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Video Article

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Abstract

Duchenne muscular dystrophy (DMD) is a severe genetic neuromuscular disorder that affects 1 in 3,500 boys, and is characterized by progressive muscle degeneration[1, 2]. In patients, the ability of resident muscle satellite cells (SCs) to regenerate damaged myofibers becomes increasingly inefficient[4]. Therefore, transplantation of muscle progenitor cells (MPCs)/myoblasts from healthy subjects is a promising therapeutic approach to DMD. A major limitation to the use of stem cell therapy, however, is a lack of reliable imaging technologies for long-term monitoring of implanted cells, and for evaluating its effectiveness. Here, we describe a non-invasive, real-time approach to evaluate the success of myoblast transplantation. This method takes advantage of a unified fusion reporter gene composed of genes (firefly luciferase [*fluc*], monomeric red fluorescent protein [*mrfp*] and sr39 thymidine kinase [*sr39tk*]) whose expression can be imaged with different imaging modalities[9, 10]. A variety of imaging modalities, including positron emission tomography (PET), single-photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), optical imaging, and high frequency 3D-ultrasound are now available, each with unique advantages and limitations[11]. Bioluminescence imaging (BLI) studies, for example, have the advantage of being relatively low cost and high-throughput. It is for this reason that, in this study, we make use of the firefly luciferase (*fluc*) reporter gene sequence contained within the fusion gene and bioluminescence imaging (BLI) for the short-term localization of viable C2C12 myoblasts following implantation into a mouse model of DMD (muscular dystrophy on the X chromosome [mdx] mouse)[12-14]. Importantly, BLI provides us with a means to examine the kinetics of labeled MPCs post-implantation, and will be useful to track cells repeatedly over time and following migration. Our reporter gene approach further allows us to merge multiple imaging modalities in a single living subject; given the tomographic nature, fine spatial resolution and ability to scale up to larger animals and humans[10,11], PET will form the basis of future work that we suggest may facilitate rapid translation of methods developed in cells to preclinical models and to clinical applications.

Video Link

The video component of this article can be found at http://www.jove.com/video/50119/

Protocol

1. Maintenance and Propagation of C2C12 Myoblasts

1. Plate C2C12 myoblasts in a 75 cm^2 flask and maintain cells in high glucose Dulbecco's Modified Eagle's Serum (HG-DMEM) supplemented with fetal bovine serum (FBS) to a final concentration of 10%. Do not allow cells to become confluent at any time as this will deplete the myoblastic population. Medium should be changed every other day. **Note:** always warm medium to 37 °C in a water bath prior to use.
2. When myoblasts become approximately 80% confluent, passage cells to a new flask. Aspirate culture medium. Wash cells with 4-5 ml Hanks Balanced Salt Solution (HBSS) to remove all traces of culture medium, which contains trypsin-inhibiting serum. Briefly rinse the cell layer with 2-3 ml 0.25% (w/v) trypsin-EDTA solution to dissociate the adherent myoblasts from the flask. Aspirate trypsin and place flask in incubator at 37 °C for 5 min.
3. During this incubation, prepare a new flask with 9 ml of HG-DMEM/10% FBS medium. After trypsinization, add 10 ml of complete medium to cells and pipette 4-5 times to ensure collection of all cells in the medium. Add 1 ml of cell suspension to the new flask and incubate in 5% CO_2 at 37 °C.

2. C2C12 Cell Transfection

1. Once cells have reached 50% confluency, transfect according to manufacturer's instructions from Invitrogen. Briefly, combine 90 µl Lipofectamine 2000 reagent with 4.5 ml OPTI-MEM medium. In another tube, combine 36 µg CMV-trifusion reporter gene DNA with 4.5 ml OPTI-MEM. Flick each tube to combine and wait 5 min. Combine contents of both tubes, mix gently and incubate at room temperature for exactly thirty minutes. **Note:** A second flask should also be set up for untransfected cells that only receive Lipofectamine and OPTI-MEM to serve as a negative control.
2. Remove medium from C2C12 cells and add 15.5 ml of fresh HG-DMEM/10%FBS medium. Add transfection medium to bring total volume to 20 ml.
3. Allow cells to transfect overnight for at least 20 hr at 37 °C.
4. Next day, aspirate transfection medium and add 10 ml HG-DMEM/10%FBS.
5. View cells under an inverted fluorescent microscope. Capture both bright field and red fluorescence images (using a TRITC filter cube; mrfp ex/em: 584/607 nm). Count the number of RFP-expressing cells viewed under fluorescence divided by total number of cells viewed under bright field for multiple fields of view to generate transfection efficiency.

