cell counter. We added the details in the protocol (line: 109) and in the material list.

Step 1.4: Please describe how the microscopy was done identify GFP expression. Please include all button clicks for software usage, parameters for image acquisitions, etc.

We added the information about excitation and emission wavelength of GFP and the magnification (line 117-120)

Step 2.1, 2.7: How are the cells counted?

The cells were counted using an automated cell counter. We added the details in the protocol (line: 138 and 158) and in the material list.

Step 2.9: What was the cell count here, how much PBS was added?

We used a volume of 1.6 x 10^5 cells / 2uL PBS. These details were added in the protocol (line: 139).

Step 3.2.4: How was the breath rate monitored?

The breath rate was monitored visually. Added in the protocol (line: 201).

Step 3.2.7: What was the betadine concentration used?

We used a 5% betadine solution. This detail was added in the protocol (line: 207).

Step 3.3.1, 6.1.1: What was the incision size?

The incision size depends on the animal's head size and form. The incision length needs to be big enough to reveal the lambda and bregma points. This is mentioned in the protocol.

Step 3.4.2: Is the target site, the marked point in step 3.3.4? If yes, please mention it here for clarity.

Yes. We clarified it in the protocol (line: 249-251).

Step 4.24: Please provide all image acquisitions setting including wavelength of the excitation and emission filter, magnification of lens, etc. The software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g., button clicks, numerical values for settings, etc.).

Thanks for the comment. We added an additional section "**initial setup"** where we describe the software steps in more detail.

Step 6.2.5, 6.2.7: Which primary and secondary antibody is used and what is its concentration? What is the total number of wells used?

We added the corresponding details to the protocol and completed the list of materials.

11. Please ensure that 3 pages of the protocol have been highlighted for filming including headings and spacings. Please ensure that the highlighted steps form a cohesive narrative as these will be used for scripting purpose.

We have added yellow highlighted sections that should be filmed.

12. Please discuss any future application of this technique.

We added potential application of all transducable cell lines in the mouse brain (L471-L472).

13. Figure 1,2: Please mention what the color bar indicates.

We added the information to figure 1 and 2.

14. Figure 2A, 2B: Does detection limit have a unit? If yes, please mention in the figure.

Yes, the numbers represent the number of transplanted cells. It has been added to the figure.

Please mention what the commas/quotes represent (e.g., 180’000).

We removed the quotes.

15. Figure 2C: What do D0, D14, etc. stand for please mention in figure legend.

It stands for days after transplantation; we added the information in the figure legend.

16. Figure 2D: Please provide the n number of the sample and please mention what does the error bar (Standard error or deviation) indicate in the figure legends.

We completed the legend of figure 2. N number is $n = 5$, data are mean ± SEM.

Reviewers' comments:
Reviewer #1:

We would like to thank Reviewer #1 for the valuable input and careful evaluation of the manuscript. We addressed the suggestions (see below) and changed/expanded the manuscript accordingly. Changes to the manuscript are highlighted in cyan.

Manuscript Summary:
In the manuscript "Transplantation and in vivo bioluminescence tracking of cells in the mouse brain", authors describe a protocol for cell tracking analysis by bioluminescence imaging of firefly luciferase expressing neural progenitor cells obtained from iPSCs administered by intraparenchymal transplantation.

Major Concerns:
This protocol might contribute to cell therapy studies for neurological disorders. Nonetheless, several aspects need to be further detailed to strengthen the its utility for potential protocol users, among which:
* Gene transduction efficacy and the BLI emission range of transduced cells should be provided as a pivotal go/no-go criteria before proceeding to the transplant.

We agree, since we do not perform a selection for transduced cells, we recommend having >50% transduction efficiency to achieve reasonable signal intensities. If this cannot be reached an enrichment method (antibiotic selection or FACS might be used before transplantation). We added this information to the manuscript in the protocol section 1.6

* Figures are important as provide an overview of the typical outcome of the procedure. In the current version figures are poorly described.

We modified the figures and added previously missing information about bars and axis labels. Furthermore, we expanded the figure legends.

Minor Concerns:
* Are the title and abstract appropriate for this methods article? The type of administered cells and the route of administration should be provided in the title

We changed the title to: "Intracerebral transplantation and in vivo bioluminescence tracking of human neural progenitor cells in the mouse brain."

* Are there any other potential applications for the method/protocol the authors could discuss? The describe protocol refers to administration of neural progenitor cells from iPSCs, but it can be applied for transplantation of other type of cells (as mentioned by the authors).

