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

Dan F. Lazar[1], Amani A. Gillette[2], Braeden L. Butler[1], Christopher T. Eggers[1], Brock F. Binkowski, Gediminas Vidugiris[1], Michael R. Slater[1], Dongping Ma[1], Terry Riss[1] and James J. Cali[1]

[1]Promega Corporation, 2800 Woods Hollow Rd, Madison, WI, 53711; [2]University of Wisconsin, Madison WI

P# 2765

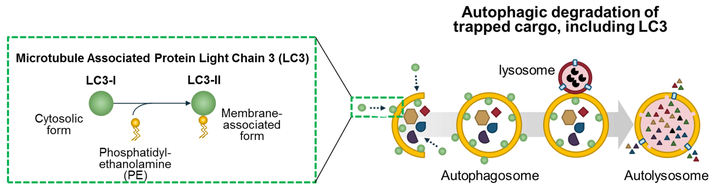
1. Introduction

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 "glow" 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.

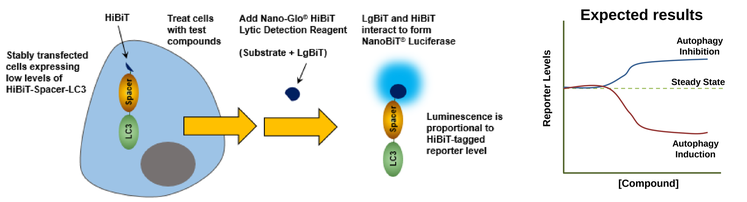
This novel assay method enables screening for modulators of autophagic flux in 2D or 3D culture model systems using a simple homogeneous assay procedure that is recorded with a plate reading luminometer.

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 enables a simple, plate reader-based method to monitor autophagic flux.**



3. How the Assay Works



Homogeneous Protocol

Treat cells expressing HiBiT-spacer-LC3 reporter

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Add Nano-Glo® HiBiT Lytic Detection Reagent

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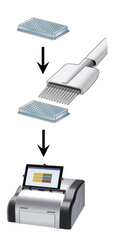
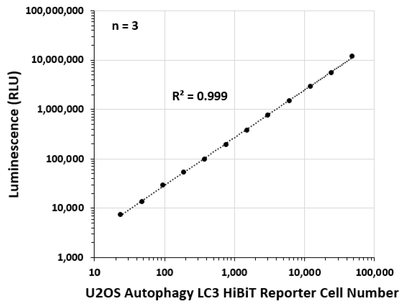
Mix and record RLU after 10 min



4. Autophagy Reporter Assay Performance

Excellent signal linearity vs. LC3 reporter concentration enables utilization of low reporter expression for plate-based assay.



5. Automated Assay Performance in 384-well Plates

- **Z' values demonstrate good performance in 384-well plates**
- **Consistent responses observed to autophagy induction and inhibition**
- **Low replicate variability (CVs = 3-5% for n = 60)**

30µl HEK293 cells (2000)
+ 10µl 4X cmpds or vehicle
40µl treatment volume

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Incubate 6 hr @ 37°C

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Add 40µl assay reagent and shake (wait 10min)

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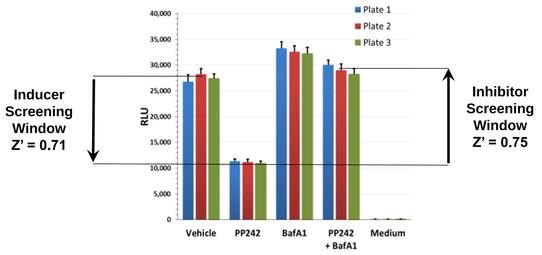
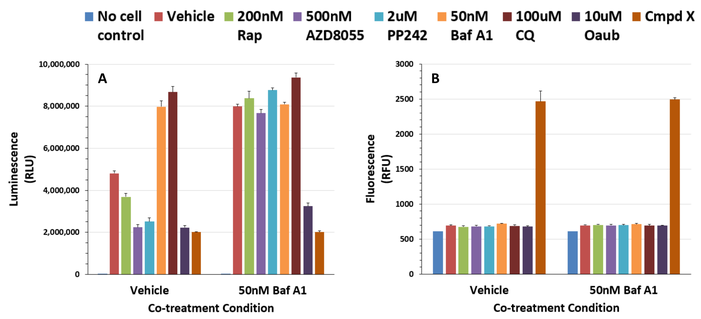
Read RLU



