

## Abstract

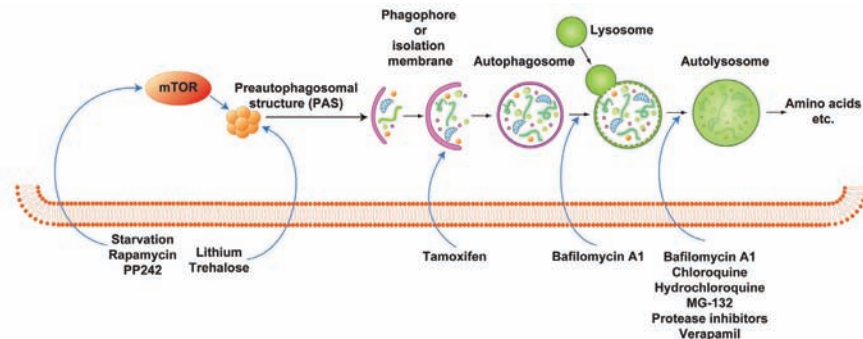
Autophagy is a normal degradative pathway that involves the sequestration of entire organelles, protein complexes, and misfolded proteins in a membrane vacuole called the autophagosome. The autophagosomal vacuole is subsequently delivered to the lysosome where it is degraded into its essential constituents and recycled back to the cytoplasm. Autophagy plays important roles in diverse biological events, including responses to nutrient limitation, tumor suppression, the immune response, and aspects of neurodegeneration. However, experimental methods to monitor this process in mammalian cells are limited and often highly qualitative. For example, LC3, a mammalian homologue of the ubiquitin-like (UBL) protein Atg8, has been used as a specific marker of autophagosomes in mammals. However, current methods to quantify autophagic activity using LC3 are time-consuming and labor-intensive, involving the physical counting of fluorescent punctate signal forming within the cells. We have developed and validated a novel cell-based autophagy assay using a 488 nm-excitable green-emitting fluorescent probe to highlight the various vacuolar components of the autophagy pathway. We demonstrated that the accumulation of autophagy probe was specifically induced by amino acid deprivation and was inhibited by 3-methyladenine, a classical inhibitor of the autophagic pathway. Furthermore, a population of this dye-labeled vesicle co-localizes with LC3. We have validated this fluorescent probe under a wide range of conditions known to modulate autophagy pathways. We have optimized the use of the EL406™ Combination Washer Dispenser to automatically aspirate media, wash cells and dispense reagents for the assay, allowing, for the first time, easy quantitation of autophagy using a convenient fluorescence microplate-based HTS format. This assay enables kinetic analysis of the autophagy pathway and is able to distinguish between increases in autophagic flux vs. autophagic vacuole accumulation.

## Introduction

When subjected to less than optimal growth conditions that threaten cell survival, eukaryotic cells employ a lysosome-mediated intracellular bulk degradation pathway for digestion of cellular contents referred to as autophagy [1]. Cytoplasmic constituents including organelles and proteins are sequestered into double-membrane autophagosomes. These autophagosomes subsequently fuse with lysosomes where their contents are degraded. Autophagy occurs at low basal levels in all cells as part of normal homeostatic-turnover of proteins and organelles. Autophagy plays an important role in maintaining the amino acid pool in times of starvation, removal of damaged proteins and organelles, tumor suppression, cellular differentiation, clearance of intracellular microbes, and regulation of immunity [1]. Autophagy is a dynamic multistep process (Figure 1) which can be regulated at several steps. Autophagy in cultured cells is typically low under normal conditions, but can be upregulated by a variety of physiological stimuli such as amino acid starvation, elevated temperature, and infection. In addition, pharmacological agents can influence autophagy either up or down.

