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Bioluminescence Imaging of Spheroids for High-throughput Longitudinal Studies on 3D Cell Culture Models†

Luca Cevenini[1‡], Maria M.Calabretta[1‡], Antonia Lopreside[1], Bruce R. Branchini[2], Tara L. Southworth[2], Elisa Michelini[1,3,4]*, Aldo Roda[1,4]*

[1]Dept. of Chemistry "G. Ciamician", University of Bologna, Via Selmi 2, 40126, Bologna, Italy

[2]Department of Chemistry, Connecticut College, New London, Connecticut, 06320, USA

[3]Health Sciences and Technologies-Interdepartmental Center for Industrial Research (HST-ICIR) University of Bologna, Bologna, Italy

[4]INBB, Istituto Nazionale di Biostrutture e Biosistemi, Viale delle Medaglie d'Oro 305, 00136-Roma, Italy

[‡]These authors contributed equally to the work

*Corresponding authors' emails: aldo.roda@unibo.it (Aldo Roda) and elisa.michelini8@unibo.it (Elisa Michelini)

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ABSTRACT

Bioluminescent (BL) cell-based assays based on two-dimensional (2D) monolayer cell cultures represent well-established bioanalytical tools for pre-clinical screening of drugs. However, cells in 2D cultures do not often reflect the morphology and functionality of living organisms, thus limiting the predictive value of 2D cell-based assays. Conversely, 3D cell models have the capability to generate the extracellular matrix and restore cell-to-cell communications; thus, they are the most suitable model to mimic in vivo physiology. In this work we developed a non-destructive real time BL imaging assay of spheroids for longitudinal studies on 3D cell models. A high-throughput BL 3D cell-based assay in micro-patterned 96 multi-well plate format is reported. The assay performance was assessed using the transcriptional regulation of nuclear factor K beta response element in human embryonic kidney (Hek293) cells. We compared concentration-response curves for tumor necrosis factor α with those obtained using conventional 2D cell cultures. One of the main advantages of this approach is the non-lysing nature of the assay, which allows for repetitive measurements on the same sample. The assay can be implemented in any laboratory equipped with basic cell culture facilities and paves the way to the development of new 3D bioluminescent cell-based assays.

INTRODUCTION

Cell-based assays represent an invaluable tool for the early stages of the drug discovery process (1). Thanks to their easy adaptability to high-throughput and high-content screenings (HTS and HCS), cell models can identify bioactive molecules interacting with molecular targets well in advance of pre-clinical studies. In particular, owing to their high sensitivity and high dynamic range, cell-based assays relying on bioluminescent (BL) reporter proteins are highly favored in HTS when compared with well-established fluorescence-based assays (2). In BL assays, a reporter protein, such as a luciferase, is expressed under the regulation of a target promoter sequence or enhancer elements, thus enabling correlation of reporter protein expression, measured as light signal, and transcriptional regulation (3).

Moreover, thanks to the availability of a palette of luciferase reporters that can nowadays compete with green fluorescent protein (GFP) and its variants, multiplexing is no limited to fluorescence detection (4,5). Multiplexed BL assays can be performed both in vitro and in vivo, i.e., in small animal non-invasive imaging (6). Different luciferases have been obtained by cloning the genes from new organisms, by mutagenesis of the genes, and by combining the N-domain and C-domains of luciferases from different species (7-10). This availability enabled the development of high-content assays and orthogonal assays, the latter relying on the use of two unrelated luciferases for monitoring the same target. This strategy was successfully applied to improve the robustness and reliability of large HTS (11,12).

Therefore BL cell-based assays based on two-dimensional (2D) monolayer cell culture models represent well-established reliable tools that improved the first steps of drug-screening, in compliance with the Replacement, Reduction, Refinement principle (3Rs) (13-15). However, cells in 2D cultures often do not reflect the morphology and functionality of their native three-dimensional

(3D) phenotypes; thus, the relevance of information obtained by the 2D assay is reduced (16). The more in vitro models represent tissue-specific functionality and, possibly, in vivo physiology, the better the prediction of the potential impact of a drug candidate before it enters animal and clinical trials. Therefore, the cell microenvironment has to mimic in vivo physiological conditions including spatial and temporal dimensions and dynamics, physical interactions, such as cell-cell contact, and the cell-extracellular matrix (17-19). Several technologies are currently available to produce such microtissues with good control of the dimensions and that exhibit tissue-like phenotypes including porous scaffolds and polymers, hydrogels, and ultra-low attachment cell culture plates (20-23). These methods take advantage of the natural self-assembly tendency that is typical of several cell types. When cells are grown as spheroids they are able to generate their extracellular matrix and communicate with other cells as in their native environment (24).

