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Real-Time Bioluminescent Tracking of Cellular Population Dynamics

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Abstract

Cellular population dynamics are routinely monitored across many diverse fields for a variety of purposes. In general, these dynamics are assayed either through the direct counting of cellular aliquots followed by extrapolation to the total population size, or through the monitoring of signal intensity from any number of externally stimulated reporter proteins. While both viable methods, here we describe a novel technique that allows for the automated, non-destructive tracking of cellular population dynamics in real-time. This method, which relies on the detection of a continuous bioluminescent signal produced through expression of the bacterial luciferase gene cassette, provides a low cost, low time-intensive means for generating additional data compared to alternative methods.

Keywords

Bacterial luciferase; *lux*; Optical imaging; Cell culture; Population tracking; Screening

1 Introduction

Monitoring the dynamics of a cultured eukaryotic cellular population can be useful for determining the presence of estrogenic [1] or androgenic compounds [2], determining drug efficacy [3], toxicity screening [4], or a host of other [5]. While data generated from this type of experiment is straightforward and highly reliable, the main detractor of this technique has been its inability to provide real time data that can be monitored in an automated fashion [6]. This restriction, however, is not a functional limitation of the assay logistics, but rather is due to the nature of the reporter systems that have been employed to visualize the subject cells. Direct cell counting provides a means to overcome this hurdle, but has proven to be prohibitive due to the large investment of time associated with the calculation of cell density in an aliquot of the total population and the related likelihood of error that is encountered when extrapolating this number to an estimate of total population size. Therefore, the use of optical reporters such as firefly luciferase or GFP remains as the preeminent method for assaying cell population dynamics. However, while these reporter systems allow for the determination of cellular population size with a high level of accuracy, neither are amenable to real-time dynamics tracking. In the case of firefly luciferase, this is because sample destruction is required prior to data acquisition in order to treat the cells with an expensive chemical luciferin that induces bio-luminescent production. GFP, on the other hand, emits a fluorescent output signal that does not require the destruction of the cell, but is limited due to the relatively high level of background fluorescence present in

eukaryotic cells during the imaging process, which results in a low signal-to-noise ratio and decreases the sensitivity of the assay [7–8].

To circumvent these detractions, cells can be tagged via expression of a modified bacterial luciferase (*lux*) gene cassette. The genes of the *lux* cassette (*luxCDABEfrp*) encode both a luciferase protein and the additional proteins capable of generating all required substrates for its function [9]. Therefore, when the full complement of *lux* genes are expressed simultaneously within a cell, it becomes capable of generating a constitutively active bioluminescent signal that can be continuously monitored across any experimentally relevant timeframe.

2 Materials

2.1 Cell Culture Medium

The exact composition of cell culture medium will be dependent on the cell line being used. In our hands this procedure has been successful regardless of the medium used, so it is therefore recommended that the cell culture medium suggested by the cell manufacturer be used throughout the course of the experiment (*see Note 1*).

2.2 Transfection Reagents

1. Opti-MEM Reduced Serum Medium (Life Technologies).
2. Lipofectamine 2000 transfection reagent (Life Technologies) (*see Note 2*).
3. Plasmid vector DNA containing the full *lux* gene cassette (*luxCDABEfrp*) in its human-optimized form (*see Note 3*).
4. Plasmid vector DNA containing only the *luxA* and *luxB* genes and their associated linker region.

2.3 Reagents for the Selection of Successfully Transfected Cells

1. Light assay reagent (*see Note 4*): 4 μ M riboflavin 5'-mono-phosphate sodium salt, 0.2 % (w/v) bovine serum albumin (BSA), 0.002 % (w/v) n-decanal, and 1U NAD(P)H:FMN Oxidoreductase protein from *Photobacterium fischeri* prepared in sterile water.
2. 1 mM β -NADPH tetrasodium salt (*see Note 4*).
3. 0.05 % Trypsin.
4. 0.0067 M Phosphate Buffered Saline (PBS), pH 6.8.
5. Appropriate selection antibiotic(s) for the vector(s) containing the full complement of *lux* genes.

2.4. Cell Culture Equipment

1. Class II biological safety cabinet.
2. Temperature controlled, CO₂ regulated incubator.

2.5 Imaging Equipment

The expressed *lux* genes generate a bioluminescent signal at a wavelength of 490 nm. Therefore, almost any standard photomultiplier tube (PMT) or charge coupled device (CCD) camera-based imaging equipment will be suitable for observing and recording the resultant bioluminescent signal (*see Note 5*). The most important consideration is that the imaging device be capable of supporting the type of plate that is used in the experiment (96-well, 384-well, etc.). For the selection of intermediately transfected cell lines, a single tube luminometer such as the LB 9509 (Berthold Technologies) will facilitate bench top screening, but the procedure can be performed in the same plate reader used for bioluminescent imaging if needed.

