Autophagic Flux Assay for HTS Measured with a Plate-Reading Luminometer

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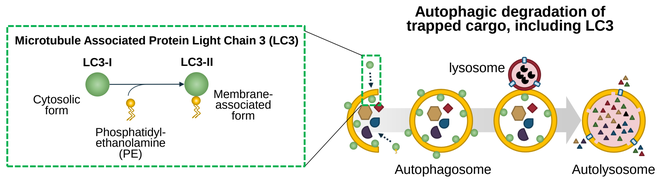
1. Introduction

We have developed a homogeneous assay to quantify autophagic flux using a standard plate-reading luminometer for detection. The assay is designed to measure changes in the level of LC3 protein involved in the process of autophagy and subject to its degradative influences. We have created U2OS and HEK293 cell lines stably expressing a fusion protein containing LC3 and an 11 amino acid fragment (HiBiT) derived from NanoLuc® luciferase. The intracellular level of this HiBiT-tagged autophagy reporter can be sensitively detected by adding a lytic reagent containing the complementing fragment (LgBiT) derived from NanoLuc® luciferase and a substrate (furimazine). LgBiT rapidly associates with HiBiT-tagged protein in the cell lysate, reconstituting a bright luminescent enzyme that produces a sustained "Glo" signal in the presence of furimazine. The detection sensitivity allows quantitation of very low levels of expression of the LC3-based reporter and avoids artifacts that might occur by excessive levels of reporter overexpression. Under basal conditions of cell culture, this HiBiT-tagged autophagy reporter is maintained at a steady-state level of expression. When the process of autophagy is stimulated, the level of reporter decreases as a result of its degradation. In contrast, when the process of autophagy is inhibited, there is a coinciding increase or buildup in the level of autophagy reporter inside the cell. Therefore, changes in assay signal determined in a microplate-based format sensitively reflect alterations in autophagic flux and can be effectively utilized for screening of test agents.

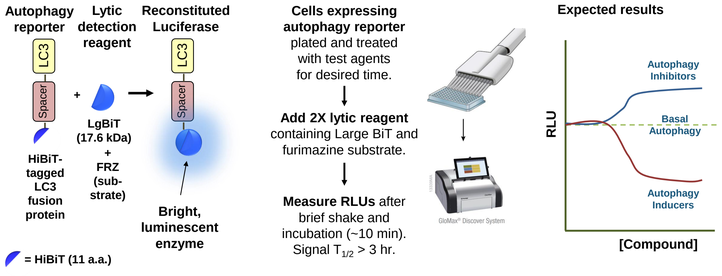
2. LC3 Protein Dynamics Provide a Useful Indicator of Autophagic Activity

- LC3 protein found predominantly as free cytosolic form (LC3-I) under basal autophagic activity.
- Induction of autophagic activity promotes LC3-PE conjugation (LC3-II) and membrane targeting.
- Substantial LC3 protein is trapped along with cargo ( ) upon closure of autophagosome.
- Subsequent degradation in the autolysosome results in a significant decrease in total LC3 protein.
- Supports a simple, plate reader-based method to quantify total LC3 to monitor autophagic flux.



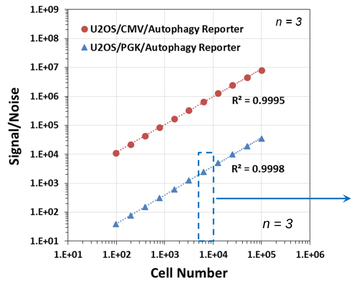
3. Bioluminescent NanoBiT™ Application Supports Homogeneous Detection of Autophagy Reporter

Simple method for plate-based quantitation of autophagic activity



4. Detection of Autophagy Reporter is Possible at Low Levels of Expression

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

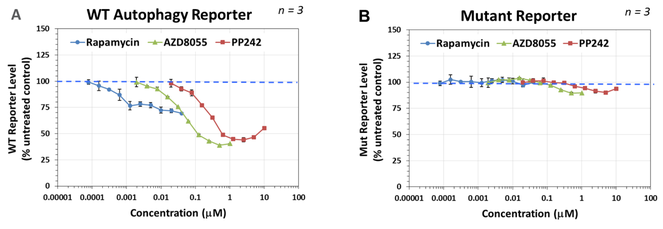


With 5,000-10,000 reporter cells plated for an autophagy assay

- S/N > 1000
- $T_{1/2}$ > 3hr

5. Dose-Dependent Induction of Autophagic Flux

- Dose-dependent induction of autophagy by mTOR inhibitors
- Stratification of compound effects is consistent with known activities
- Low replicate variability in 96-well assay format
- Negligible response of mutant reporter (contains LC3 G120A mutation)