Several 3D cell-based assays have been reported, although most of them rely on viability and morphology endpoints with fluorescent readouts (18, 24-25). The transition from BL 2D cell-based assays to 3D is not trivial since most current BL assays were designed and optimized for monolayer or suspension cultures. To the best of our knowledge, BL non-lysing approaches have not yet been reported for imaging of live spheroids. Here, we report a non-invasive real time imaging assay of 3D cell cultures for longitudinal and high-throughput studies that can be easy implemented for screening of drugs and new molecules of interest.

MATERIALS AND METHODS

Materials. The Human Embryonic Kidney HEK293 cell line was obtained from ATCC (American Type Culture Collection [ATCC], Manassas, VA, USA). Dulbecco's modified Eagle's medium high glucose (DMEM), fetal bovine serum (FBS), L-Glutamine, penicillin and streptomycin were from Carlo Erba Reagents (Cornaredo, Milano, Italy). The restriction enzymes

required for cloning were from Fermentas (Vilnius, Lithuania). Tumor necrosis factor-α (TNFα) was from Sigma-Aldrich (St. Louis, MO, USA) and plasmid extraction kit, FuGENE HD transfection reagent and D-luciferin were from Promega (Madison, WI, USA). The PLG2 luciferase gene, previously obtained and described (10) was cloned into the pCDNA3 vector and pGL4.322[luc2P/NF-κB-RE/Hygro] backbone to replace Luc2P luciferase, yielding vectors named pCMV_PLG2 and pNFkB_PLG2.

2D cell cultures. Hek293 cells were grown in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin. Cells were plated in black, clear bottom 96-well plates at a density of $2x10^4$ cells per well, with 100 µl of complete growth medium.

3D cell cultures. Hek293 spheroids were obtained using 96 well micro-space round bottom cell culture plates with a non-adherent surface generously provided by Elplasia^{TM}, Kuraray, Japan. Before cell seeding, 100 µL of complete culture medium was added to each well followed by 200 µL of cell suspension ($2x10^4$ cells per well). The plate was then incubated at 37°C with 5% CO_2. Spheroids growth was monitored every 24 h for a period of 4 days.

Characterization of 2D and 3D Hek293 cultures expressing PLG2 luciferase. The day after seeding, cells grown in 2D and 3D format were transfected with 0.10 µg of pCMV_PLG2 according to the manufacturer's instructions, using a FUGENE HD:DNA ratio of 3:1 and incubated at 37°C with 5% CO_2. At 24 h post transfection, kinetic measurements (10 min with 200 ms integration time) were obtained in 96-well plates with a Varioskan Flash luminometer (Thermo Fisher Scientific, Waltham, MA, USA) after automatic injection of 100 µL of 1 mM D-Luciferin citrate solution pH 5.0.

Analogously, at 24 h post transfection, BL emission spectra were recorded from 450 to 750 nm, at 2 nm intervals with 100 ms integration time, with a 30s delay after D-luciferin addition. All experiments were performed in triplicate and repeated at least three times.

2D and 3D bioluminescence imaging of Hek293 cells expressing PLG2 luciferase. Cells grown for 24 h in 2D and 3D microplates were transfected with 0.10 µg of pCMV_PLG2 using a FUGENE HD:DNA ratio of 3:1 and incubated for 24 h at 37°C with 5% CO_2. BL imaging was performed using an inverted microscope (Olympus CK40) connected to an electron multiplying charge coupled device (EM-CCD) camera (ImagEM-X2, Hamamatsu). Images of 2D and 3D cell cultures were acquired using 10X (Olympus A10PL) and 4X (SPlan4SL) objectives with an integration time of 30 sec, at a gain level set to 500, after the addition of 100 µL of 1 mM D-luciferin substrate in citrate buffer pH 5.0. Overlay images were obtained using HCImage software (v 4.1.2.0). All experiments were performed in triplicate and repeated at least three times.

