Video Article

Autonomously Bioluminescent Mammalian Cells for Continuous and Real-time Monitoring of Cytotoxicity

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Abstract

Mammalian cell-based *in vitro* assays have been widely employed as alternatives to animal testing for toxicological studies but have been limited due to the high monetary and time costs of parallel sample preparation that are necessitated due to the destructive nature of firefly luciferase-based screening methods. This video describes the utilization of autonomously bioluminescent mammalian cells, which do not require the destructive addition of a luciferin substrate, as an inexpensive and facile method for monitoring the cytotoxic effects of a compound of interest. Mammalian cells stably expressing the full bacterial bioluminescence (*luxCDABEfrp*) gene cassette autonomously produce an optical signal that peaks at 490 nm without the addition of an expensive and possibly interfering luciferin substrate, excitation by an external energy source, or destruction of the sample that is traditionally performed during optical imaging procedures. This independence from external stimulation places the burden for maintaining the bioluminescent reaction solely on the cell, meaning that the resultant signal is only detected during active metabolism. This characteristic makes the *lux*-expressing cell line an excellent candidate for use as a biosentinel against cytotoxic effects because changes in bioluminescent production are indicative of adverse effects on cellular growth and metabolism. Similarly, the autonomous nature and lack of required sample destruction permits repeated imaging of the same sample in real-time throughout the period of toxicant exposure and can be performed across multiple samples using existing imaging equipment in an automated fashion.

Video Link

The video component of this article can be found at https://www.jove.com/video/50972/

Introduction

In the U.S., pharmaceuticals and other products intended for human consumption require extensive assessment before they are approved for consumer use by the Food and Drug Administration. The financial burden for performing this testing is placed on the developer[1], which substantially increases the cost of new compound development and therefore translates into an increased cost for consumers. While traditionally much of this screening has utilized animal subjects to act as proxies for human hosts, this has proven to be a large financial burden, with an estimated $2.8 billion spent annually on ADME/Tox (adsorption, distribution, metabolism, excretion, and toxicity) screening alone[2] and mounting evidence suggesting that animal models cannot reliably predict human toxicological responses[3]. Therefore, *in vitro* human cell culture-based testing has gained popularity over the past two decades because of its relatively lower cost, higher throughput, and better representation of human bioavailability and toxicology[4]. Current cell culture-based toxicity screening methods employ various endpoints, such as the measurement of ATP levels, screening the activity of endogenously available cytoplasmic enzymes, probing the integrity of the cellular membrane, or tracking the level of mitochondrial activity, to evaluate cellular viability[5,6]. However, regardless of the endpoint chosen, these methods all require the destruction of the sample before measurements can be taken, thus only producing data at a single time point. As a result, large numbers of samples need to be prepared and treated in parallel for basic toxicological kinetics studies, again adding to the cost and labor required for new compound development. Alternatively, assays using secreted luciferase such as *Gaussia* luciferase[7], *Vargula* luciferase[8], and *Metridia* luciferase[9] have been developed that eliminate the need for cell lysis and require a fraction of the media for endpoint measurement, however, these are still limited to sampling at predetermined time points and also require the addition of exogenous light-activating substrates.

To avoid the detriment of requisite sample destruction as well as to eliminate the cost of substrates, a human cell line has been engineered that expresses the full bacterial bioluminescence (*lux*) gene cassette (*luxCDABEfrp*) to allow for continuous monitoring of live cells that is similar to fluorescent-dye based live cell imaging, but without the additional photon-activating and microscopic investigation procedures. This cell line is capable of constitutively producing an optical signal for continuous, direct detection without the need for external stimulation, thus avoiding destruction of the sample. Mechanistically, the bioluminescent signal generated from these cells results when the *luxAB*-formed luciferase enzyme catalyzes the oxidation of a long chain fatty aldehyde (synthesized and regenerated by the *luxCDE* gene products using endogenous substrates) in the presence of reduced riboflavin phosphate ($FMNH_2$, which is recycled from FMN by the *frp* gene product) and molecular oxygen[10]. Expression of the *lux* cassette in the host cell therefore enables light to be produced and detected without cellular destruction or

exogenous substrate addition. Similarly, the interaction between the *lux* genes and the endogenously available FMN, and O_2 cosubstrates, and the requirement for maintenance of an environment that can support the conversion of FMN to $FMNH_2$, ensures that the resulting bioluminescent signal can only be detected from living, metabolically active cells.

