1266-B

Assessing Autophagic Flux in 2D and 3D Cell Culture Models with a Novel Plate-Based Assay

James J. Cali,, Braeden L. Butler, Gediminas Vidugiris, Dongping Ma, Michael R. Slater, and Dan F. Lazar

Promega Corporation, 2800 Woods Hollow Rd, Madison, WI, 53711

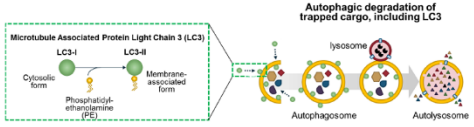
1. Introduction

We have developed a homogeneous plate-based assay to measure autophagic flux that works in 2D and 3D cell culture models. The LC3 protein was tagged on its N-terminus with a spacer sequence and a small subunit of a shrimp-derived luciferase (HiBiT). When stably expressed at low to moderate levels in mammalian cell lines, this novel LC3-based reporter is processed through the autophagic pathway. The cellular level of the autophagy reporter is determined by addition of a lytic detection reagent containing a large subunit of luciferase (LgBiT) and a luminogenic substrate. LgBiT rapidly associates with HiBiT in the cell lysate producing an active NanoBiT luciferase that generates a luminescent signal proportional to the amount of autophagy reporter. Cells stably expressing the autophagy reporter and treated with stimulators of autophagy will show a decreased luminescent signal. Treatment with inhibitors of autophagy results in a buildup in the level of LC3-based reporter and thus a higher luminescent signal. The autophagic flux assay can be multiplexed (on the same sample) with a cytotoxicity assay to serve as a control to detect cytotoxic effects of test compounds. The assay has been shown to have excellent performance in an automated 384 well high throughput screening format using U2OS and HEK293 autophagy reporter cells. The luminescent signal is stable for hours enabling batch processing of multiple 96- or 384-well plates in the same experiment. Both induction and inhibition of autophagic activity was easily observable following reference compound treatment of HEK293 cells grown as 3D spheroids.

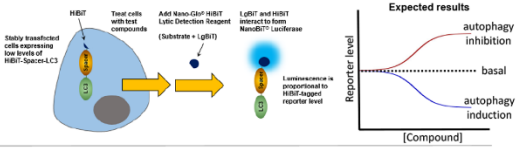
This novel assay method enables screening for modulators of autophagic flux in 2D or 3D culture model systems using a simple homogeneous assay procedure that is recorded with a plate reading luminometer.

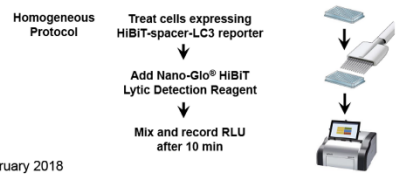
2. LC3 protein dynamics provide a useful indicator of autophagic activity

- **LC3 autophagy marker protein exists mostly as free cytosolic form (LC3-I) under basal conditions.**
- **Induction of autophagic activity promotes LC3-PE conjugation (LC3-II) and membrane targeting.**
- **Substantial LC3-II protein is trapped with cargo upon closure of autophagosome.**
- **Subsequent degradation in the autolysosome results in a decrease in total LC3 protein.**
- **HiBiT-tagged LC3 reporter is a luminogenic LC3 surrogate that enables a simple, plate reader-based method to monitor autophagic flux.**