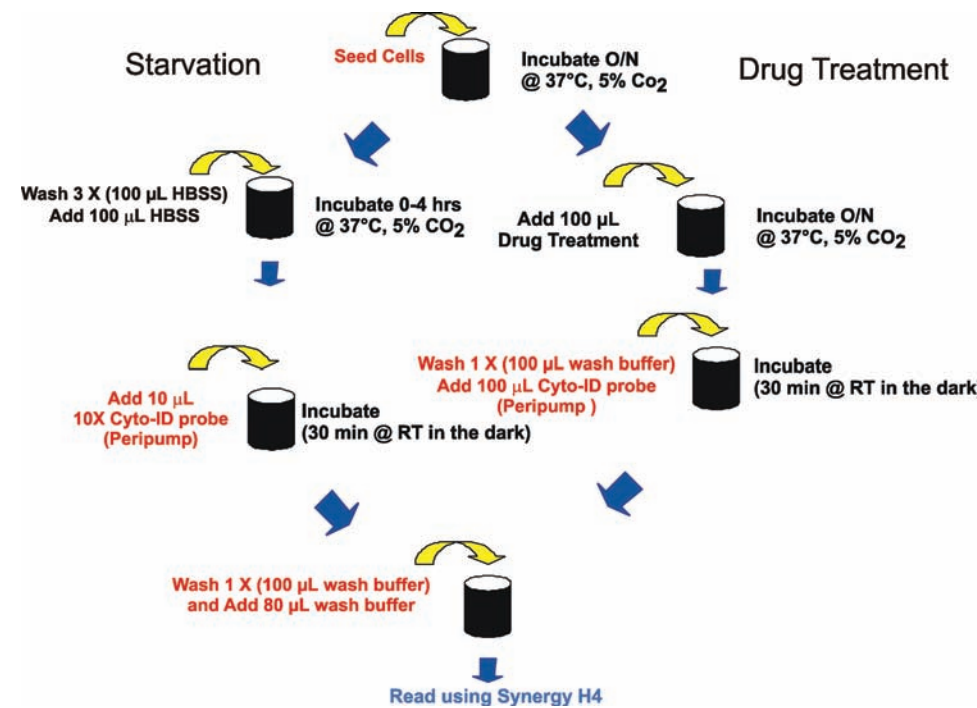
At the molecular level, several conserved factors are associated with autophagy. TOR kinase, the target of Rapamycin, is the major inhibitory signal that shuts off autophagy in the presence of growth factors [2]. The PI3k/Akt signaling pathway connects the receptor tyrosine kinases to TOR activation, thus repressing autophagy in response to insulin-like and other growth factors. The 5'-AMP-activated kinase (AMPK), which responds to low energy conditions and eukaryotic initiation factor (eIF2α), which responds to nutrient starvation are two of the many regulatory molecules [2]. Downstream of TOR there are more than 20 conserved ATG genes in yeast that are essential for autophagy [3]. Phosphatidylethanolamine conjugation of Microtubule-associated protein 1A/1B light chain protein 3 (LC3) results in the recruitment and translocation of this cytosolic protein to the autophagosomal membrane, making the conjugated form of LC3 (LC3-II) a specific marker for the autophagosome [4]. Tracer compounds that co-localize with LC3-II could be used to monitor autophagy.

## Autophagy



**Figure 1** – Schematic depiction of Autophagy. Cytosolic material is sequestered by an expanding membrane sac, the phagophore, resulting in the formation of a double membrane vesicle, an autophagosome. The outer membrane of the phagosome subsequently fuses with the lysosome and the internal material is degraded in the autolysosome. Various regulators of autophagy are also depicted.