These requirements have previously been exploited to demonstrate that *lux*-based bioluminescent output correlates strongly with cellular population size[11] and that toxic compound exposure impairs autobioluminescent production in a dose-response fashion[12]. Here we use a previously characterized autobioluminescent human embryonic kidney (HEK293) cell line[11] to demonstrate the automated toxicological screening of an antibiotic of the bleomycin family with known DNA damaging activity as a representative example to validate the application of autobioluminescent mammalian cells for toxicity testing.

Protocol

1. Cell Preparation

1. Recover a vial of bioluminescent HEK293 cells from liquid nitrogen frozen stock and grow them in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 0.01 mM nonessential amino acids, 1x antibiotic-antimycotic, and 0.01 mM sodium pyruvate in a T_{75} tissue culture flask at 37 °C and 5% CO_2.
   Note: The media and supplemental components will vary based on cell lines and therefore should be chosen accordingly.
2. Refresh medium every 2-3 days until reaching ~80% confluence.
   Note: The amount of cells required will be based on the experimental design. If needed, more cells can be obtained by subculturing.
3. Harvest cells for testing.
   1. Remove the medium and wash the cells with phosphate buffered saline (PBS) by gently swirling the flask and then discarding the spent PBS.
   2. Add trypsin and incubate at 37 °C for 2 min, or until the cells have detached from the flask.
   3. Collect the detached cells in PBS and transfer them into a clean centrifuge tube.
      Note: If nonadherent cell lines are used, separate cells by centrifugation and wash once with PBS.
4. Determine the total cell count using a hemacytometer or other cell counting system.
5. Centrifuge the cell suspension at 300 x g for 5 min. Discard the supernatant and resuspend the cells in fresh, prewarmed medium.
6. Seed equal numbers of cells into individual wells of an opaque multi-well plate. In this example, plate 5 x 10^5 cells in a 1 ml volume into each well of a black 24-well plate. Plate an equal volume of medium in triplicate wells to act as the negative control.
   Note: The number of cells plated in each well is flexible and can be optimized for individual experiments. For each bioluminescent cell line, experimentally determine the relationship between cell number and bioluminescent output, as well as the minimal detectable cell count prior to any toxicity tests. To do this, measure bioluminescence across a wide range of population sizes (for example, 1 x 10^3 to 1 x 10^6 cells) under standardized imaging conditions.
7. Treat cells with testing compound(s) as described below.

2. Chemical Preparation

1. Prepare the chemical being tested as a concentrated stock solution. In this example, use a 100 mg/ml stock solution of an antibiotic of the bleomycin family.
   Note: To minimize potential vehicle-based toxic effects, especially if an organic solvent is used as a vehicle for chemical addition, it is recommended that the stock concentration be at least 1,000 times the desired post-application concentration.
2. Add the prepared chemical stock directly to the cells. In this example, dose the antibiotic of interest at final concentrations of 100, 200, 300, and 400 µg/ml in triplicate wells. Leave three wells of cells untreated as controls.
   Note: The final concentration of the target chemical should be selected based on specific experimental goals. A wide range of concentrations is normally tested to obtain a full spectrum of the toxic effects.

3. Imaging and Data Analysis

1. Immediately following chemical addition, place the plate in the imaging chamber of appropriate instrument for image acquisition and bioluminescence measurement.
2. Measure bioluminescence every 15 min over a 24 hr period using a 10 min integration time for each reading.
   Note: The integration time used in this example is on a per-plate basis and can be adjusted for measurement on a per-well basis using other luminometers of choice. The integration time can also be adjusted based on the chosen bioluminescent cell line. Because bioluminescence is produced autonomously without cell destruction or any external stimulation, readings can be taken at any desired time point to fulfill specific experimental objectives.
3. Quantify the bioluminescent intensity from each well by identifying a region of interest using compatible software. Display the light output either as the total flux (photons/second) or the average radiance (photons/second/cm^2/steridian) of each sample.