3 Methods

3.1 Development of an Intermediately Transfected Cell Line

Because the *lux* gene cassette consists of six separate gene productions, and all six of these are required for bioluminescent production, a two-step transfection process is recommended. The first step will introduce only the *luxA* and *luxB* genes in order to provide an area of homology that significantly improves the efficiency and speed of autubioluminescent cell line development (*see Note 6*). All steps should be performed in a class II biological safety cabinet to prevent contamination of cell cultures unless otherwise stated.

1. Perform a kill curve on the untransfected stock of the cell line that will receive the *lux* cassette genes (*see Note 7*).
2. The day prior to transfection, trypsinize cells from their flask and obtain a total cell count using a hemocytometer, Coulter counter, or other cell counting system.
3. Resuspend the harvested cells in two wells of a six-well plate (*see Note 8*) in a 2 ml volume of the appropriate medium so that they will be ~85 % confluent at the time of transfection (*see Note 9*).
4. Allow the cells to incubate at 37 °C and 5 % CO₂ for 24 h.
5. In a 2 ml micro-centrifuge tube, add 10 µl of Lipofectamine 2000 to 140 µl of pre-warmed Opti-MEM medium.
6. Prepare a second 2 ml micro-centrifuge tube by adding 2.5 µg of the vector containing only the *luxA* and *luxB* genes and bringing the total volume to 150 µl with pre-warmed Opti-MEM medium.
7. Remove the full 150 µl volume from one of the two microcentrifuge tubes and combine into the remaining tube. Mix by gently flicking the tube.
8. Allow the combined mixture to incubate at room temperature for 5 min.
9. Carefully pipette 250 µl of the combined mixture into one of the two wells of the six-well plate (*see Note 10*). The second well will be left unmodified as a negative control.
10. Incubate the cells at 37 °C and 5 % CO₂ for 24 h.

3.2 Selection of the Intermediately Transfected Cell Line

Cells should be kept in an incubator at 37 °C and 5 % CO₂ except during manipulation.

1. Trypsinize the transfected cells to detach them from the six-well plate, then dilute them at a ratio of 1:100 with fresh medium, and replate 2 ml volumes into five of the six wells of a new six-well plate.
2. Repeat this process with the negative control well and replate into the remaining well. Return the cells to the incubator for 24 h.
3. Twenty-four hours post plating, remove the medium from all wells with serological pipettes, then wash the cells once with 1 ml of PBS and refresh with 2 ml of the appropriate medium containing selective antibiotic.
4. Monitor the cells daily and refresh with selective medium every 3rd day until all cells in the negative control well have died and distinct colonies have formed in the remaining five wells.
5. Transfer surviving colonies into individual wells of a 24-well plate in a 1 ml volume of the selective medium and return to the incubator.
6. Upon reaching 80 % confluence, trypsinize the cells from their wells and transfer them to T₂₅ flasks for continued growth.
7. When cells in a T₂₅ flask reach confluence, passage them at a ratio of 1:5 into a new T₂₅ flask, and harvest the remainder of the cells by transferring the spent medium to a 15 ml centrifuge tube and centrifuging at 300 × *g* for 7 min.
8. Remove the supernatant, resuspend the cell pellet in 1 ml of PBS and transfer to a microcentrifuge tube. Place the new tube immediately on ice.
9. Perform a protein extraction of the cell pellet by repeating three cycles of 30 s submersion in liquid nitrogen, followed by 3 min thawing in a 37 °C water bath.
10. Centrifuge the extracted sample at 14,000 × *g* in a bench top microfuge for 10 min and transfer the resulting supernatant to a new micro-centrifuge tube. Remove an aliquot of the extracted protein solution and perform a BCA protein assay to determine the overall concentration of soluble protein in the sample, and then place the tube on ice until it is ready to be processed (*see* **Notes 11** and **12**).
11. In an appropriate cuvette for the luminometer, combine 400 µl of the extracted protein with 500 µl of the light assay reagent.
12. Spike in 100 µl of the NADPH solution and immediately measure the luminescent output using a 1 s integration time. This result should be normalized to the total soluble protein concentration as determined using a standard protein determination assay (for example BCA assay) in order to control for variations in cell number during harvesting.
13. Select the intermediately transfected cell line whose protein extract produced the greatest normalized level of luminescent output for use in developing a stable autoluminescent cell line. This will ensure that the *luxAB* genes were

successfully integrated into the cellular genome and provide homologous recombination insertion points for the full *lux* cassette that are preselected for an improved likelihood of expression.

