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Noninvasive Assessment of Cell Fate and Biology in Transplanted Mesenchymal Stem Cells

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Summary

Recently, molecular imaging has become a *conditio sine qua non* for cell-based regenerative medicine. Developments in molecular imaging techniques, such as reporter gene technology, have increasingly enabled the noninvasive assessment of the fate and biology of cells after cardiovascular applications. In this context, bioluminescence imaging is the most commonly used imaging modality in small animal models of preclinical studies. Here, we present a detailed protocol of a reporter gene imaging approach for monitoring the viability and biology of Mesenchymal Stem Cells transplanted in a mouse model of myocardial ischemia reperfusion injury.

Keywords

Stem Cells; Molecular Imaging; Reporter Gene; Bioluminescence; Luciferase; Tracking; Monitoring; Heart

1. Introduction

In the first decade of the 21st century we have been pelted with all sort of cell-based therapies in clinical cardiovascular practice[1–4] stimulated to a good degree by the promising results of some preclinical studies[5–8]. A wide range of adult stem cell types have been used, including Mesenchymal Stromal Cells (MSCs)[9,10], Bone Marrow-derived Mononuclear Cells (BMMNCs)[4] and Hematopoietic Stem Cells (HSCs)[11,12]. However, the bottom line has been a deflated enthusiasm due to insufficient retention and engraftment of the cells, which may limit their regenerative capacity[13–15]. Thus, the need to further investigate *in vivo* the kinetics of cell integration into the host tissue (both in preclinical and clinical settings) has become critical for the advancement of regenerative medicine. Until recently, assessment of the fate of transplanted stem cells has relied on traditional *ex vivo* assays and molecular techniques (i.e., histology, western blotting)[16]. Although these methods are easy to carry out for the molecular biologists and do not require special instruments, they involve invasive procedures and are limited in their capacity to monitor temporal changes in the living subject. This has inspired the development of novel research

strategies devoted to improve the delivery as well as the biology of transplanted stem cells. Within this context, the advent of molecular imaging represents a far-reaching milestone for the noninvasive monitoring of cell fate *in vivo*[17–20].

Direct (fluorophores[21], superparamagnetic iron oxide particles[22], radioisotopes[23,24]) and indirect (reporter genes) labelling[16,25] of stem cells may be used to assess their short and long-term distribution, along with their viability, proliferation and functional interaction with the host microenvironment.

When choosing the appropriate technique for any experimental design, it is critical to keep in mind the biological and biochemical properties as well as the sensitivity of each strategy. While direct labelling is used to monitor cell fate only shortly after transplantation, mainly due to the progressive dilution of the signal - as a consequence of cell division - and cell toxicity issues - which may vary depending on the agent and doses used, reporter gene imaging allows long-term assessment of cell fate with longitudinal and repetitive imaging[26,27]. Using this strategy, cells are engineered to over-express or produce *de novo* an enzyme, receptor or protein: when this protein interacts with an exogenously administered substrate, it results in a signal that can be used to distinguish not only implanted cells from endogenous cells with high specificity, but also modulations in intracellular functions[28]. However, it is critical to keep in mind that the manipulation of DNA sequences may alter the biology of transplanted stem cells. Furthermore, transgene expression is a very complex process, that involves many molecular steps: this may limit the amount of protein produced as well as the strength of the signal.

During the last ten years, using a reporter gene strategies designed to express the firefly luciferase (Fluc) or renilla luciferase (Rluc) gene, Bioluminescence Imaging (BLI) has been successfully adopted to monitor *in vivo* stem cell viability and engraftment[29,18,30] as well as to study transplanted stem cell biology and its interaction with the microenvironment[28] (Figure 1). Due to the lack of spatial resolution and tissue depth penetration, the use of BLI is restricted to small animal studies (rats and mice)[26]. However, this strategy can be adapted to clinically used imaging modalities, such as Positron Emission Tomography (PET) or Single-Photon Emission Computed Tomography (SPECT), using - for instance - thymidine kinase (TK) or human sodium-iodide symporter (hNIS) as reporter genes, respectively[19,27]. Therefore, BLI may be considered as a starting step in the development of novel imaging strategies in high-throughput preclinical studies.