Representative Results

In this study, the dynamics of autobioluminescent HEK293 cells were monitored continuously over a 24 hr period in response to antibiotic exposure (**Figure 1**). The toxic effects of this antibiotic, which is a member of the bleomycin family known to kill living cells by binding to and cleaving DNA[13], were demonstrated via a decrease in bioluminescent production compared to untreated cells, which can be directly visualized through the pseudocolor images (**Figure 2**). The autobioluminescent nature of these cells, which permitted the repeated parallel screening

of samples under varying treatment conditions, allowed for comparison of different concentrations at any given time point for easy dose-response analysis. For example, bioluminescence produced from cells after 15 hr, 18 hr, and 20 hr of treatment was plotted against chemical concentrations in **Figure 3**, showing that increasing antibiotic treatment resulted in the reduction of bioluminescent production in a dose-response manner.

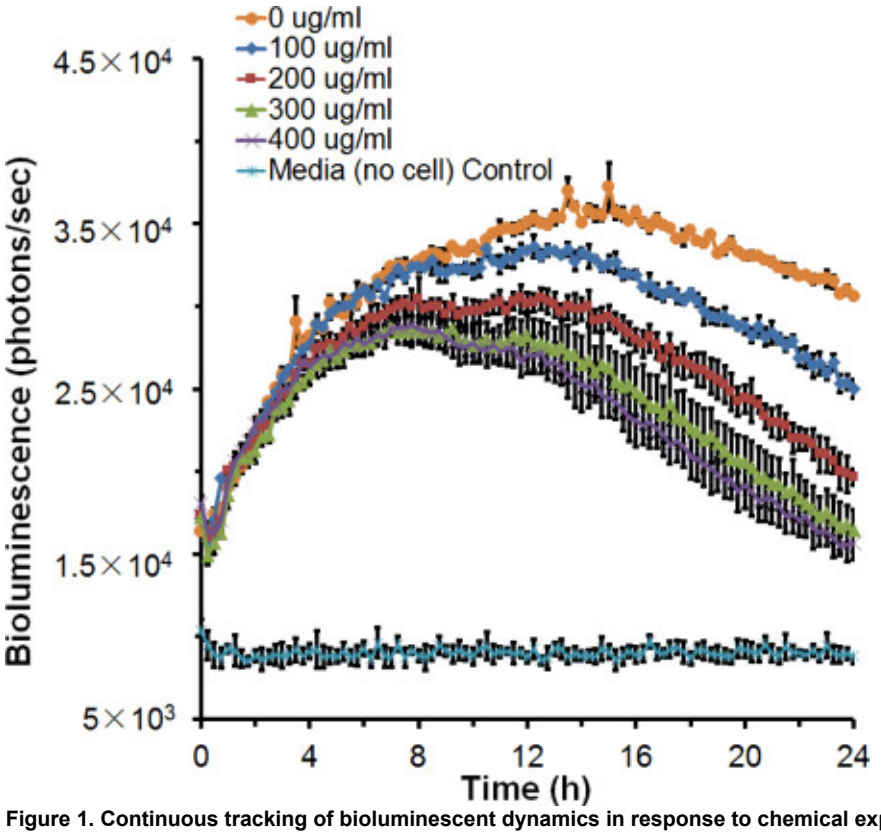

Figure 1. Continuous tracking of bioluminescent dynamics in response to chemical exposure. Constitutively bioluminescent HEK293 cells were exposed to various concentrations of an antibiotic of the bleomycin family. Bioluminescent production was monitored over a 24 hr period of exposure (representative data for technical triplicates on one plate, mean ± standard error) (reprint from [14]).