3. Assessment of Cell Survivability/MTT Assay

1. One day prior to transfection, plate $1x10^5$ C2C12 cells into each well in a 24-well plate. Cells should be plated in 500 µl volumes in HG-DMEM/10%FBS .
2. Next day, transfect myoblasts overnight as previously described. Follow manufacturer's suggestions for transfection reagent volumes for a 24-well plate. Incubate a set of wells with Lipofectamine only (no DNA) as a control. View cells under fluorescence to ensure that proper transfection has occurred.
3. Remove transfection medium and incubate myoblasts with 5 mg/ml thiazolyl blue tetrazolium bromide (MTT) in HG-DMEM/10% FBS. Add D-luciferin to eight of the transfected wells, and incubate at 37 °C for four hours.
4. Remove medium from wells. Solubilize blue formazan crystals by adding 180 µl isopropanol to each well. Shake at 37 °C for 15 min.
5. Avoiding any precipitate, pipette solution into a 96-well plate and read absorbance at 575 nm.

4. Preparation of Myoblasts for Transplant

1. Remove medium, wash myoblasts with HBSS, and trypsinize cells as outlined in 1.1.
2. Re-suspend cells in 4 ml of complete medium.
3. Using a hemocytometer, count cells to generate volumes containing 10^6 myoblasts. Pipette volume into sterile 1.5 ml microtubes. With a transfection efficiency of ~10%, these values indicate that 100,000 luciferase-expressing cells are detectable on the GE ExploreOptix scanner after transplant.
4. Attain a final injection volume of 15 µl, containing 10^6 C2C12 cells. If necessary, centrifuge microtubes at 2,000 rpm for one minute. Carefully aspirate supernatant with a pipette. Re-suspend cells in 15 µl of HG-DMEM lacking FBS.

5. Cell Implantation

1. Anesthetize mouse with 2% isoflurane/2%O_2. Pluck hair from the dorsal hind limb area. Maintain anesthesia at 1.5%isoflurane/2%O_2.
2. Ensure C2C12 myoblasts are well suspended. With the mouse in a prone position, extend the hind limb and use an insulin syringe to inject cells directly into the lateral head of the gastrocnemius muscle at a 30 ° angle. Inject transfected myoblasts into the right hind limb and untransfected myoblasts into the contralateral (left) hind limb.
3. Perform a baseline bioluminescence scan: Quickly transfer mouse to stage in the optical scanner, laying the mouse in a prone position. Hook up the anesthetic line. To ensure the mouse remains anesthetized, a second person should be present to hook up the anesthetic line while the other places the animal in the scanner.
4. Gently extend the hind limb so the area of injection is visible. Tape hind limbs in place with a gentle adhesive such as medical tape.
5. Close the chamber and ensure that no light can access the interior of the scanner, as this will increase the background signal detected. The scanner should be set to parameters included in your manufacturer's instructions for bioluminescence imaging. Specifically, ensure "no laser" is selected.
6. Draw a region of interest (ROI) around the plucked area and start scan.

6. Injection of Fluc Substrate, D-luciferin, into Mdx Mouse

1. While the mouse is anesthetized intraperitoneally inject 150 mg/kg of firefly luciferase substrate, D-luciferin (from a 40 mg/ml stock solution, made up according to manufacturer's instructions).
2. Recover mouse and allow a 15 min uptake period before preparing mouse for the next scan.

7. BLI to Target Luc-expressing MPCs Following Implant into Mouse Models of DMD

1. After the uptake period, anesthetize mouse again, as described in 5.1.
2. Transfer the mouse back to the optical scanner and perform another bioluminescence scan in the same manner as the background scan.
3. Although a 20 min uptake period should provide maximal signal intensity, a subsequent scan may be performed.

4. Upon completion of image acquisition, sacrifice the mouse according to guidelines set by your Institutional Animal Ethics Committee and the Canadian Council on Animal Care (CCAC). Isolate hind limb muscle and place immediately in 10% formalin to fix for paraffin embedment. Perform immunohistochemistry staining for luciferase to confirm intramuscular injection of myoblasts.

Representative Results

Upon 50-60% confluency, C2C12 myoblasts were transiently transfected with the above-mentioned fusion reporter gene construct composed of firefly luciferase [*fluc*], monomeric red fluorescent protein [*mrfp*] and sr39 thymidine kinase [*sr39tk*](**Figure 1A**). Transfection efficiency was calculated via fluorescence microscopy (**Figures 1B,C**), making use of the *mrfp* sequence in our reporter construct. Cell survivability was not affected by labeling with the BLI substrate, D-luciferin (**Figure 1D**). Following transfection, approximately 100,000 mrfp-expressing myoblasts were implanted intramuscularly into the gastrocnemius muscle of mdx mice (determined previously, manuscript submitted); 100,000 untransfected cells were similarly implanted into the contralateral hind limb as a control. Immediately following cell implantation, mice were injected intraperitoneally (IP) with 150 mg/kg D-luciferin. Following an uptake period of ~20 min, mice were imaged on a small animal optical scanner (GE ExplorOptix black box that is equipped for live animal bioluminescence and fluorescence). As previously demonstrated, both *in vitro* and *post-implantation* (manuscript submitted) uptake of D-luciferin was specific to fluc-expressing myoblasts, with no detectable bioluminescence in untransfected cells (**Figure 2**). Immunohistochemistry confirmed intramuscular transplantation of myoblasts (**Figure 3**)