This is correct. Generally, all transducible cell sources can be used for in vivo applications to the mouse brain. We added this information in the abstract and introduction.

* Are all the materials and equipment needed listed in the table? (Please note that any basic lab materials or equipment do not need to be listed, e.g. pipettes.) Define the manufacturer rather than the distributor for each product.

We added and updated the information to in the material list.

* Do you think the steps listed in the procedure would lead to the described outcome? The possibility of detection by BLI depends on the number of engrafted luciferase-expressing cells. Minimal requirements for BLI expression must be defined.

This is correct. In our experience the minimal detected cell number is around 6000 cells transplanted to the cortex. However, these numbers strongly depend on the strength of the promoter, transduction efficacy and the region of transplantation. We discuss these aspects in the discussion L489-L492; and we have expanded this paragraph.

* Are the steps listed in the procedure clearly explained? In part.

We expanded the explanation throughout the manuscript and expanded the material list.

* Are any important steps missing from the procedure? More in depth definition of the coordinates for stereotaxic intraparenchymal transplantation should be provided

The coordinates were chosen based on the Franklin and Paxinos (2008) Mouse Brain Atlas. We added this to the protocol. Generally, we recommend to perform pilot studies with a dye to confirm the correct region for transplantation. We added this information to the L233-L234

* Are appropriate controls suggested? Controls (NSG-control in figure 2D) should be described more in depth and analysis on control samples included in Figure 2E

We added the information to the representative results (L443-444) and the discussion (L472-L478). Furthermore, the figure legends have been expanded

* Are all the critical steps highlighted? Yes

Thank you.

* Is there any additional information that would be useful to include? As mentioned above: definition of cell BLI emission range and coordinates for transplant should be clearly mentioned

We added some more details regarding the transplantation coordinates and the BLI setup. We added a new section "**Initial setup 4.2.1**" where we describe the different parameters.

* Are the anticipated results reasonable, and if so, are they useful to readers? Yes

Thank you.

* Are any important references missing and are the included references useful? No

Thank you.

Additional issues:
* Clearly define MOI used for transduction.

We added this information.

* Line 79. "Neural progenitor cells (NPCs) are differentiation from iPSCs as previously described". Revise and briefly include more information on the procedure.

We added this information in Line 87.

* Line 87: "prepackaged viral vectors". What do you mean with "prepackaged"?

The manufacturer offers the described construct in two versions, as a plasmid or as a pre-packaged viral vector. This viral vector can be directly applied to the cells. Please see for more information here: https://www.systembio.com/pll-ef1a-rfluc-t2a-gfp-mpgk-puro-lenti-labeler-lentivector-plasmid-pre-packaged-virus

* No need for video for cell preparation procedure.

We believe it is important to see the preparation of the cells before transplantation. Wrong handling may result in substantially lower cell viability / overall success of the method. We will carefully discuss this with the JoVE team.

* Suggested manufactured dose for D-luciferin solution is 150 mg/kg: how did you proceed with the optimization for the suggested dose (300 mg/kg)?

Substrate dose was chosen based on **Aswendt et al., 2013**. They determined a novel and efficient protocol with ip injection **of 300 mg/kg.** This novel protocol turned out to be twice as sensitive as the conventionally applied "standard protocol".

Reference: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3566035/

* Provide full description for each reagent

We completed the list of materials.

* Figure 1: time after transduction and transplant should be defined. Include cell ex vivo BLI imaging. Define min e max values in pseudocolor scale bar.

We freeze the cells after transduction and expansion. Therefore, the time of transplantation can be therefore chosen as required, we clarified this in L129-L132. We added a figure legend and defined the min and max of the scale bar in the Figures 1 and 2.

* Figure 2B: time of the analysis after transplant should be defined.

We added this information to figure legend 2B. It was an acute measurement 1 h after transplantation to determine the resolution limit.

* Figure 2E: time of the analysis after transplant should be defined. Time course and control samples in immunofluorescent analysis must be included.

We added this information. The tissue has been analysed 5 weeks after cell transplantation.

* Line 367: "a stable bioluminescence signal up to 6 weeks" in Figure 2C shows not stable signal; not for 6 weeks.

We removed the word “stable” and corrected the timeline to 5 weeks.

Reviewer #2:

We would like to thank the Reviewer #2 for his valuable input and careful evaluation of the manuscript. We addressed the suggestions (see below) and changed/expanded the manuscript accordingly. Changes to the manuscript are highlighted in cyan.

Manuscript Summary:
Very well written. It will be interesting to many experimental bench scientists with Jove video recording procedures.