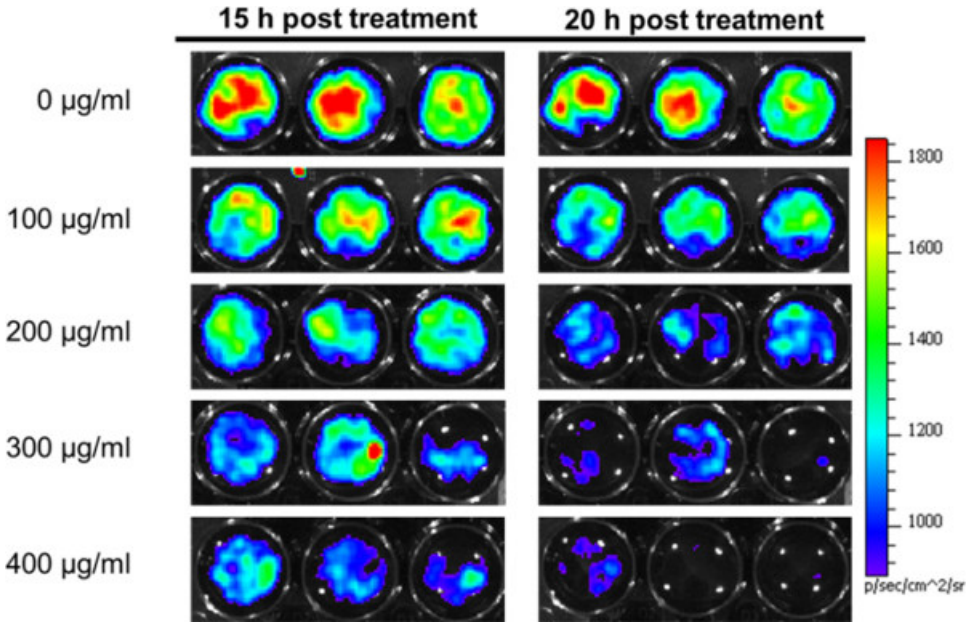

Figure 2. Pseudocolor images of treated cells. Antibiotic treated cells displayed a reduced bioluminescent signal compared to untreated control.

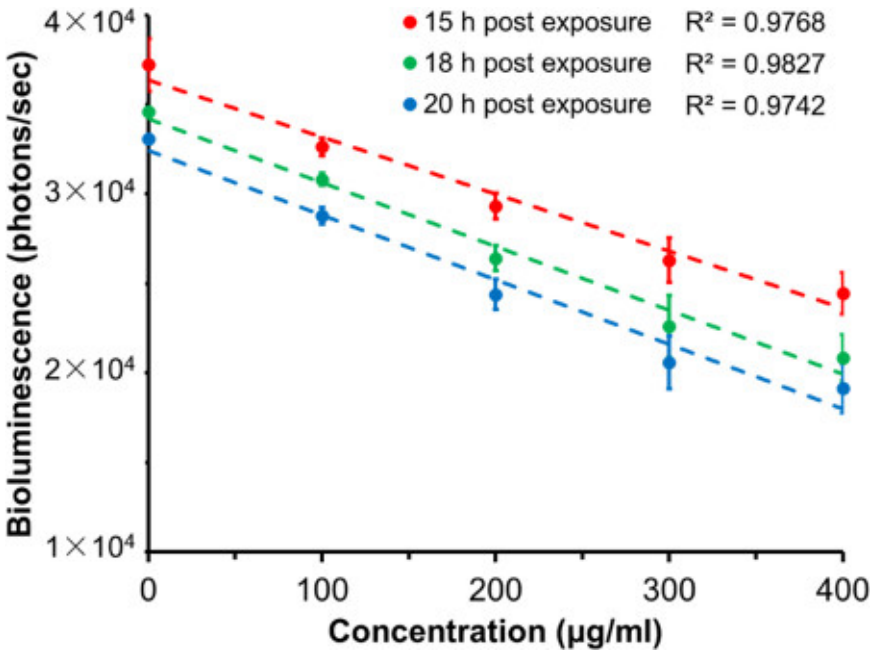

Figure 3. Dose-response of chemical exposure. The bioluminescent output after 15 hr, 18 hr, and 20 hr of exposure showed that increasing antibiotic treatment resulted in the reduction of light production in a dose-response manner (representative data for technical triplicates on one plate, mean ± standard error). R^2 value is calculated for linear regression.