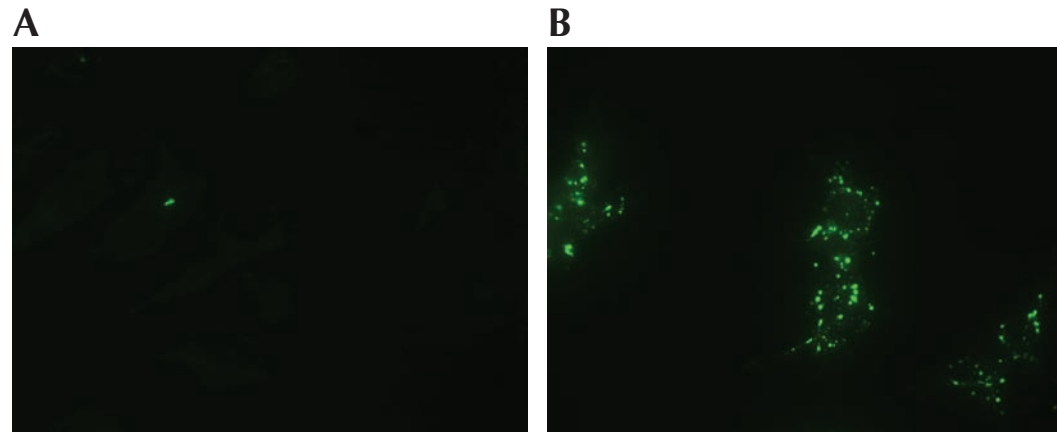
## Assay Process



**Figure 2** – Schematic of the Automated Cyto-ID™ Assay Process Carried out by the EL406 Washer Dispenser (Red text) and Synergy™ H4 Reader (Blue text)

For all experiments, HepG2 cells were seeded into 96-well plates at 20,000 cells per well (100 µL) and allowed to attach overnight. The following morning the cells were amino acid starved for up to 4 hours or treated with increasing doses of various compounds. Amino acid starved cells were washed 3 times with HBSS and 100 µL HBSS added to each well. After starvation 10 µL of Cyto-ID/Hoechst 33342 concentrate (10x) was added directly to the wells. Drug treated cells were exposed for 18-hours and then washed (PBS + 2% FBS) and Cyto-ID Green and Hoechst 33342 dyes were added using the EL406. In both procedures, after a 30 minute incubation, excess dye was removed by washing followed by a final addition of 80 µL of wash solution. The fluorescence was then determined using a Synergy H4 reader. Cyto-ID Green dye (green fluorescence) was measured using an excitation of 480 nm and an emission of 530 nm, while Hoechst dye (blue fluorescence) was determined with an excitation of 340 nm and an emission of 480. Cyto-ID data was normalized against the Hoechst signal and the fold change from untreated calculated and plotted.

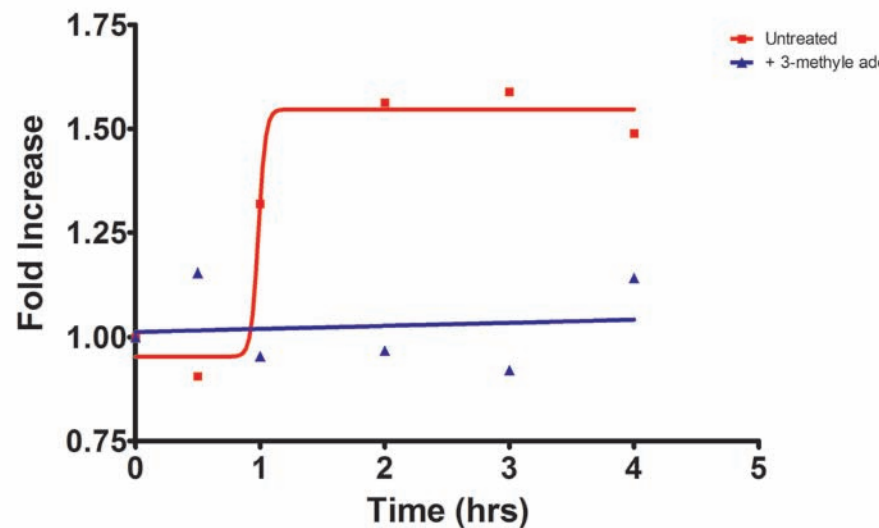
## Phagosome Formation



**Figure 3** – Cyto-ID localization in the perinuclear region of the cell. Figure A depicts untreated cells, while figure B shows staining after 4 hour starvation.

When the CELLestial dye Cyto-ID is incorporated into cells, the accumulation of the fluorescent probe is typically observed in spherical vacuoles in the perinuclear region of the cell or in foci distributed throughout the cytoplasm depending on the cell type. Cyto-ID staining has been shown to co-localize with LC3 when autophagy has been induced in HeLa cells transfected with RFP-LC3 (data not shown).