Spheroid analysis. Brightfield images of Hek293 spheroids were analyzed using ImageJ version 1.51d software to calculate the projected area (*A*) and perimeter (*P*) of each spheroid (26). A sphericity factor, named ϕ, was then calculated as follows:

$$\phi = \frac{\pi \ x \sqrt{\frac{4A}{\pi}}}{P} \qquad (1)$$

3D bioluminescence imaging assay for inflammatory pathway activation. A 3D assay for inflammatory activity was developed by employing 3-day old Hek293 spheroids. Briefly, Hek293 cells were plated in 96-well micro-space round bottom cell culture plates (Elplasia) at a concentration of $2x10^4$ cells/well in a total volume of 100 µL of medium. After 72 h, cells were transfected with 0.10 µg of plasmid pNFkB_PLG2 per well using a FUGENE HD:DNA ratio of 3:1. At 48 h post transfection, medium was changed and cells were treated in triplicate with 50 µL of TNFα solutions in culture medium (0.1-20 ng/mL) or with 50 µL of culture medium as a control. After 4 h incubation at 37°C, 100 µL of 1 mM D-luciferin substrate in citrate buffer pH 5.0 (27) was added to each well. Images were acquired with the ImagEMX2 EMCCD camera with an integration time of 30 sec, gain 500, using 4X objectives. Bioluminescence images were quantified using ImageJ software; a region of interest (ROI) was manually designed around each spheroid and its BL intensity was calculated with respect to the projected area (corrected BL emission). The mean of corrected BL emissions obtained from 20 spheroids was used for the dose-response curve generated using GraphPad Prism software. All measurements were performed in triplicate and repeated at least three times.

RESULTS AND DISCUSSION

Characterization of PLG2 luciferase expressed in 3D cell cultures/spheroids

A new enhanced chimeric luciferase mutant, named PLG2, was selected as a reporter protein for engineering spheroids. PLG2 is characterized by improved spectral and physical properties, i.e., enhanced activity compared with the *Photinus pyralis* wild-type luciferase (PpyWT) (about 35%), absence of red-shifting of bioluminescence at low pH (~6.5), and improved

thermostability (24 h vs. 20 min at 37 °C). Stability to pH and temperature is a desirable feature for BL reporters to be used within cells or living animals, where 37°C and low pH conditions, e.g., as consequence of cell metabolism, are commonly found. This luciferase has a K_m value of 52 ± 6 µM for D-luciferin, which is about 3-fold higher than the value of PpyWT, and a K_m value for Mg–ATP very similar to PpyWT (79±5 µM vs 86±7 µM, respectively). For evaluating the potential application of PLG2 luciferase in spheroid-based assays, two main issues were considered: the possibility to perform bioluminescence measurements in non-lysing conditions, to enable repetitive measurements for longitudinal studies, and likely biodistribution issues that are not encountered with monolayer or suspension cultures. To this end, we first characterized PLG2 expression in spheroids to evaluate emission spectra, kinetics and highlight possible problems that could circumvent BL detection in 3D cultures, such as substrate and oxygen availability issues at the core of spheroids. As expected, the emission spectra did not significantly change when compared with those obtained with Hek293 monolayer cultures (Fig. 1a). The maximum emission wavelength was 556nm in both 2D and 3D cultures and a minor broadening effect was reported in the 3D format with bandwidth at half-maximal intensity of 71 nm vs 64 nm obtained in 2D cultures.