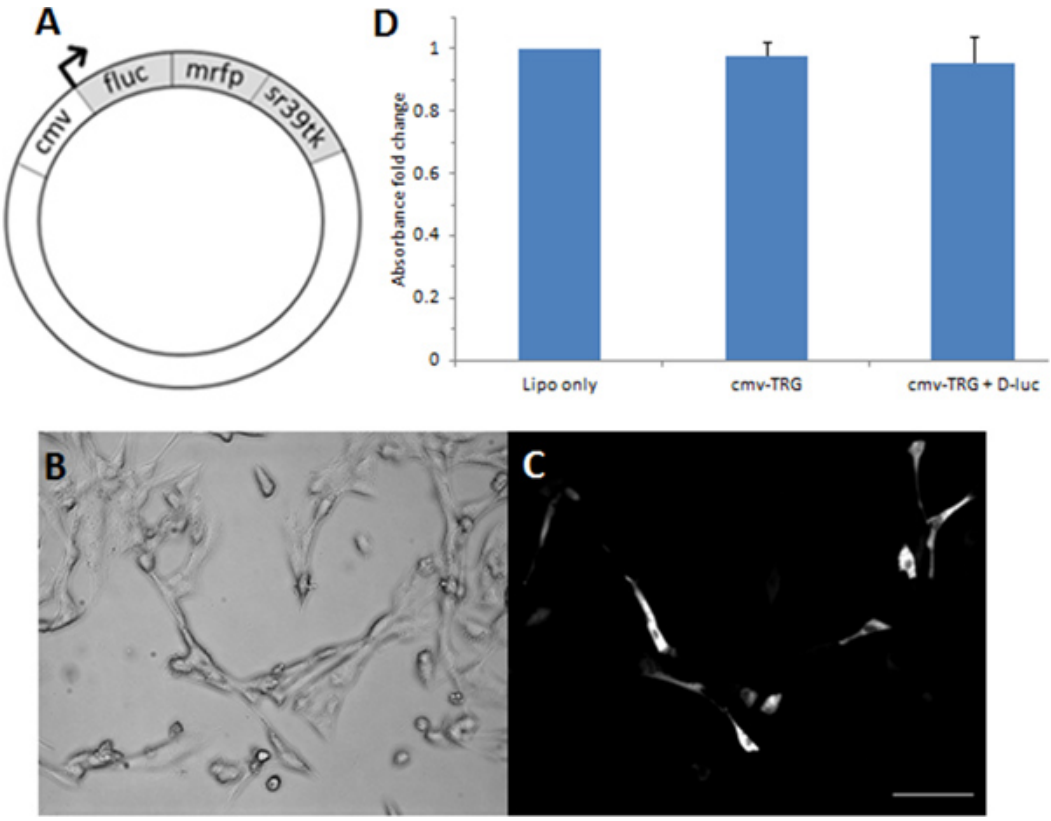

Figure 1. Schematic of CMV-trifusion reporter construct **(A)**; brightfield/fluorescence images of C2C12 myoblasts transfected with the trifusion reporter plasmid **(B,C)**; MTT assay to assess C2C12 cell survivability following labeling with BLI substrate, D-luciferin **(D)**.