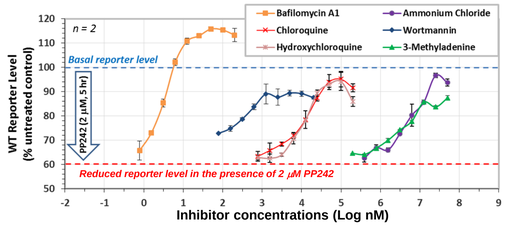


Compound treatment (8 hr) of U2OS cells with low reporter expression

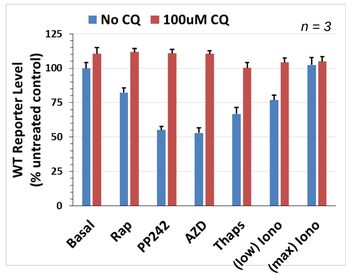
6. Dose-Dependent Inhibition of Autophagic Flux

- Effect of pathway inhibitors in the presence of an autophagy inducer (PP242)
- Stratification of compound effects is consistent with known potencies
- Dose-dependent response independent of compound MOA

PP242 (inducer) vs. autophagy inhibitor cotreatment (5 hr) of U2OS reporter cells



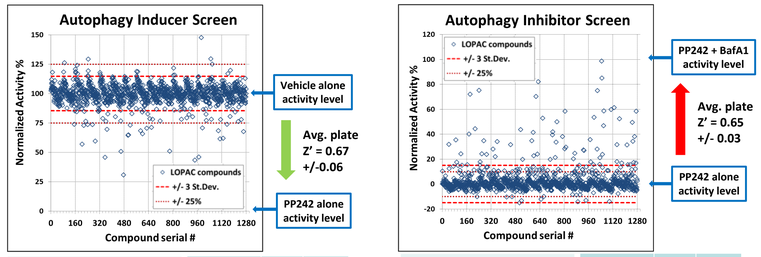
7. Autophagy Reporter Response to Various Inducers in Presence and Absence of Autophagy Inhibitor



- Responses to a panel of autophagy inducers consistent with literature
- Consistent blockade of responses by chloroquine (CQ), an autophagy inhibitor
- Ionomycin "max" concentration kills cells rapidly, preventing autophagic response
- Multiplex with CellTox™ Green is possible for same-well cytotoxicity assessment (data not shown)

Compound treatment (8 hr) of U2OS reporter cell line

8. LOPAC Screen in Low Volume 384-Well Plates



0.005μl cmpds
+ 8.0μl HEK293 cells (1000)
8.0μl treatment vol
Incubate 6hr @ 37ºC
Add 8.0μl assay reagent and shake (wait 10min)
Read RLUs

autophagy induction	# hits	hit rate
>25%	26	2.0%
>40%	8	0.6%

0.005μl cmpds
+ 1.0μl PP242 (8X) cells
+ 7.0μl HEK293 cells (1000)
8.0μl treatment vol
Incubate 6hr @ 37ºC
Add 8.0μl assay reagent and shake (wait 10min)
Read RLUs

autophagy inhibition	# hits	hit rate
>25%	44	3.4%
>40%	23	1.8%

9. Conclusions

NanoBiT™ application enables autophagic flux assay development
- Bioluminescent, homogeneous, plate reader assay
- HiBiT-tagged LC3-based reporter easily detected at low levels
- Excellent signal linearity over wide range of reporter levels

Directional, quantitative determination of autophagic flux
- Autophagy stimulation → decreased reporter levels
- Autophagy inhibition → increased reporter levels
- Both upstream and downstream inhibitors easily detected
- S/N > 1000 and signal $T_{1/2}$ > 3hr in autophagy assay

Novel, plate reader assay enables efficient screening of test agents for impact on autophagic flux

Challenge for other assay methods

Autophagic Flux Regulation	Plate-based assay signal (RLUs)	Puncta formation rate
Stimulation	↓	↑
Upstream Inhibition	↑	↓
Downstream Inhibition	↑	↑

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