Those values are consistent with those previously reported (10) in monolayer cultures. Bioluminescence emission kinetics were measured by adding a non-lysing formulation of D-luciferin previously optimized for live cell imaging (28). Cells grown either in 2D and 3D culture format showed a glow-type emission with a peak 35s after substrate addition and the BL signal decreased by half after 5 min (Fig. 1b). Thus, the emission kinetics obtained with 3D cell culture are suitable for BL imaging. An optimal acquisition time window was identified between 25 and 55s, when the mean BL signal is 97±3% of maximum emission. However, despite the same number of cells were seeded in 3D and 2D format, BL intensities

per well obtained with spheroids are approximately half of those obtained with 2D cultures. This may be partially explained by the inability of D-luciferin solution to penetrate spheroids in depth, thus only external cell layers contribute to BL emission. More studies will be required to address this point. Moreover, photon losses and scattering effects of PLG2 green emission must be considered. This aspect will be further investigated using a microscope equipped with optical emission filters and a combination of red- and green-emitting luciferases respectively expressed in the core region and in surface layers, to quantitatively assess and optimize the substrate concentration and distribution within the spheroids.

The typical output from BL imaging of Hek293 cells ($2.0x10^4$ cells/well) cultured on conventional 96-well plates and transfected to constitutively express PLG2 is shown in Fig. 2a-c. Cells grew as a monolayer and were imaged before confluency using a 20X objective to identify individual cells (about 800 cells with an average dimension of 20±5 μm). Pseudocolor overlay was then used to quantify single cell emission corrected by the size of each cell. BL imaging also allows direct visualization of the transfection efficiency and to evaluate the metabolic activity of the cells. Indeed, despite that all cells are transfected with a plasmid for the constitutive expression of the same PLG2 luciferase, BL intensities might be quite different due to the metabolic state of individual cells, which can affect the rate of protein expression and degradation, ATP content, etc.).

Bioluminescence imaging of spheroids was performed using clear bottom 96-well micro-space culture plates, where the bottom of each well contains micro patterned cone-shaped holes (about 130 micro-spaces) treated with poly(2-hydroxyethyl methacrylate) to create a low-adhesion surface. Cells grown in such plates are forced to interact with each other forming multicellular aggregates within few hours, evolving into spheroids of homogeneous morphology during incubation for several days. The dimension and compactness of the

spheroids can be defined by selecting the initial cell number and incubation time in the 3D culture plate. A seeding cell density of $4x10^4$ cells was found to be suitable for efficient formation of spheroids with an average diameter of 210±25 µm by day 3 (Fig. 2d). The obtained spheroid size is suitable to avoid necrosis in the core and to maintain the functionality of the cells within the aggregate (29). The sphericity factor (ϕ) of 30 spheroids on day 3 was calculated according to equation (1) obtaining an average value of 0.943±0.007. This is close to the icosahedron value (0.939) and indicates that the aggregates are quite uniform and of nearly spherical shape.

Since PLG2 luciferase requires oxygen for BL emission, one issue is related to oxygen availability at the core of spheroid. According to a previous report (30), if we consider a partial pressure of 150 mm Hg for oxygen at 37°C, the outer layer of each spheroid, considered to have a depth of 10–20 µm, approximately reaches complete saturation (90%), and no oxygen limitations are observed with spheroids with an average diameter of 100 µm. A spheroid with a diameter of 150 µm contains less than 2% of cells within a hypoxic core, and 98% of cells composing the spheroids have sufficient oxygen for BL reactions. The BL imaging of Hek293 spheroids expressing PLG2 luciferase (Fig. 2e-f) with a 4X objective allows simultaneous recording of the BL emission from at least 20 spheroids in each field. Then, ROIs are manually selected around each spheroid to quantify the BL emission and corresponding surface area, to correct the signal from each spheroid according to its dimension.

In contrast with confocal fluorescence microscopy, BL emission cannot currently be scanned in z-axis to make a 3D reconstruction of emitted light. In the present configuration, i.e. using micro molded plates to create spheroids and an inverted microscope, it is only possible to focus on a few cells on the bottom surface of the spheroids, while BL emission originates in the upper

portion of the aggregate. This results in more blurred images compared with the 2D format. Nevertheless, since the aim is to determine the global distribution and biological effect of a given treatment/target in a more in-vivo like format, BL imaging of spheroids can represent a convenient, easy to use approach.

