Video Article

In vitro and in vivo Bioluminescence Reporter Gene Imaging of Human Embryonic Stem Cells

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Abstract

The discovery of human embryonic stem cells (hESCs) has dramatically increased the tools available to medical scientists interested in regenerative medicine. However, direct injection of hESCs, and cells differentiated from hESCs, into living organisms has thus far been hampered by significant cell death, teratoma formation, and host immune rejection. Understanding the in vivo hESC behavior after transplantation requires novel imaging techniques to longitudinally monitor hESC localization, proliferation, and viability. Molecular imaging has given investigators a high-throughput, inexpensive, and sensitive means for tracking in vivo cell proliferation over days, weeks, and even months. This advancement has significantly increased the understanding of the spatio-temporal kinetics of hESC engraftment, proliferation, and teratoma-formation in living subjects.

A major advance in molecular imaging has been the extension of noninvasive reporter gene assays from molecular and cellular biology into in vivo multi-modality imaging platforms. These reporter genes, under control of engineered promoters and enhancers that take advantage of the host cell s transcriptional machinery, are introduced into cells using a variety of vector and non-vector methods. Once in the cell, reporter genes can be transcribed either constitutively or only under specific biological or cellular conditions, depending on the type of promoter used. Transcription and translation of reporter genes into bioactive proteins is then detected with sensitive, noninvasive instrumentation (e.g., CCD cameras) using signal-generating probes such as D-luciferin.

To avoid the need for excitatory light to track stem cells in vivo as is required for fluorescence imaging, bioluminescence reporter gene imaging systems require only an exogenously administered probe to induce light emission. Firefly luciferase, derived from the firefly Photinus pyralis, encodes an enzyme that catalyzes D-luciferin to the optically active metabolite, oxyluciferin. Optical activity can then be monitored with an external CCD camera. Stably transduced cells that carry the reporter construct within their chromosomal DNA will pass the reporter construct DNA to daughter cells, allowing for longitudinal monitoring of hESC survival and proliferation in vivo. Furthermore, because expression of the reporter gene product is required for signal generation, only viable parent and daughter cells will create bioluminescence signal; apoptotic or dead cells will not.

In this video, the specific materials and methods needed for tracking stem cell proliferation and teratoma formation with bioluminescence imaging will be described.

Protocol

1. **Construction of Double Fusion Reporter Gene**
    1. In order to perform bioluminescence imaging of human embryonic stem cells, you first need to obtain cells that stably express a luciferase reporter gene such as firefly luciferase driven by a constitutive promoter like Ubiquitin or EF1a.
    2. The focus of this protocol is on reporter gene applications, so detailed procedures are not provided here. However, our lab's general strategy is to use a double fusion construct vector that contains firefly luciferase (fluc) and enhanced green fluorescent protein (egfp) separated by a spacer within pCDNA 3.1+.
    3. Briefly, our double fusion gene was originally located downstream of the cytomegalovirus promoter in pCDNA 3.1(+), so the 3.3 kbp fragment was excised using NdeI and NotI digestion and blunt-end ligated into the multiple cloning site of a self-inactivating (SIN) lentiviral vector, under control of a Ubiquitin promoter.

2. **Lentiviral Transduction**
    1. hESCs can be transduced 3-5 days after passage at a multiplicity of infection (MOI) of 10 (viral titer of approximately 107 incubated with 106 cells).
    2. The thawed viral stock can be added directly to fresh hESC media.
    3. Refresh the media 12 and 24 hours later.
    4. After 48 h, transduction efficiency can by qualitatively assessed using fluorescence microscopy. Subsequently, FACS can be used to isolate infected cells.

3. **Culturing hESCs and Introduction to Imaging Set-up**
    1. Culture your luciferase positive hESCs in feeder-free conditions. We typically follow the standard WiCell protocol and use 6-well plates coated in growth factor reduced Matrigel, using either conditioned media or commercial, feeder-free media.
    2. To detect the luciferase positive cells, you will need to have the reporter probe D-luciferin already prepared. To do this, aliquot luciferin in 1-1.5 mL preparations at a final concentration of 45 mg/mL. Keep the aliquots at -20°C when not in use and avoid exposure to light by covering with paper towel when not in storage.
    3. In order to visualize the luciferase positive cells, we use IVIS 50 and IVIS 200 Imaging Systems (Xenogen Corporation, Alameda, CA). This latter system includes an integrated isoflourane apparatus and induction chamber for temporary anesthesia of small animals.

4. **In vitro bioluminescence imaging of hESCs**
    1. For in vitro bioluminescence imaging, it is important to maintain sterile conditions. Therefore, the imaging system should be sterile, preferably in a cell culture room.

2. Before imaging, remove the cell media and add just enough PBS to cover the cells. For example, add 1mL PBS to each well of a 6-well plate containing your hESC cultures.
3. The ratio of D-Luciferin to PBS should be 1:100, so add 10 µL of thawed D-Luciferin to each well of the 6-well plate.
4. Wait one minute, then take an image using an exposure time of 1 second. If signal is weak, increase the exposure interval and try again.
5. The bioluminescent signal reflects cell number, so quantitation assays can be performed that correlate signal with different cell numbers.