Here, we describe in detail the materials and methods necessary to perform *in vivo* and *ex vivo* BLI of MSCs transplanted to the myocardium of a mouse model of ischemia/reperfusion injury.

2. Materials

Prepare and store all reagents at 4°C or on ice, unless stated otherwise. Prepare at the same time all the necessary controls. Carefully follow all waste disposal regulations for waste materials.

2.1 Cell Culture Components

1. Culture media: Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), 2 mM L-Glutamine, 1% Penicillin/Streptomycin.
2. Trypsin: trypsin-EDTA (0.25%).
3. Buffered solution: Phosphate Buffered Saline (PBS), pH 7.4.
4. Tissue culture flasks: T25, T75 or T175 flasks, 24-well plates.
5. CO₂ incubator for cell culture at 37°C and 5% CO₂.

2.2 Reporter Gene Technology

a. Transient Transfection (Plasmid Vectors)

1. Early-passage MSCs (*see* Note 1).
2. Effectene Transfection Reagent (Qiagen): Buffer EC (15 ml), Enhancer (1 mg/ml, 0.8 ml), Effectene Transfection Reagent (1 mg/ml, 1 ml). Store at 4°C.
3. Plasmid vectors: CMV-Fluc or CMV-Rluc for monitoring of cell viability and engraftment; X-Fluc (X = pathway-specific promoter) for monitoring of cellular functions; Null-Fluc (promoter-less vector) as a control vector.

b. Stable Infection (Retroviral Vectors)

1. Early-passage MSCs (*see* Note 1).
2. Retroviral vectors: CMV-Fluc or CMV-Rluc for monitoring of cell viability and engraftment; X-Fluc (X = pathway-specific promoter) for monitoring of cellular functions; Null-Fluc (promoter-less vector) as a control vector (*see* Note 2).
3. Polybrene Infection/Transfection Reagent. Store at –20°C (*see* Note 3).
4. Antibiotic selection: Geneticin Selective Antibiotic (G418 Sulfate, Gibco) could be used as selective antibiotic in the concentration range of 200 – 500 µg/ml for most mammalian cells in case the retroviral vector has the correspondent resistance gene (*see* Note 4).

2.3 Surgical Materials

1. Surgical tools (Fine Science): Delicate Forceps (Smooth/Angled 45°/9 cm) and Bonn-Strabismus Scissors (Straight/Blunt-Blunt/9 cm) for skin and muscle, Cohan-Vannas Spring Scissors (Curved/Sharp/5.7 cm/6 mm Cutting Edge) for ribs interspace, Chest retractor, Moria MC31 Iris Forceps (Serrated/Curved/10

¹Young cells are required for better efficiency of transfection.

²Recently, novel strategies have been developed for stable genomic integration to facilitate targeted editing of the genome by creating double-strand breaks in DNA at user-specified locations. These technologies use DNA-binding proteins such as Zinc Finger Nucleases (ZFN), Transcription activator-like effector nucleases (TALEN) or Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR)/Cas9 systems[31].

³The efficiency of retroviral infection is enhanced significantly in MSCs, by including polybrene during the infection.

⁴This step allows the positive selection of those cells that were efficiently infected by the retroviral vector. An alternative could be Puromycin (1–5 µg/ml).

cm) and Moria MC31/B Iris Forceps (Smooth/Curved/10 cm) for pericardium removal, Castroviejo Micro Needle Holders (Curved/9cm/with Lock) and two Suture Tying Forceps (10 cm) for left anterior descending coronary artery (LAD) ligation, Halsey Micro Needle Holder (Tungsten Carbide/Straight/Serrated/12.5 cm/with Lock) for suturing muscle and skin (*see Note 5*).

- 2 Glass beads sterilizer.
- 3 Heating pad for animal surgery and animal recovery (*see Note 6*).
- 4 Anesthetic: Isoflurane.
- 5 Lactated ringers for fluids replacement.
- 6 Analgesic: Buprenorphine.
- 7 Puralube Vet Ointment.
- 8 Pre-operative hair removal: depilatory cream
- 8 Endotracheal tube (20-gauge, ¼”).
- 9 Intubation panel.
- 10 Small animals laryngoscope.
- 11 Physio Suite with MouseVent Automatic Ventilator module and MouseSTAT Pulse Oximeter/Heart Rate module (Kent Scientific).
- 12 Surgical microscope (Stereo Microscope Leica M125, Leica Microsystems).
- 13 Povidone Iodine applicators and Ethanol pads.
- 14 Sutures: 9-0 Ethilon suture, 6-0 Silk suture and 6-0 Vicryl suture.
- 15 UltiCare Insulin Syringe U-100, 30-GAUGE × ½”.
- 16 Antibiotic treatment: Triple Antibiotic Ointment.
- 17 Cotton Swabs and gauzes.