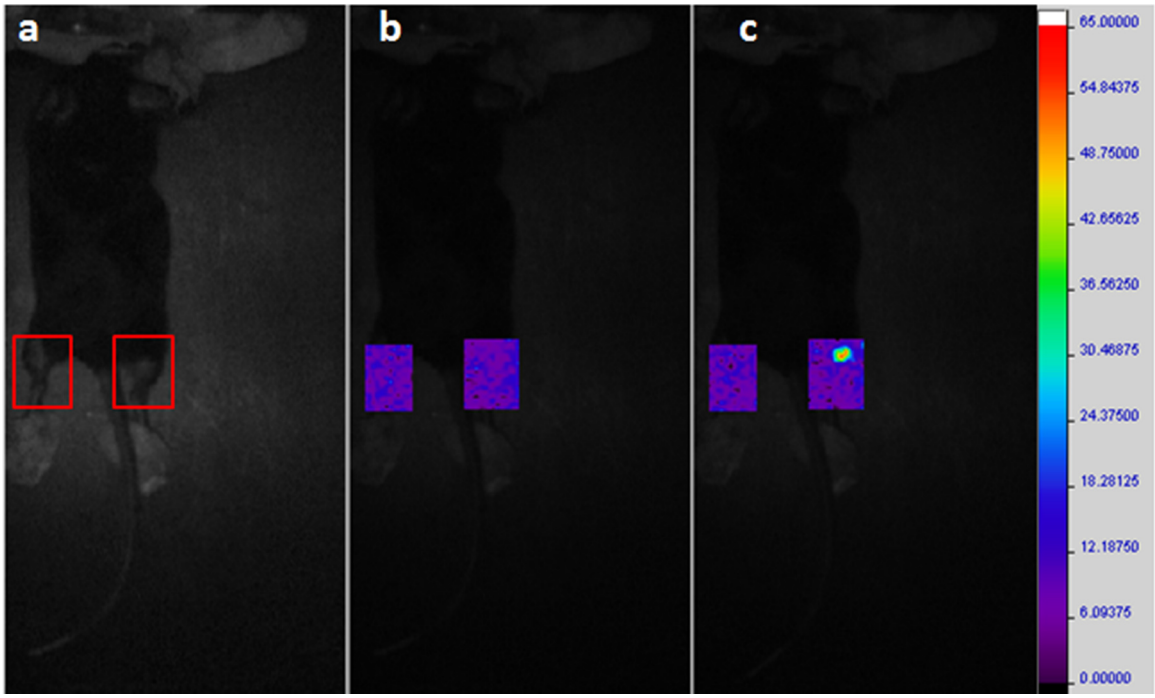

Figure 2. Bioluminescence imaging (BLI). A region of interest (ROI) is drawn to enclose the plucked hind limb area where myoblasts are injected **(A)**. Bioluminescence is not detected during a background scan **(B)**. At 23 min after injection of D-luciferin, a clear signal is detected from the right hind limb where luciferase-expressing myoblasts are injected. No bioluminescence is detected in the contralateral hind limb injected with untransfected myoblasts **(C)**. Click here to view larger figure.

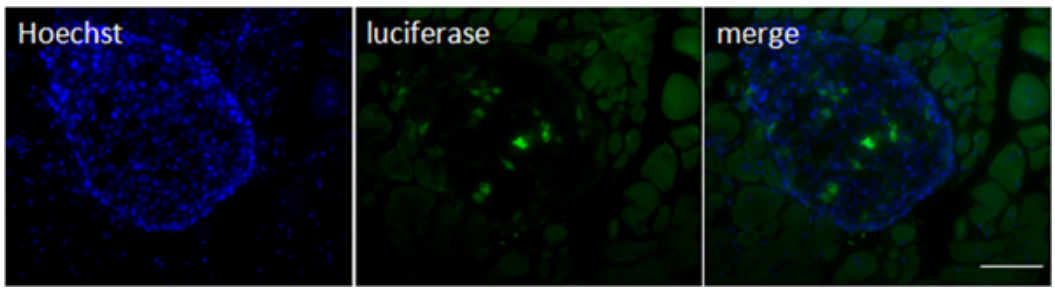

Figure 3. IHC using a firefly luciferase antibody confirms intramuscular implantation of transfected C2C12 cells.

Discussion

In this study, we have described a fast and reliable molecular imaging, reporter gene approach to non-invasively target myoblasts/MPCs following transplantation. While this study demonstrates the *short-term localization* of transplanted MPCs via bioluminescense imaging (BLI), the *manner* in which cells are targeted can, in fact, be easily applied to a longitudinal assessment of cell engraftment, through the implantation of cells that stably express the reporter gene. To this end, our group has generated transgenic mouse lines that harbor the unified reporter gene. Only cells expressing the reporter gene oxidize D-luciferin to produce photons for visualization using BLI. Since oxidation of D-luciferin is dependent on gene expression, this is a powerful technology with which to non-invasively image *viable* transplanted cells. Muscle tissue harvested from these transgenic mice and satellite cells (SCs) isolated via FACS can indeed be targeted following implantation into mdx mice. Additionally, we can track their differentiation status through the use of a muscle-specific promoter, further heightening the usefulness and importance of molecular imaging technologies, such as presented herein, to the field of DMD research (manuscript submitted). In addition to its rapidity and low-cost, BLI is non-toxic, making it an attractive choice for frequent imaging of small animals. This feature, as well as its high specificity, will be invaluable in refining myoblast replacement therapies in pre-clinical disease models of Duchenne muscular dystrophy before advancing to clinically-applicable studies involving technologies such as PET.

Disclosures

Authors have nothing to disclose.

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