Minor Concerns:
Recommended to try more brain penetrable luciferase substrate, like cycluc1. At least mention it in discussion.

Cycluc references:1. 1]. Synthetic luciferin, CycLuc1, improves bioluminescence imaging for intracranial glioblastoma xenografts. 10.1158/1538-7445.AM2018-4112 2. In Vivo Optical Imaging of Myelination Events in a Myelin Basic Protein Promoter-Driven Luciferase Transgenic Mouse Model, ASN Neuro . Jan-Dec 2018;10:1759091418777329. doi: 10.1177/1759091418777329.

This is a great advice. We added this information into the protocol 4.1.2 L300 and discussion L504.

Reviewer #3:

We would like to thank Reviewer #3 for the valuable input and careful evaluation of the manuscript. We addressed the suggestions (see below) and changed/expanded the manuscript accordingly. Changes to the manuscript are highlighted in cyan.

Manuscript Summary:
The paper describes a method to transplant tagged cells (neural progenitor cells, NPCs) into the CNS. The cells, transduced with a lentiviral vector to express luciferase and eGFP allow longitudinal studies of the cell fate by means of bioluminescence imaging, or the exact localization in the brain by means of fluorescent hystological analyses. The authors provide examples showing that in immunodeficient animals the NPCs express luciferase up to 6 weeks following transplantation.
The method deserves publication when minor issues will be fixed.

Minor Concerns:
1. Abstract. The authors claim that the method is "non-invasive". Nevertheless, to transplant the cells it is necessary a intraparenchymal injection, which is an invasive procedure. I suggest to remove the claim.

We agree. We changed it to minimally invasive.

2. Protocol 1.1 change "differentiation" with "differentiated".

We changed it accordingly.

3. Protocol 1.2 The description of the medium is very hard to understand. Please fix.

We added two tables (Table 1,2 in Protocol 1.2) with example volumes for the medium to make it more comprehensive.

4. Protocol 1.3 It should be stated the Biosafety level of containment necessary to perform viral transduction of the cells.

We added the following note: “Note (III): Work with Lentivirus requires the compliance with the local guidelines for biosafety level 2 (BSL-2) products. ”

5. Protocol 1.4 No change of medium is needed after 72h? Please comment or add details.

We added: “..., while keeping daily media changes (protocol 1.4)”

6. Protocol 1.5 After successful transduction, what happens to the cells? I suppose that the cells will be stocked in LN2: if this is the case add the procedure necessary to freeze the cells.

We added this information to Protocol 1.6. Yes the cells are frozen at -150°C.

7. Protocol 2.3 Which is the biosafety level of containment of the safety cabinet? Please add the information. If operations should be performed in BSL-2 cabinets please always give this information and describe the hazard.

We added the following note: “Note: Work with lentivirally-transduced cells requires BSL-2. However, washing and passaging cells removes viral particles from the medium. Information about when a transfer from BSL-2 to BSL-1 is permitted should be inquired at the local authorities (Protocol 2.3)”

8. Protocol 3.2.1 Why only female animals were used for the protocol? Do you expect different results in male mice? Please comment

We performed the procedure with over 100 mice of different sex and genotype. We did not observe any differences between the sex. We added this information in 3.2.1

9. Protocol 3.2.2 Please specify which "pain killer" should be used, and why.

Carprofen was used (5 mg/kg body weight). This was added to the protocol.

10. Protocol 3.2.5 The use of toe pinches should be performed to check if deep anesthesia is reached. Therefore this step should be placed before 3.2.4.

Thanks for the input. We changed the order of these steps.

11. Protocol 3.3.6 Specify how to "calculate the depth coordinates" In this protocol DV -0.8 mm is used. How did you identify this depth as the optimal?

The coordinates were taken from the **Franklin & Paxinos (2008) Mouse Brain Atlas.** We added this to the protocol.

Reference: https://www.elsevier.com/books/paxinos-and-franklins-the-mouse-brain-in-stereotaxic-coordinates-compact/franklin/978-0-12-816159-3

12. Protocol 3.4.1 The protocol states to draw 2 to 3 ul cell suspension. This is a huge range. It is not specified (at 3.4.5) if the whole volume should be injected, or if it is necessary to inject a max volume of 2ul.

Thanks for the input. We injected an **exact amount of 2ul.** We clarified this step in the protocol (line: 3.4.5).

13. Protocol 3.5.1. Apply NaCl solution to what? Please specify

NaCl is applied to the exposed skull to prevent it from drying out. We added this detail to the protocol (line: 232).