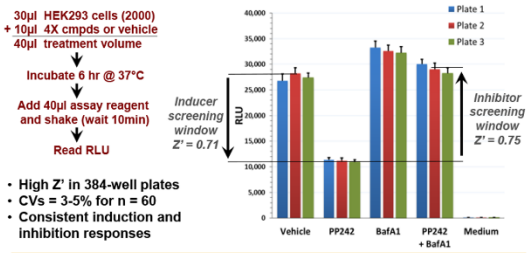


3. How the assay works



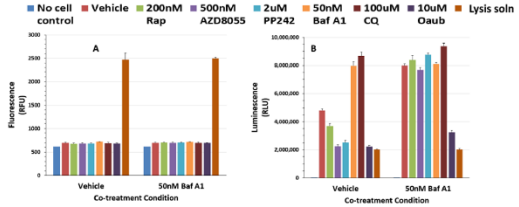


4. Automated assay in 384-well plates



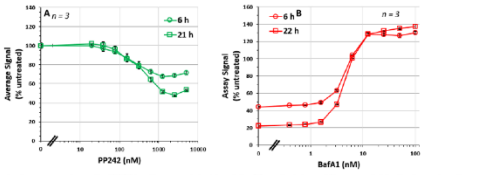
- **High Z' in 384-well plates**
- **CVs = 3-5% for n = 60**
- **Consistent induction and inhibition responses**

5. MOA confirmation for autophagy modulators



Autophagy induction blockade by BafA1 and cytotoxicity assessment. U2OS autophagy reporter cells (8000/well) in 96-well plates in medium with CellTox™ Green Dye. Attached cells were treated for 21 hr with test comps ± Baf A1. **(A)** Cytotoxicity was first assessed by CellTox™ Green fluorescence (only lysis soln killed cells). **(B)** Nano-Glo® HiBiT Lytic Rgnt added and RLU measured after 10 min. Treatments induced or inhibited autophagy as expected.

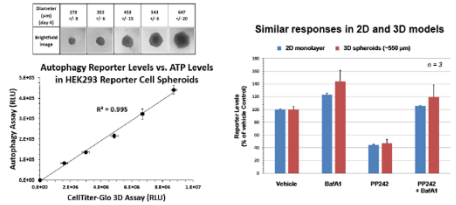
6. Dose-dependent modulation of autophagic flux



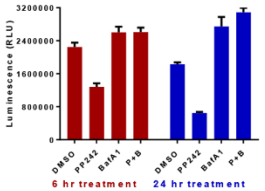
U2OS autophagy reporter cells (8000/well) were plated in white 96-well plates. After overnight attachment, cells were treated for the indicated times with (A) increasing concentrations of a reference autophagy inducer, PP242, or (B) increasing concentrations of a reference autophagy inhibitor, Bafilomycin A1, in the presence of a fixed concentration of PP242 (2µM). Nano-Glo® HiBiT Lytic Reagent was added and luminescence measured after 10 minutes. Assay signal is normalized to time-matched, vehicle-treated controls.

7. Autophagy assay applied to 3D cell culture

- **Autophagy assay signal from different sizes of HEK293 spheroids is proportional to ATP viability marker measured using CellTiter-Glo® 3D Assay in parallel wells**
- **longer mixing time with detection reagent enhanced spheroid lysis**



8. Autophagy reporter transient expression by BacMam transduction



Modulation of transiently expressed autophagy reporter activity. BacMam viral particles harboring the autophagy reporter cDNA were applied (6.25% vol/vol) to U2OS cells (5000/100µL/well) in a 96 well plate. 24 hrs later vehicle (DMSO), PP242 (2µM), BafA1 (50 nM), or PP242 + BafA1 were applied for 6 or 24 hrs before adding Nano-Glo® HiBiT Lytic Rgnt and measuring luminescence. Similar results were obtain with HEK293 cells.

9. Conclusions

NanoBiT™ luciferase technology enables detection of autophagic flux

- The LC3-based autophagy reporter can be stably expressed in desired cell lines
- The autophagy reporter HiBiT subunit is measured by adding LgBiT subunit to reconstitute a bright luciferase activity
- Homogeneous assay protocol: add reagent, mix, measure signal on plate reader
- Stimulation of autophagy results in signal reduction
- Inhibition of autophagy results in signal increase

Assay performance

- Signal is linear over wide range of reporter levels
- Half-life of luminescent signal is greater than 3 hours
- Quality assessment by Z' indicates suitability for HTS for autophagy modulators

This novel multi-well plate assay detects changes in autophagic flux and enables efficient screening for autophagy modulators in both 2D and 3D cell culture models.

February 2018

Corresponding author: jim.cali@promega.com