5. **Preparation of mouse and injecting cells for in vivo imaging**
   1. Reporter gene imaging provides a high-throughput, inexpensive and sensitive method for tracking cells after transplantation over days, weeks, and even months. This is especially important since transplanted stem cells frequently form teratomas, are rejected by the host's immune system, or simply die.
   2. The simplest method for imaging teratoma formation in vivo is to inject hESCs that express the firefly luciferase reporter gene into the backs of SCID mice and acquire bioluminescent images with the IVIS system.
   3. When you're ready to transplant the cells, use dispase or collagenase to loosen the cells, wash several times, and suspend them in a 1:1 mixture of growth factor reduced-matrigel and DMEM. Typically we first mix the cells with chilled DMEM and then add in the matrigel. For each subcutaneous injection site, we inject 200,000 cells suspended in a 50-100 ul volume of the matrigel/DMEM mixture. The cell number can be adjusted depending on your application. Remember to keep everything on ice prior to injection.
   4. Once the cells are ready, anesthetize the mouse by placing it in the induction box with isoflurane flow turned on. After 1-5 minutes, the mouse is asleep and can be placed on the operating table with continuous isoflurane.
   5. Because fur naturally auto-fluoresces and may obscure the bioluminescent image, we will need to remove the hair from the back side of the mouse. The easiest way to do this is to use an electric shaver, but you can also use hair-removal gels. Wipe with alcohol to sterilize the skin afterwards
   6. Next, draw up your cell suspension in a syringe. 23 to 27 gauge needles work best since they will not clog up with cells. If the needle is too large (<23 gauge) the needle tract may leave a large hole through which cells can leak back out.
   7. Inject the cells under the skin into the mouse's back. Because the skin is quite loose, use your thumb and forefinger to pinch and stretch out the area you want to inject. Inject just under the skin, taking care not to puncture too deeply. Also, try to keep the needle from sliding out of the injection site while depressing the plunger: this will prevent creating a hole in the skin through which the cells can leak if you try to inject again.
   8. After the cells are injected, wait a few hours to allow the mouse to wake up and run around before re-anesthetizing for bioluminescence imaging. Doing so avoids isoflurane toxicity.

6. **In vivo bioluminescence imaging of transplanted cells**
   1. For whole animal bioluminescence imaging, we do an intraperitoneal injection of D-luciferin at 375 mg/kg body weight. Weigh the animal to calculate the dosage prior to injection.
   2. Inject the D-luciferin in the peritoneum, just off the midline. Be careful to not go too deep or you can damage internal organs. If the mouse begins to wake up, place it back in the knockout box and wait a minute or two. For mice, the maximum bioluminescence usually occurs 15-40 mins after injection.
   3. Remember to place matte black paper in the imaging box to help absorb any light not emitted by the hESCs. Place the mouse (backside up) in the imaging chamber with isoflurane, on top of the black paper. Start with an exposure time of 10 seconds. If the signal is saturated, try decreasing the exposure time; if too weak, increase the exposure.
   4. Once the signal exposure time has been optimized for your mouse, begin taking images every minute until the signal reaches a maximum. This is best done by using the imaging software to select "regions of interest (ROI)" that cover your injection sites. By monitoring the signal intensity at each ROI you can easily determine when the signal begins to decrease, indicating that the maximum signal intensity has been reached. The maximum signal should be used as your final data.
   5. When you are satisfied with your images, remove the mouse from isoflurane and allow it to wake up in its cage. Usually, the mouse should wake up within 15 minutes.
   6. Bioluminescence reporter gene imaging can be repeated daily, weekly and monthly for as long as the hESCs survive in the animal. Signal from a developing teratoma will increase exponentially over time, typically on the order of weeks, as the hESCs begin to proliferate.
   7. After the desired time course of bioluminescence images is acquired, the animal may be sacrificed and tissue sections used for histology.

Discussion

Compared to other modalities such as PET and MRI, bioluminescence has limited spatial resolution and reduced tissue penetration due to the relatively weak energy of emitted photons (2-3 eV); for these reasons it has thus far not been applicable in large animals. However, bioluminescence has the advantage of being low-cost, high-throughput, and non-invasive, making it highly desirable for in vivo stem cell tracking in small animals. Non-bioluminescence reporter genes such as PET and fluorescence constructs may be used in conjunction with luciferase to create a fusion reporter gene that is composed of different domains containing the individual reporter genes. For example, our group uses a fusion construct containing fluc, monomeric red fluorescent protein (mrfp), and herpes simplex virus truncated thymidine kinase (ttk, a PET reporter gene) for multi-modality tracking of stem cell behavior in small animals. Over time, stably integrated reporter genes may be subject to gene silencing by the endogenous chromosomal machinery. A reporter gene's susceptibility to gene silencing is closely related to the choice of promoter driving its expression. For instance, the cytomegalovirus promoter (pCMV) is quickly silenced in hESCs. Our lab has had good success with the human ubiquitin-C promoter (pUbiC) to drive expression of a double fusion construct in multiple hESC cell lines, and have observed minimal signal loss over time.

In conclusion, before hESC-derived cell regeneration becomes clinically relevant, several basic biologic hurdles must be overcome - namely cell death or apoptosis following transplantation of differentiated cells, teratoma formation from undifferentiated cells, and immune rejection by the host organism. These and other challenges highlight the need for tracking hESC engraftment, survival, and proliferation within the recipient organism. The development of molecular imaging techniques such as the firefly luciferase reporter gene and ultra-sensitive CCD cameras has enabled non-invasive, repetitive assessment of cell location, migration, proliferation, and differentiation in vivo. Technologies such as these will help push translation of hESC biology from the lab towards clinical applications.

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