2.4 Bioluminescence Imaging Technique

1. D-Luciferin Firefly, Potassium Salt: dissolve D-Luciferin in PBS without calcium and magnesium to a final concentration of 10 mg/ml. Filter sterilize the solution through a 0.2 µm filter. Store at –20°C (*see Note 7*).
2. 29-gauge insulin syringe.
3. Anesthetic: Isoflurane.
4. Cooled charge-coupled device camera (Xenogen)

⁵Autoclave all instruments before surgical session. Sterilize instruments in between animals using a glass beads sterilizer to avoid infections.

⁶Do not overheat the pad as it may cause burns to the animal.

⁷The reagent is light sensitive. Minimize exposure to light.

2.5 Luminometry Components

1. 5× Passive Lysis Buffer (Promega, store at -20°C): dilute in H_2O and keep on ice.
2. Tissue homogenizer (IKA RW20 digital)
3. Luciferase Assay Reagent (substrate of Fluc, Promega). Aliquot and store at -80°C (*see* Note 7).
4. Coelenterazine (Biotium, 1 mg): dissolve in 1 ml Ethanol to prepare a stock solution of $1\text{ }\mu\text{g}/\mu\text{l}$. Aliquot $13\text{ }\mu\text{l}$ per tube into black microtubes to shield from light. Store at -80°C (*see* Note 7). Before use, add 1.3 ml of PBS and vortex. Keep on ice.
5. Luminometer (Turner Designs 20/20)

3. Methods

3.1 Reporter Gene Labelling of Mesenchymal Stem Cells

For plasmid transfection:

1. Plate MSCs at a density of 1×10^4 cells per cm^2 18 hours before the transfection in T25 culture flasks.
2. Following manufacturer's instructions mix plasmid DNA (2.5–3 μg) with Buffer EC and Enhancer (The ratio of DNA to Enhancer is 1 μg DNA to 8 μl Enhancer). Vortex briefly and incubate for 5 min at room temperature (*see* Note 8).
3. Add Effectene Reagent (in our hands, for MSCs the ratio of DNA to Effectene is 1 μg DNA to 10 μl Effectene. This should be optimized for every new cell line and DNA construct used). Vortex and incubate for 10 min at room temperature.
4. Add culture media and mix by pipetting up and down several times.
5. Add the transfection cocktail onto the cells and incubate for 6 hours at 37°C .
6. Wash the cells once with PBS and add fresh culture media.
7. After 24h incubation at 37°C , prepare the cells for the injection.

For retroviral infection:

1. Plate MSCs at a density of 1×10^4 cells per cm^2 24 hours before the infection in 24-well plate.
2. Change media and infect MSCs with a multiplicity of infection (MOI) of 10 in the presence of Polybrene Infection/Transfection Reagent (8 $\mu\text{g}/\text{ml}$).
3. Incubate the cells overnight at 37°C .
4. Change the media every day. When cells reach 70% confluency, split them 1:5 into selection media (according to the resistance gene of the viral vector)

⁸The enhancer condenses the DNA molecules and the buffer provides optimal salt conditions for efficient DNA condensation.