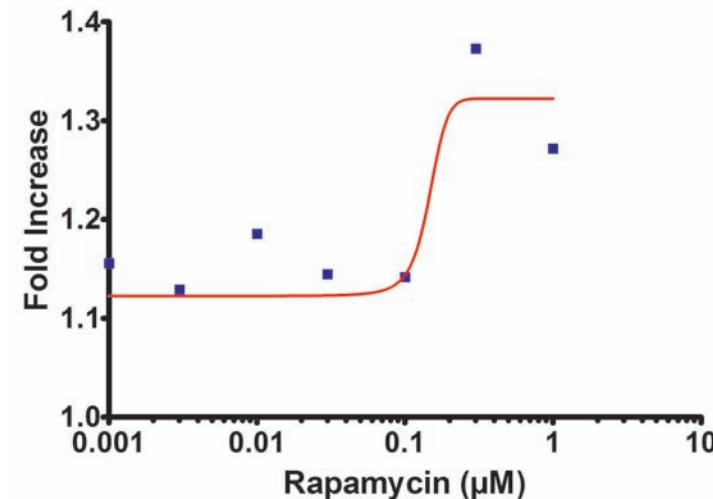
## Nutrition



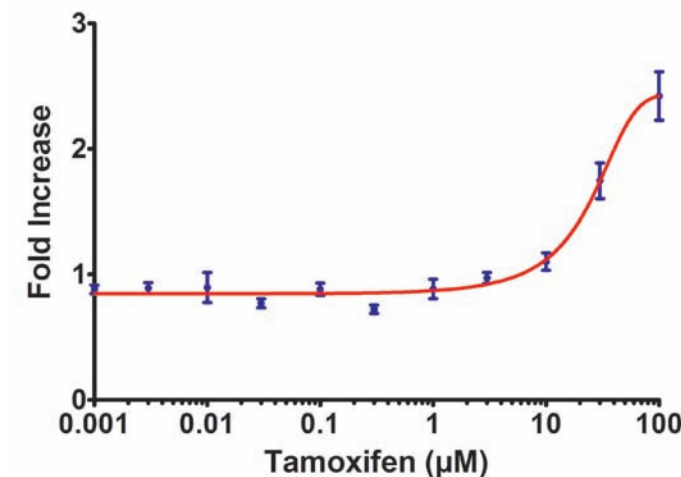
**Figure 4** – Effect of Starvation on Cyto-ID Signal.

A number of different effectors of autophagy were tested using the CELLestial Dye, Cyto-ID in combination with the EL406 Washer Dispenser system. Amino acid starvation for as little as 1 hours demonstrates an increase in Cyto-ID signal as compared to the untreated control (Figure 4). The addition of 3-methyl adenine at the time of starvation, a known inhibitor of autophagy, abolishes this increase. (Fig 4). Overnight incubation of HepG2 cells with Rapamycin, which inhibits mTOR kinase, also results in an increase in Cyto-ID signal (Figure 5). Likewise ATP-competitive inhibitors of mTOR such as PP242 will also increase Cyto-ID signal (Table 1). Tamoxifen, which increases the intracellular level of ceramide and abolishes the inhibitory effect of PI3K, can increase Cyto-ID signal at concentrations above 1 µM with a 16 hour exposure (Figure 6). Verapamil is a small molecule that passively diffuses into the lysosome and becomes trapped upon protonation. Verapamil causes an increase in lysosomal pH, which inhibits lysosome function and blocks fusion with the autophagosome. Cellular exposure to concentrations of 10 µM or greater resulted in an increase in Cyto-ID signal.