3.3 Development of the Fully Transfected Autobioluminescent Cell Line

Follow **steps 1–10** of Subheading 3.1, however, using 2.5 µg of the plasmid DNA containing the full complement of the *lux* genes rather than the plasmid DNA containing only the *luxA* and *luxB* genes in **step 6** (*see Note 13*).

3.4 Selection of Fully Transfected Autobioluminescent Cell Lines

Cells should be kept in an incubator at 37 °C and 5 % CO₂ except during manipulation.

1. Trypsinize the transfected cells to detach them from the six-well plate, then dilute them at a ratio of 1:100 with fresh medium, and replate 2 ml volumes into five of the six wells of a new six-well plate.
2. Repeat this process with the negative control well and replate into the remaining well. Return the cells to the incubator for 24 h.
3. Twenty-four hours post plating, remove the medium from all wells with serological pipettes, then wash the cells once with 1 ml of PBS and refresh with 2 ml of the appropriate medium containing selective antibiotic.
4. Monitor the cells daily and refresh with selective medium every 3rd day until all cells in the negative control well have died and distinct colonies have formed in the remaining five wells.
5. Transfer surviving colonies into individual wells of a 24-well plate in a 1 ml volume of the selective medium and return to the incubator.
6. Upon reaching 80 % confluence, trypsinize the cells from their wells and transfer them to individual T₂₅ flasks for continued growth.
7. When cells in the T₂₅ flask reach confluence, passage them at a ratio of 1:5 into a new T₂₅ flask. Harvest the remainder of the cells, count them to determine the total cell number, and then transfer them to a 15 ml centrifuge tube and centrifuge at 300× *g* for 7 min.
8. Resuspend the cell pellet in 1 ml of the appropriate medium and transfer immediately to an individual well of a 24-well plate (*see Note 14*).
9. Assay the cells for autobioluminescent production in a platereader using a 1 min read time every 30 min for 24 h (Fig. 1)(*see Note 15*) and normalize the bioluminescent readings to the total cell number in each well.
10. The cell line displaying the greatest level of normalized autobioluminescent production should then be selected for all future experiments (*see Note 16*).

3.5 Imaging Autobioluminescent Cells

1. Allow a T₂₅ flask of autobioluminescent cells to grow to 75 % confluence under standard growth conditions.
2. Trypsinize the actively growing cells to detach them from the T₂₅ flask and obtain total cell counts using a hemocytometer, Coulter counter, or other cell counting system.
3. Resuspend 5×10^4 autobioluminescent cells into triplicate wells of a 24-well plate (*see Note 17*), along with an equal number of untransfected cells (also in triplicate) to act as the negative control.
4. Incubate the cells at 37 °C and 5 % CO₂ for 2 h to allow the cells to attach to the plate (*see Note 18*).
5. If required, treat the cells with your compound(s) of interest (*see Notes 19 and 20*).
6. Put the plate into the plate reader and measure the autobioluminescence continuously over a 24 h period using a 10 s read time (Fig. 1) (*see Notes 21 and 22*).

4 Notes

1. If available, it is advantageous to choose a version of the appropriate medium that does not contain phenol red. It has been observed that the presence of phenol red in the medium can lead to a slight absorption of resulting autobioluminescent signal. However, this reduction in signal strength is very minor, and if phenol red is a necessary component of the appropriate medium its negative absorption effects will be less than those resulting from growth in sub-optimal medium.
2. Alternative transfection reagents based on polycationic liposomes are theoretically viable options for this protocol; however, all of our work to date has utilized Lipofectamine 2000 and this protocol is therefore optimized for use with this reagent. If a different reagent is used, consult the manufacturer to determine necessary changes to the transfection protocol.
3. A human optimized version of the *lux* gene cassette consists of a vector or series of vectors that contains the *lux A*, *luxB*, *luxC*, *luxD*, and *luxE* genes of *Photobacterium luminescens* and the *frp* gene of *Vibrio harveyi* in their human codon-optimized forms. While these genes do not necessarily need to be housed on a single plasmid to generate a bioluminescent phenotype [10], it is much easier and faster to generate bioluminescent cell lines by using a single vector containing each of the required *lux* genes under the control of a single strong promoter and linked via viral 2A elements.
4. This must be made fresh before each experiment.
5. While any standard plate reader or CCD camera-based detection system can be used, an instrument with integrated incubation capabilities will significantly improve the automated nature of the imaging experiment. Special attention should be paid to the humidity level that can be maintained within the imaging chamber

because an instrument that maintains an elevated temperature (i.e., 37 °C) but does not control for increased humidity will hasten the rate at which the medium evaporates. This is especially true as well volume decreases during higher throughput experimental designs (i.e., with a 96-well plate).