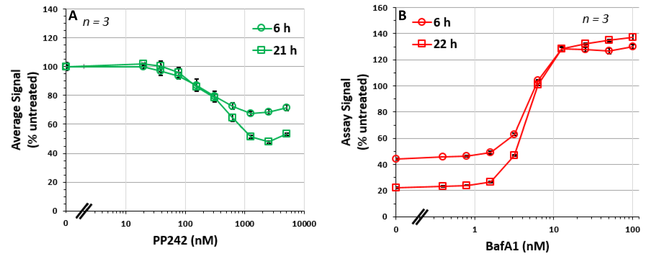
6. MOA Confirmation for Autophagy Modulators



Confirmation of autophagy induction by Bafilomycin A1 blockade and cytotoxicity assessment. U2OS autophagy reporter cells (8000/well) were plated into white, 96-well plates in medium containing CellTox™ Green Dye. After overnight attachment, cells were treated for 21 hr with test compounds both with and without the reference autophagy inhibitor, Bafilomycin A1. **(A)** Nano-Glo® HiBiT Lytic Reagent was added and luminescence measured after 10 minutes. **(B)** Prior to determination of autophagy reporter levels, cytotoxicity assessment was performed by measuring CellTox™ Green fluorescence with only Compound X causing apparent cell death.

7. Dose-Dependent Modulation of Autophagic Flux

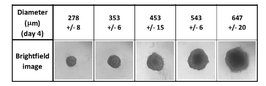
Easily distinguish autophagy induction from inhibition.

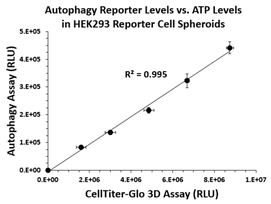


U2OS autophagy reporter cells (8000/well) were plated in white 96-well plates. After overnight attachment, cells were treated for the indicated times with (A) increasing concentrations of a reference autophagy inducer, PP242, or (B) increasing concentrations of a reference autophagy inhibitor, Bafilomycin 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.

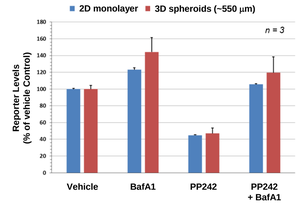
8. Autophagy Assay Applied to 3D Cell Culture

- **Autophagy assay reporter signal from different sizes of HEK293 spheroids is proportional to ATP as a viability marker measured using CellTiter-Glo® 3D Assay in parallel wells**
- **After addition of autophagy detection reagent, longer mixing time was used to ensure disruption of spheroids**





Similar responses in 2D and 3D models



9. Conclusions

NanoBiT™ luciferase technology enables detection of autophagic flux

- HiBiT-spacer-LC3 autophagy reporter is stably expressed in desired cell line
- HiBiT subunit is quantified by addition of LgBiT subunit to reconstitute a bright luciferase activity
- The assay protocol is homogeneous (add reagent; mix sample; record using plate reader)
- Stimulation of autophagy results in decrease in signal
- Inhibition of autophagy results in increase in signal

Assay performance is excellent

- Signal is linear over wide range of reporter levels
- Signal to noise is greater than 1000
- Half-life of luminescent signal is greater than 3 hours
- Suitable for automated high throughput screening to identify modulators of autophagy
- Can be used to detect autophagy reporter in 3D culture model

This novel plate reader assay to detect autophagy reporter enables efficient screening of test agents for impact on autophagic flux in both 2D and 3D cell culture models.

Corresponding author: terry.riss@promega.com