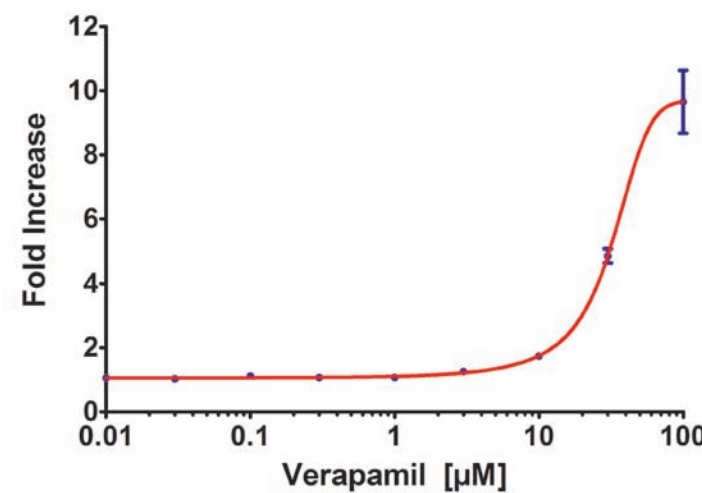
## Pharmacology



**Figure 5** – Effect of Rapamycin on Cyto-ID signal.



**Figure 6** – Effect of Tamoxifen on Cyto-ID signal.



**Figure 7** – Effect of Verapamil on Cyto-ID signal.

## EL406 Washer Dispenser



**Figure 8** – EL406 Washer Dispenser

The EL406 Washer Dispenser combines several existing fluid handling technologies into one instrument. The patented dual-action manifold provides full plate washer functionality 96- and 384- and 1536-well microplates. The priming trough has been replaced with a sonicator bath to provide easy cleaning of the aspiration and dispense-tubes of the washer manifold. Immediately adjacent to the full plate washer manifold is an eight channel peristaltic pump capable dispenser uses autoclavable cassettes that come in different sizes to optimize fluid dispense accuracy and precision. In addition to a low dead volume, reagents can be also be recovered by reversing the direction of the peristaltic pump. Two optional syringe pump dispensers can also be utilized to dispense additional reagents.

## Validated Compounds

Treatment	Target	Effect	µM used	Induction Time (hrs)	Cell Line
Starvation	Inhibits mammalian target of rapamycin (mTOR)	Activates autophagy	N/A	1~4	HeLa & Jurkat
Rapamycin	Inhibits mammalian target of rapamycin (mTOR)	Activates autophagy	0.2	6~18	HeLa & Jurkat
PP242	ATP-competitive inhibitor of mTOR	Activates autophagy	1	18	HeLa
Lithium	Inhibits IMPase and reduce inositol and IP <sub>3</sub> levels; mTOR-independent	Activates autophagy	10,000	18	HeLa & Jurkat
Trehalose	Unknown, mTOR-independent	Activates autophagy	50,000	6	HeLa & Jurkat
Bafilomycin A1	Inhibits Vacuolar-ATPase	Inhibits autophagy	6~9*10 <sup>-3</sup>	18	HeLa & Jurkat
Chloroquine	Alkalinizes Lysosomal pH	Inhibits autophagy	10~50	18	HeLa & Jurkat
Tamoxifen	Increases the intracellular level of ceramide and abolishes the inhibitory effect of PI3K	Activates autophagy	4~10	6~18	HeLa & Jurkat
Verapamil	Ca <sup>2+</sup> channel blocker; reduces intracellular Ca <sup>2+</sup> levels; mTOR-independent	Activates autophagy	40~100	18	HeLa & Jurkat
Hydroxychloroquine	Alkalinizes Lysosomal pH	Inhibits autophagy	10	18	HeLa & Jurkat
Loperamide	Ca <sup>2+</sup> channel blocker; reduces intra-cytosolic Ca <sup>2+</sup> levels; mTOR-independent	Activates autophagy	5	18	HeLa
Clonidine	Imidazoline-1 receptor agonist; reduces cAMP levels; mTOR-independent	Activates autophagy	100	18	HeLa
MG-132	Selective proteasome inhibitor	Induce aggresome	2~5	18	HeLa & Jurkat
Epoxomicin	Selective proteasome inhibitor	Induce aggresome	0.5	18	HeLa
Lactacystin	Selective proteasome inhibitor	Induce aggresome	4		
Velcade®	Selective proteasome inhibitor	Induce aggresome	0.5	18	HeLa
amyloid beta peptide 1~42	Induce oxidative stress	Induce aggresome	25	18	SK-N-SH
Norclomipramine	Alkalinizes Lysosomal pH	Inhibits autophagy	5~20	18	HeLa

**Table 1** – Assay Validated Compounds

## Conclusions

1. The CELLestial™ Cyto-ID Kit provides an easy means to screen compounds for the induction or inhibition of Autophagy.
2. The described assay is rapid, sensitive and specific. It is also compatible with standard high-throughput microplate-based screening workflows.
3. The EL406 Washer Dispenser is capable of automating the different fluid handling steps of several different CELLestial assays.