5. Prepare the cells for the injection.

3.2 Surgical protocol for induction of ischemia/reperfusion injury and cell delivery

1. The day before the surgical procedure, shave the chest area of the mouse and depilate with depilatory cream. This will minimize the stress the day of the surgery.
2. Before starting the surgery, clean the working area, surgical tools and accessories. Turn on the instrument sterilizer, the heating pad so that the overlying surgery panel can warm up, and the heating plate with recovery cage on top of it.
3. Turn on the anesthesia system, with inflow to the induction chamber open, at 4% isoflurane and a flow rate of approximately 2 liters/min.
4. Place the mouse in the induction chamber. When the mouse becomes unconscious (within 1–2 min), weigh and mark it.
5. Open the flow to the ventilation cone. Place the mouse on the intubation panel and place cone near mouse's face to keep anesthetized during intubation.
6. Inject 1 ml of Lactated ringers subcutaneously in the back to keep the animal hydrated.
7. Inject buprenorphine (0.1 mg/kg body weight) subcutaneously in the shoulder.
8. Put a dab of ointment (Puralube Vet Ointment) directly on the eyes to prevent drying during surgery.
9. Intubation procedure: place the mouse in a supine position, fixed at its front teeth by a piece of suture, and tape its tail. With the laryngoscope visualize the trachea. Insert the endotracheal tube between the two vocal cords (*see* Note 9).
10. Connect the tube to the ventilation machine. Input the animal weight: the machine will calculate the optimal respiration rate and tidal volume. Reduce flow rate to 1 liters/min. Secure the ventilation tubing on the surgery panel with a tape.
11. Once the animal is breathing with the ventilator, keep isoflurane at 1.5–1.8%.
12. To position the animal suitably for the surgery, fix both forelimbs to the right side of the animal; fix the right hind leg in parallel with the tail and the left hind leg turned to the right side. The orientation from the surgeon's viewpoint is horizontal, tail right, head left.
13. Cover the shaved area with Povidone-Iodine using a cotton tip applicator. Clean with alcohol.

⁹The mouse should be deeply anesthetized to avoid pharyngeal reflex (gag reflex), which makes the intubation procedure more difficult. Insert the endotracheal tube only if the trachea and the vocal cords are clearly visible. Do not try to force entry, but change the position of the tube tip. The tube tip is angled and should face upward. Do not perform more than 3 attempts as the trachea may be damaged irreversibly.

14. Focus the microscope on the left side of the chest area.
15. With blunt scissors and forceps, make a 1.5 cm long skin incision over the left thorax area, just 1 mm below the first nipple visible from the left axilla.
16. Loosen the skin from the connective tissue/muscle layers by blunt dissection (prodding the scissors under the skin and opening them).
17. Make an incision of 1 cm in the muscles between the 4th and the 5th intercostal space. Using Cohan-Vannas Spring Scissors, perforate and cut the intercostal muscle layer 1 mm away from the sternum (*see* Note 10).
18. Retract the chest.
19. Zoom in on the heart. Open the pericardium using two pairs of small rounded forceps. Expose the upper and middle parts of the left ventricle with its partly overlying auricle (atrium) and blood vessels.
20. Localize the LAD (*see* Note 11) (Figure 2).
21. Ligate the LAD distal to the left auricle, 2 mm below the atrium edge (*see* Note 12): hold a 9-0 ethilon suture with a small needle holder and insert it shallowly into the myocardium, enclosing the LAD and approximately four times its diameter of surrounding myocardium (*see* Note 13). Tightly compress the LAD in its middle third by a thin piece of plastic tubing secured by the suture. Confirm ischemia by the appearance of pallor over the anteroapical LV myocardium, along with hypokinesia/akinesia (Figure 2) (*see* Note 14).
22. After 35 min of ischemia, cut the suture and remove the tubing to allow reperfusion (Figure 2).
23. After about 10 min of reperfusion, using a 30-gauge insulin syringe, inject cells into the myocardial wall. Inject around 4×10^5 cells in 30 μ l of PBS (2 spots of 15 μ l each) in the peri-infarct area (Figure 2).
24. Remove the retractor.
25. Decrease anesthesia to 1–1.2% isoflurane.
26. Insert the chest tube (24-gauge) through the skin, the muscles and between the 3rd and the 4th intercostal space.
27. Close the thoracic wall in three layers: first the ribs with individual 6-0 silk sutures, then the muscles with running 6-0 vicryl suture and, finally, close the skin with running 6-0 vicryl sutures.

¹⁰Choose the intercostal space for thoracotomy based on curvature, after first rib that is less curved than the rib above. Be careful avoiding contact with the lungs.

¹¹This requires some expertise. If necessary, carefully lift the atrium. The left anterior descending coronary artery is bright red to orange/pink, as opposed to the veins, which are dark red, is pulsatile and runs from below the left auricle to the apex.