14. Protocol 4.2.3 I suggest to change "wait approx. 5 minutes" to "wait 5 minutes". In my opinion it is very important to keep a precise timing for biodistribution of luciferin to compare results.

We adapted the protocol in 4.2.3 accordingly.

15. Protocol 4.2.4 Please remove the "approx." - see issue 14. Please specify that the acquisition parameters are optimized for bioluminescence imaging on a Lumina III instrument. Since you were very detailed with the instrument settings, you should also add a

section, at the very beginning of this paragraph, describing the preliminary procedures for the set up of the instrument (e.g. acquisition of the background, etc). Which is the used FOV? please report the distance between camera and mouse.

"Approx." was removed. We further specified the instrument settings and the corresponding preliminary procedures. We added a new section "**Initial setup**" were we describe all the necessary hardware details.

16. Protocol 5.6 Please report the temperature of the ringer solution

We added this information in protocol 5.6.

17. Protocol 5.7 Please report the temperature of PFA

We added this information in protocol 5.7.

18. Protocol 6.1.6 Are the slices collected and stored in plain PBS, or preservative agents are added?

The brains are collected and stored in plain PBS (1X) on 4° (short term). After cutting we add a preservative/cryoprotectant solution and keep the slices at -20°C. We added this information in 6.2.2.

19. Discussion, line 413. Again, intraparenchymal injection is an invasive procedure, therefore the protocol isn't "non-invasive".

We changed it to minimally invasive as in the abstract.

Reviewer #4:

We would like to thank Reviewer #4 for the valuable input and careful evaluation of the manuscript. We addressed the suggestions (see below) and changed/expanded the manuscript accordingly. Changes to the manuscript are highlighted in cyan.

Manuscript Summary:
The manuscript describes the protocol for injection and visualization of bioluminescence-labelled stem cells in the mouse brain. Although the idea and the procedure is not entirely new, the detailed protocol would be useful for the interesteg research community.

Major Concerns:
None.

Minor Concerns:
1. iPSC are of human origin. This is an important aspect of the protocol and should be mentioned throughout the article, and the aspects of combining human cells with the mouse host discussed.

We agree. This issue has been added to the discussion in line 473-478. We also clarified the use of immune deficient NSG mice in the representative results line 443 – 444.

2. It seems that the native GFP is visualized in the histology sections. The aspects how to preserve the original GFP fluorescence and omit the imaging via anti-GFP antibodies would be welcomed.

This is correct. We preserve the GFP expression of cells using 4% Paraformaldehyde solution during the perfusion of the mouse (to avoid GFP signal loss or diffusion). Moreover, sections are preserved in cryoprotectant solution at -20 for long term storage. We added this information in Protocol 5.8 Notes and Protocol 6.2.

3. Line 144 - it is true that the painkiller was defined later in the table among Pharmaceuticals and Reagents, but maybe for those places in the manuscript when exact dosage is mentioned would be beneficial to name the substance as well, please apply throughout (e.g. line 261 - IVIS Lumina III), BTW I think it is carprofen, and not carprofenum.

Thanks for the input. We specified it in the protocol.

4. Knowing the flow and assuming the injection lasted 5 min, one can calculate you had injected 1ul in the brain. Anyhow, the volume can be stated, in particular as it is discussed later on.

In the protocol, we suggest that the needle should be left in place for 5 minutes following injection, to prevent reflux. The injection itself lasts between 7 and 12 minutes, depending on the flow rate. We clarified this aspect in the protocol.

5. line 288 - typo - stopclock.

Changed “stopcock” to “stopclock”

6. line 87 - was there any puromycin selection?

No. We achieve relatively high transduction efficacy 65-95%. However, we are aware that different cell lines may have lower transduction efficacies. Therefore, we added protocol 1.6 with a note that puromycin selection can be applied if transduction efficacy is below 50%.

7. Discussion - the first part of the discussion repeats the introduction and can be commited. I would prefer to specifically address the 5 bullet points described in the Instructions. Risks and how they could be mitigated would be beneficial. Discussing the relation between promoter strength and number of detected cells could be added. The relevance of intact or damaged BBB as well.

We expanded the discussion accordingly. We added 1) the relevance of promoter strength in different cell types, 2) the use of immue deficient mice vs. immune competent vs. humanized mice, 3) Brain regions of transplantation, 4) Alternative substrates.

8. Line 361 - Figure 2D - number of transplanted cells for the given graph should be given. NSG acronym should be defined.

We added this information in the figure legend of Figure 2D