Video Article

Tracking Dynamics of Muscle Engraftment in Small Animals by *In Vivo* Fluorescent Imaging

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Abstract

Muscular dystrophies are a group of degenerative muscle diseases characterized by progressive loss of contractile muscle cells. Currently, there is no curative treatment available. Recent advances in stem cell biology have generated new hopes for the development of effective cell based therapies to treat these diseases. Transplantation of various types of stem cells labeled with fluorescent proteins into muscles of dystrophic animal models has been used broadly in the field. A non-invasive technique with the capability to track the transplanted cell fate longitudinally can further our ability to evaluate muscle engraftment by transplanted cells more accurately and efficiently.

In the present study, we demonstrate that *in vivo* fluorescence imaging is a sensitive and reliable method for tracking transplanted GFP (Green Fluorescent Protein)-labeled cells in mouse skeletal muscles. Despite the concern about background due to the use of an external light necessary for excitation of fluorescent protein, we found that by using either nude mouse or eliminating hair with hair removal reagents much of this problem is eliminated. Using a CCD camera, the fluorescent signal can be detected in the tibialis anterior (TA) muscle after injection of 5 x 10^5 cells from either GFP transgenic mice or eGFP transduced myoblast culture. For more superficial muscles such as the extensor digitorum longus (EDL), injection of fewer cells produces a detectable signal. Signal intensity can be measured and quantified as the number of emitted photons per second in a region of interest (ROI). Since the acquired images show clear boundaries demarcating the engrafted area, the size of the ROI can be measured as well. If the legs are positioned consistently every time, the changes in total number of photons per second per muscle and the size of the ROI reflect the changes in the number of engrafted cells and the size of the engrafted area. Therefore the changes in the same muscle over time are quantifiable. *In vivo* fluorescent imaging technique has been used primarily to track the growth of tumorogenic cells, our study shows that it is a powerful tool that enables us to track the fate of transplanted stem cells.

Video Link

The video component of this article can be found at https://www.jove.com/video/1388/

Protocol

Cell preparation

1. Satellite cell isolation
    1. Under anesthetic, the limb muscles of GFP transgenic mice (C57BL/ Ka-βactin-EGFP) are removed. After cutting into small pieces, satellite cells are enzymatically dissociated by incubating the minced tissue with 2% dispase collagenase solution for 1 hour at 37°C.
    2. Neutralize the enzyme digestion with growth media containing Ham F-10 and 20% FBS, dissociate the muscle by repeated triturating with a Pasteur pipette. The cells are filtered through a cell strainer (ø70-100μm) and then plated on a non-coated dish for 1 hour at 37°C.
    3. Gently remove the supernatant and plate them on a collagen coated dish. Repeat this step 1 hour later to purify the myoblasts. (pre-plate)
    4. Change the media and plate the cells in time. The final purified myoblasts are cultured in medium containing Ham F-10, 20% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (growth medium) at 37°C with 5% CO2, 95% air.
2. Culture and expand the primary myoblasts in growth medium with addition of basic FGF of 10 ng/ml.
3. For all the *in vivo* experiments, we inject 5x10^5 cells per TA muscle.
4. When harvesting the myoblasts, we detach the cells by using 0.25% trypsin/EDTA and wash them with PBS twice. The cell numbers are counted with a hemocytometer and the cells are resuspended in HBSS at a concentration of 5x10^4 cells/μl. The concentrated cells in HBSS are placed on ice and injected within 1 hour after collection.

In Vivo Imaging

1. Male mdx mice at the age of 4-6 weeks are purchased from Jackson lab and maintained at an animal housing facility.
2. After about one week of accommodation to the environment, the mice are ready to be implanted with the cultured cells.
3. Before cell transplantation, the mouse is anesthetized by an intraperitoneal (i.p.) injection of the mixture of Ketamine (100 mg/kg) and Xylazine (10 mg/kg). Then the hair around the TA muscle area is removed by applying Nair to the leg and waiting for 30 seconds to wipe it clean with distilled water.
4. While the mouse is still under anesthesia, $5x10^5$ cells in 10µl HBSS are slowly injected into each TA muscle at 3 points using a 30 gauge needle.
5. After step 4, the mouse can be regularly imaged by *in vivo* fluorescence imaging. The fluorescence imaging station NightOwl LB981 (Berthold Technologies, Inc.), equipped with a high sensitive charge-coupled camera, is used to obtain images from the hinder legs of the mice.
6. On the day of *in vivo* imaging, we first check the hair regrowth on the TA muscle. If necessary, we re-apply Nair to remove the hair as described above, after anesthetizing the mouse with the mixture of Ketamine and Xylazine.
7. While the mouse is under anesthesia, we place the mouse in a prone position on a piece of black non-fluorescent paper (black Artagain paper from Strathmore). To fully expose the TA muscle for better imaging, we tape the hind paws to the paper.
8. We place the mouse in the imaging chamber and position the hind leg to be imaged at the center of field of view of the camera. We first switch out the emission filter to take a traditional gray-scale photographic picture of the leg with an exposure time of 5 seconds. Readjust the position of the mouse and focus of the camera if necessary. The imaging parameters including exposure time (1,250 ms), specimen height (0.9 cm) etc. are kept constant in the experimental process.
9. We then switch in the appropriate emission filter for the green fluorescent protein wavelength. We take fluorescence image with an exposure time of 1,250 ms and a binning number of 1. After imaging, the mouse is removed from the imaging chamber to a cage to wait recovery.
10. For analysis, we draw a region of interest (ROI) over the fluorescent signals to measure their intensity in terms of photons per second. We also measure the size of the ROI in terms of number of pixels as another quantification value. The imaging experiments can be repeated every week or more frequently over a few months, if needed, to acquire longitudinal data.

At the end of experiments, the mouse is euthanized and the TA muscle is harvested for histology analysis.

Discussion

We set up a reliable and stable imaging platform for tracking the fluorescence labeled transplanted cells in host skeletal muscle in this study. GFP-labeled myoblasts from GFP transgenic mouse stands for a type of adult stem cells that will be candidates for cell therapy in the future. A constant manipulation including cell number, cell injection, clear background, imaging position and machine parameter is important for gaining high quality image and especially for quantitative analysis.

Fluorescence imaging has many applications in biomedical research as it is non-invasive, easy to use, and has a high throughput. In addition fluorescence imaging can provide a quantitative measurement based on the photon counts, though due to the scattering, attenuation and absorption, light cannot penetrate a large distance in tissue, which is a shortcoming of the technique. Efforts have been underway to use red or near-infrared reporter to increase the penetration depth of light.

Another advantage of fluorescence imaging is that it is translatable to clinics if the specific fluorescent reporter is approved for human use. Compared with MRI, CT, and PET, fluorescence imaging has high flexibility as it does not require expensive hardware and imaging agents. One example is fluorescence-based endoscope and micro-endoscope that have been used in clinics. By combining fluorescence imaging with other modalities, we expect to achieve the capability to acquire both anatomic and functional information about the underlying biological processes.

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