3D Bioluminescence assay for inflammatory pathway activation

To evaluate the feasibility of using 3D bioluminescence imaging of Hek293 spheroids for upgrading 2D drug screening, we first developed a 3D assay for inflammatory activity. Three day-old Hek293 spheroids, transfected with a reporter construct in which the PLG2 luciferase is placed under the control of the NFkB (Nuclear Factor kB) response element, were incubated with different concentrations of TNFα (concentration range 0.1-10 ng/mL) for 5 h. The binding of TNFα to its specific endogenous receptor (TNFR) activates the intracellular inflammatory pathway, leading to PLG2 expression.

Hek293 spheroids of uniform size and shape (Fig. 3a-d) had BL emission that increased with TNFα concentration in a dose dependent matter. Noticeably, the BL emission from individual spheroids was not homogeneous. This distribution is actually expected since cells have been transfected directly in 3D; thus, the cell response is affected by the diffusion of the transfection complex within the aggregates. We choose to transfect 1 day-old already formed aggregates (mean dimension 120$\pm$15 μm, estimated 350 cells/spheroid) to simulate the delivery of the nano complex (i.e. plasmid DNA/ FugeneHD cationic polymer) and visualize its biodistribution and cell response after treatment, based on PLG2 expression. To evaluate the response of all the cells composing the spheroid, the establishment of stable cell lines will be required.

Dose-response curves for TNFα were obtained with both monolayer cultures and spheroids, obtaining EC50 values of 2.6±0.4 and 3.5±0.5 ng/ml, respectively (Fig. 4). Compared with the 2D format, a higher NFkB basal activation (4.1±0.3 fold) was found in 3D spheroids. This is consistent with results obtained by Jack et al., who reported the presence of an intra-spheroid cytokine signaling that propagated inside the aggregate inducing NFkB and JNK pathways (31).

CONCLUSIONS

A high-throughput bioluminescence assay based on micro-patterned multi-well plates is reported. The feasibility of the assay was tested using the well-known transcriptional regulation of the nuclear factor k beta (NFkB) response element in human embryonic kidney Hek293 cells. We obtained concentration-response curves and compared them with those obtained using conventional 2D cell cultures. One of the main advantages of this approach is the non-lysing nature of the assay, which allows for repetitive measurements on the same sample. The assay can be implemented in any laboratory equipped with basic cell culture facilities and paves the way to the development of new 3D assays in fields ranging from drug screening to drug delivery.

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SUPPORTING INFORMATION

Supporting Information is available in the online version of this article:

Figure S1. Schematic map (Vector NTI) of inflammation reporter vector pNFkB_PLG2 used to transfect Hek293T cells. The PLG2 luciferse coding sequence (1647bp) was cloned under the control of five copies of an NFkB response element (NFkB-RE) and a minimal CMV promoter from pGL4.32[luc2P/NF-kB-RE/Hygro]. Cloning was confirmed by restriction map analysis and DNA sequencing.

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Figure legends

Figure 1. (a) Normalized emission spectra of PLG2 luciferase expressed in spheroids (solid line) and in 2D monolayer cell cultures (dotted line). Spectra were obtained in non-lysing conditions using D-luciferin as the substrate, as described in the Materials and Methods. (b) Comparison of BL intensities and kinetic profiles obtained with Hek293 cells (2x104 per well) expressing PLG2 luciferase and cultured to form spheroids (solid line) or Hek293 cells (2x104 per well) grown in 2D monolayer cell cultures (dotted line) in 96 well plates.

Figure 2. Brightfield, bioluminescence and pseudocolor overlay images of Hek293 cells expressing PLG2 luciferase, grown in 2D (a,b,c) or in 3D microplate format (d,e,f).

Figure 3. 3D bioluminescent assay for inflammatory pathway activation. Bioluminescence imaging of Hek293 spheroids (4X objective, 30 s acquisition) transfected with 0.10 µg of reporter plasmid pNFkB_PLG2 and treated, 48 h post-transfection with medium only as a control (a) or 0.1, 1.0 or 10 ng/mL TNFα solutions (b,c,d). Magnification bar is 500 µm.

Figure 4. Dose-response curves obtained in 2D (dotted line) or 3D (solid line) cell-based assays. Hek293 cells were incubated for 4 h at 37°C with the indicated concentrations of TNFα, using PLG2 as a reporter under the control of NFkB-response element. BL measurements were obtained after the addition of 1 mM D-luciferin.