6. This step can be skipped (moving straight to Subheading 3.3); however, this will significantly reduce the efficiency of the transfection procedure. This is not recommended unless the goal is to develop transiently transfected autoluminescent cell lines for short term (16–36 h) experiments.
7. It is very important that a new kill curve be conducted prior to the start of the transfection protocol. The resistance of cell lines to antibiotics can vary greatly both from lab to lab and within the same line as it ages.
8. The size of the plate can be varied as needed; however, the amount of both DNA and Lipofectamine 2000 that are used must then be scaled appropriately. For information on scaling the amount of DNA and Lipofectamine 2000 when alternative size plates are used, consult the manufacturer's manual at the time of purchase.
9. For a standard six-well plate, we have found that plating between 4×10^5 and 6×10^5 cells will provide optimal confluence for transfection at 24 h post plating.
10. Care must be taken not to dislodge the plated cells. It is therefore advisable to gently swirl the medium within the well while adding the mixture solution dropwise. This ensures that the attached cells are not adversely affected, as well as evenly distributes the mixture solution within the well.
11. This extracted protein sample can be stored at -80°C or in liquid nitrogen indefinitely if needed. It is not recommended that the sample be stored at -20°C or 4°C for longer than 1 h, or loss of protein activity can occur that will invalidate the results of the impending light assay. Following transfer to ice, the sample should be processed as quickly as possible to insure the best results.
12. It is not necessary to wait until the results of the BCA assay are known to proceed. The light assay can be performed immediately and, when complete, can be normalized using the resulting BCA data.
13. Note that a second kill curve must be performed on the intermediately transfected (*luxAB* containing) cell line in order to determine its resistance to the antibiotic selection marker that will be used for selection of the stably transfected autoluminescent cell line.
14. This step can be performed in any size plate as needed; however, the volume of medium should be scaled appropriately to account for the change in well size. In general, it is not recommended that plates with fewer than 24 wells be used, since the higher concentration of cells across a lower area provides for improved signal detection. In addition, an opaque plate (white or black) should be used, as the use of a clear plate can lead to signal contamination during screening.

15. The read time can be adjusted based on the sensitivity of the plate reader; however, it is not recommended that times be shorter than 10 s or longer than 10 min. The 24 h duration may be much longer than is necessary to determine which lines are autoluminescent, but a gradual increase in bioluminescent production is often observed following transfer to the 24-well plate as the cells adapt to the new environment and this time period can serve to determine which cell lines have stable, rather than transient, autoluminescent production (Fig. 1).
16. This cell line is now stably transfected and can be stored in liquid nitrogen for future use if needed.
17. The number of cells required is dependent on the size of the well being used. For assays performed in 24-well plates, the minimum number of cells that allow for reliable detection is 5×10^4 . However, for smaller wells a lower cell number can be used and detected with the same reading window time (i.e., 1×10^4 cells can be imaged in a 96-well plate). More cells can always be included to increase the autoluminescent signal if the cell line was not efficiently transfected. If a CCD camera-based instrument is used, a minimum number of 5×10^3 cells should be used for assays performed in 24-well plates.
18. This step is optional if the attachment of cells is integral to the experimental design. Readings can be taken immediately if attachment is not required. The attachment time varies greatly with the cell line employed and may have to be determined empirically due to the effects of gene integration during the transfection reactions.
19. The compound being tested should first be prepared as a concentrated stock solution. The final concentration, which will be based on the requirements of the experimental design, should therefore be determined based on what its concentration will be post-application.
20. If an organic solvent is used to prepare the compound, the volume of compound solution added to the cells should not exceed 0.2 % of the total volume of medium in each well to prevent vehicle-based cytotoxic effects that could potentially mask any compound-mediated effects.
21. The reading window can be adjusted based on the requirements of the experimental design. While imaging for between 10 s and 1 min is common in PMT-based plate readers, if a CCD camera-based instrument is being used to obtain pseudocolor images, a longer integration time of up to 10 min may be used to improve the quality of the visual image.
22. Up to 24 h continuous reading is recommended for testing acute toxicity or other fast-acting effects. If longer term monitoring is necessary (i.e., for determining the proliferative effect of a target compound) the cells can be imaged once every 24 h and should be kept in the incubator between readings.

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Fig. 1.

The *lux* cassette genes allow for continuous bioluminescent production, permitting facile detection of positively transfected stable cell lines and providing an elegant means for tracking population changes over time