	***lux*-based assay**	***luc*-based assay**
Treatment levels tested	5	5
Time points assayed	97	6
Samples prepared	15 (3/treatment level)	90 (3/treatment level/time point)
Reagent cost	$0	Cost for luciferin and/or luciferase
Hands on time	~ 0.5 h	~ 1 h
Sample destruction required?	No	Yes

Table 1. Representative comparison of the demonstrated toxicity screen using either autobioluminescent (*lux*-based) human cells or a traditional firefly luciferase (*luc*)-based assay in a 96-well plate format.

Discussion

This method demonstrates the use of autonomously bioluminescent mammalian cells as an *in vitro* cytotoxicity screening assay that allows live cells to be continuously monitored over their lifetime. This protocol is flexible and can be modified to accommodate specific experimental conditions as required. For example, the experiment presented here is suitable for tracking acute toxic effects, but can be adapted to assess slow-acting or long term effects by repeatedly imaging at increased time intervals (*i.e.* every 24 hr) and maintaining the cells under standard incubation conditions between readings. In addition, the integration time can be adjusted based on the bioluminescent cell line. A preliminary experiment of imaging with various integration times can be performed to determine the optimal reading window. The 10 min integration used in this example was chosen to highlight the dynamic range of bioluminescent production across all treatment conditions, however a shorter reading frame can be applied depending on the application. Similarly, while a 24-well plate is used for demonstration in this example, 96- or 384-well plates can be substituted for higher throughput applications. Although the representative results shown here demonstrate technical triplicates on a single plate, it is important to note that independent assays on different plates should be performed to establish statistical significance between treatment levels when testing a compound of interest. Moreover, because this assay can be adapted for use in a variety of instruments with

varying detection sensitivities, it is recommended that acceptable signal-to-noise and signal-to-background ratios should be determined on a per-instrument basis.

It was previously determined that the minimal detectable cell number in a 24-well plate was approximately 1.5 x 10^4 cells under the imaging conditions described here[15]. However, it is important to note that the minimal number of cells required for reliable detection varies with well size, and that the use of smaller wells reduces the number of cells required for detection by increasing the number of bioluminescent cells per unit area. Therefore, it is strongly recommended that the relationship between bioluminescent output and population size be determined under the desired imaging conditions prior to screening. In addition, a photomultiplier tube-based plate reader can also be used for data acquisition in place of the IVIS Lumina imaging system, but at the detriment of obtaining pseudocolor images. Regardless of the imaging instrument employed, an opaque plate should be used to minimize signal loss and/or bioluminescent contamination of adjacent wells due to photons traversing the plate.

Compared to other commonly employed cell culture-based approaches, this method offers the advantage that data acquisition can be performed in a fully automated fashion since the need for sample destruction or substrate addition is eliminated. It also allows for the continuous tracking of dynamic effects upon the same cell population over a prolonged period of exposure, which provides an enhanced resolution of toxicological kinetics without the concurrent increase in monetary or time costs that is necessitated when using cell lysis-requiring methods (**Table 1**). However, an important limitation of this method is that cell type-specific testing will require the stable transfection of the *lux* cassette into each cell type of interest in order to generate an autobioluminescent phenotype for screening. Although this need only be performed once since the cells can subsequently be stored in liquid nitrogen for future use, the transfection procedure is a potentially time-consuming endeavor.

Despite this prerequisite, the protocol outlined here is simple and inexpensive, requires minimal hands-on work from laboratory personnel, does not require any additional reagents, and generates data that can be directly used for toxicological interpretation. For these reasons, we conclude that autobioluminescent mammalian cell lines can be used as a high-throughput assay for rapid preliminary screening of a large number of candidates to select compounds that require more detailed downstream evaluation.

Disclosures

D.M. Close, S.A. Ripp, and G.S. Sayler are founders and owners of 490 BioTech, Inc.

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