¹²If the heart rate is too high, temporarily increase the anesthesia to 2.5% isoflurane.

¹³Avoid entering the LV cavity, but go deep enough to see the LAD pulsate over the needle.

¹⁴For a murine model of myocardial infarction perform a permanent ligation of the LAD: close the suture using a double surgeon's knot, fixed with two extra half hitches. The heart region below the knot should become pale in few seconds. The knot is not released.

28. Aspirate air from the chest with a 1 ml tuberculin syringe and remove the chest tube.
29. Clean the incision area. Apply the triple antibiotic ointment to the incision area.
30. Turn off the isoflurane and allow the animal to recover increasing the flow rate (oxygen only) to 2 liters/min. The animal should be able to recover in less than 1 min (*see* Note 15).
31. Extubate the animal as soon as it begins fighting the tube and breathing on its own.
32. Place the animal into a clean cage on the heating pad and monitor frequently for any sign of discomfort.
33. Administer buprenorphine (0.1 mg/kg body weight) subcutaneously every 8–12 hours for at least 3 days post surgery.

3.3 In Vivo Bioluminescence Imaging of Transplanted Mesenchymal Stem Cells

- 1 Shave the chest area of the mouse and depilate with depilatory cream (*see* Note 16).
- 2 Turn on the anesthesia system, with inflow to the induction chamber open, at 4% isoflurane and a flow rate of approximately 2 liters/min.
- 4 Place the mouse in the induction chamber.
- 5 When the mouse becomes unconscious, inject intraperitoneally 100 µl of the reporter substrate D-Luciferin Firefly, using a 29-gauge insulin syringe.
- 6 Place the animal in the cooled charge-coupled device camera (Figure 3) in a supine position. During imaging procedure keep isoflurane at 1–1.5% (*see* Note 17).
- 7 Image animal for 20 min using one-minute high sensitivity, acquisition scans (Figure 3).
- 8 Quantify bioluminescence as total radiance (photons/s/cm²/sr) over the area of the heart, using a region of interest kept at constant size for all scans (*see* Note 18).
- 9 After the imaging session is complete, let the animal recover in a cage placed on a warm pad (*see* Note 19).

¹⁵The surgical procedure should last no more than 75 min.

¹⁶The presence of fur may decrease the amount of detectable signal.

¹⁷High doses of isoflurane may affect cellular metabolism in the living subject. The interaction between substrate (D-luciferin) and reporter protein (Firefly Luciferase) requires oxygen, magnesium and ATP, all co-factors that are available only in viable and metabolically active cells. Therefore, it is critical to avoid deep sedation.

¹⁸Due to its absorption/emission properties, the Renilla Luciferase signal cannot be reliably detected by *in vivo* BLI. Thus, the use of *ex vivo* luminometry is recommended.

¹⁹The imaging procedure should not cause any kind of discomfort to the animal.

3.4 Ex Vivo Luminometry of Heart Homogenates

1. Sacrifice the animal with an overdose of CO₂.
2. Immediately harvest and weight the organs.
3. Wash twice with PBS.
4. Add lysis buffer (4 ml/g harvested tissue).
5. Homogenize for 30 sec.
6. Centrifuge at 14,000 rpm for 15 min at 4° C.
7. Assay 20 µl supernatant with 100 µl substrate (Luciferase Assay Reagent or Coelenterazine) on luminometer. Be sure to vortex each sample before assaying.

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Figure 1. Reporter gene technology for bioluminescence imaging

D-Luciferin, a light-emitting compound, is the substrate of the firefly luciferase reporter gene. In presence of oxygen, adenosine triphosphate (ATP) and magnesium, the enzyme luciferase catalyzes the oxidation of D-Luciferin to form oxyluciferin in an electronically excited state. The reaction releases a photon of light as oxyluciferin returns to the ground state. These photons can be detected, collected, and quantified by a CCD camera.

Figure 2. Ischemia/reperfusion injury and cell delivery

Schematic representation of the surgical procedure for the induction of an ischemia/reperfusion injury in mice followed by intra-myocardial transplantation of Mesenchymal Stem Cells. LA: left atrium, RA: right atrium, LV: left ventricle, LAD: left anterior descending coronary artery, MSCs: Mesenchymal Stem Cells.