Visualizing subcellular vesicles to quantitate autophagy in neuronal cells

Cyto-ID® Autophagy Detection Kit (ENZ-51031)

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OVERVIEW OF THE BIOLOGY

Autophagy is an intracellular catabolic process that sequesters and degrades proteins and organelles that have either been recognized as faulty or are simply no longer needed by the cell. Autophagy supports normal cellular processes by carrying out the "housekeeping" function of removing invasive microorganisms, misfolded proteins, and degraded organelles. Autophagy is essential to the maintenance of cell homeostasis and, in addition to its role as a mechanism of cell material turnover, autophagy supports the response of an organism to challenges such as starvation by adjusting rates of energy consumption and material re-utilization.

Both beneficial and harmful roles for autophagy have been discovered in cancer, infectious disease, diabetes, neurodegenerative disease, and other conditions. The role of autophagy is of particular interest in the study of neuronal cells, where it helps to maintain protein structure and function over long axonal distances. Unlike the cells of other tissues, the cytosolic contents of neurons are not routinely diluted by mitosis. Altered autophagy and accumulation of cellular toxins are seen in Alzheimer's disease, Parkinson's disease, and other neurological disorders.[2] These observations have led to the consideration of the various mechanisms of autophagy as potential areas of therapeutic interventions. Quantitation of the effects of experimental variables on this process is an essential element of research that may lead to the development of safe and effective treatments targeting autophagy.

VISUALIZING AUTOPHAGY

Autophagy can be visualized and characterized even in heterogeneous cell populations using readily available reagents. Cyto-ID® Autophagy Reagent (Enzo Life Sciences, ENZ-51031) selectively stains the membranes of phagophores, autophagosomes, and autolysosomes. It has also been shown to co-localize with the autophagosome protein LC3-II. It has a fluorescence excitation/emission profile of 495/519 and can be used with a standard FITC filter set. Destructive autophagosome fusions with lysosomes can be detected with the aid of lysosome-specific fluorescent visualization. In the examples that follow, LysoTracker Deep Red with excitation/emission wavelengths of 647/668 was used to characterize lysosomal activity. A standard Cy5 filter set can be used to detect emissions from LysoTracker Deep Red.

BENEFITS

- Accurate segmentation of unlabeled neurites
- Deeper insights into mechanisms of neurodegenerative disease
- Precise discrimination of similar subcellular structures
- Rich data generated by measuring multiple biologies in one well

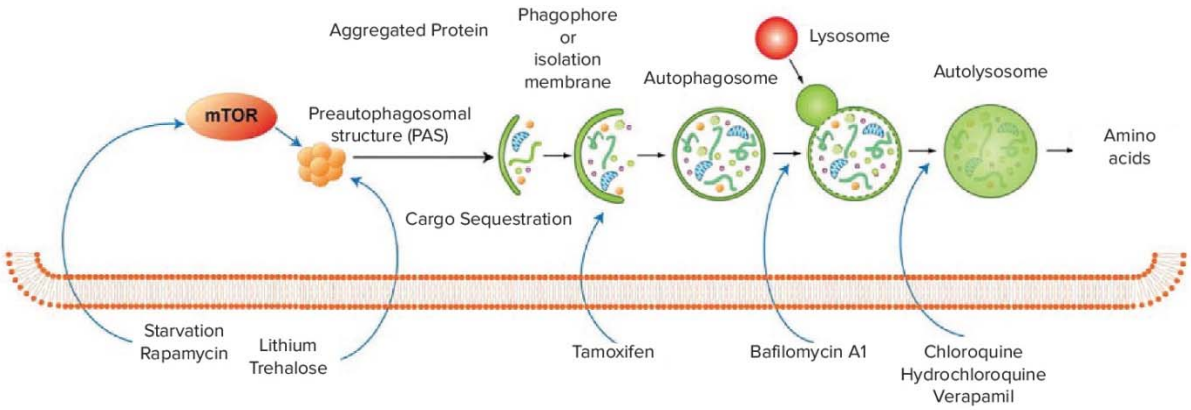

Figure 1: The process of autophagy begins when an internal or external signal promotes the formation of a phagophore, a spherical double-membrane sequestering structure. Microtubule-associated protein 1 light chain 3 alpha (LC3) stimulates elongation of the phagophore, which begins to engulf cytoplasmic targets. In a process mediated by autophagy-related (ATG) proteins, the phagophore closes around the targets to become an autophagosome. The autophagosome then fuses with a lysosome, exposing its contents and its own membrane to degradation by hydrolytic enzymes.[1]

QUANTITATION METHODS

The level of Cyto-ID Green staining of autophagosomes and LysoTracker Deep Red staining of lysosomes in live cells provides information about the mechanism of a compound's stimulation or inhibition of the autophagy pathway. This can be accomplished using the ImageXpress® Micro XLS System, a high content screening system for widefield fluorescence or brightfield imaging of fixed or live cells, tissues, and small organisms. MetaXpress® software analyzes images using either pre-configured applications or customized modules to characterize phenotypic changes and produce specific outputs. A Custom Module enables the simultaneous detection, measurement and area summation of multiple stained bodies, such as nuclei, autophagosomes, and lysosomes. Measured values can be plotted over time or against experimental conditions such as a range of drug compound concentrations.

INCREASED QUANTITY OF AUTOPHAGOSOMES INDICATES CELL DISTRESS

In the examples that follow, iCell (Cellular Dynamics Intl.) human-induced pluripotent stem cell (iPSC) derived neurons and rat PC-12 cells were used to demonstrate the utility of testing for multiple cellular responses in each well of a 384-well microplate.

HUMAN NEURONS

Neurons were plated at 5000 cells/well and grown as following the manufacturer's recommendations. After 5 days, cells were treated with a dilution series of compounds prepared in maintenance media. After 24 hours of compound incubation, staining for lysosomes, nuclei and autophagosomes was performed and live cells were imaged using ImageXpress Micro XLS Widefield Automated System with the chamber heated to 37°C. Images were acquired from 2-4 sites per well using either a 60X Plan Fluor or 40X Plan Apo objective. Image analysis was accomplished using MetaXpress software to quantitate the effects of exposure to experimental compounds over a range of concentrations. While example images show cells at 60X magnification for illustrative purposes, the data in the graphs in Figures 3-6 are from the same experiments but utilizing 40X magnification. The lower magnification produces images of sufficient quality for measurement of intracellular vesicles while offering the benefit of capturing more cells within each field of view.

RAT PC-12 CELLS

PC-12 cells were plated at 2000 cells/well and cultured overnight before treatment with a dilution series of compounds prepared in complete media. After 24 hours, staining for lysosomes, nuclei and autophagosomes was performed and live cells were imaged at 37°C. Images were acquired from 2-4 sites per well at 40X magnification. Image analysis was accomplished using MetaXpress software to quantitate the effects of exposure to experimental compounds over a range of concentrations.

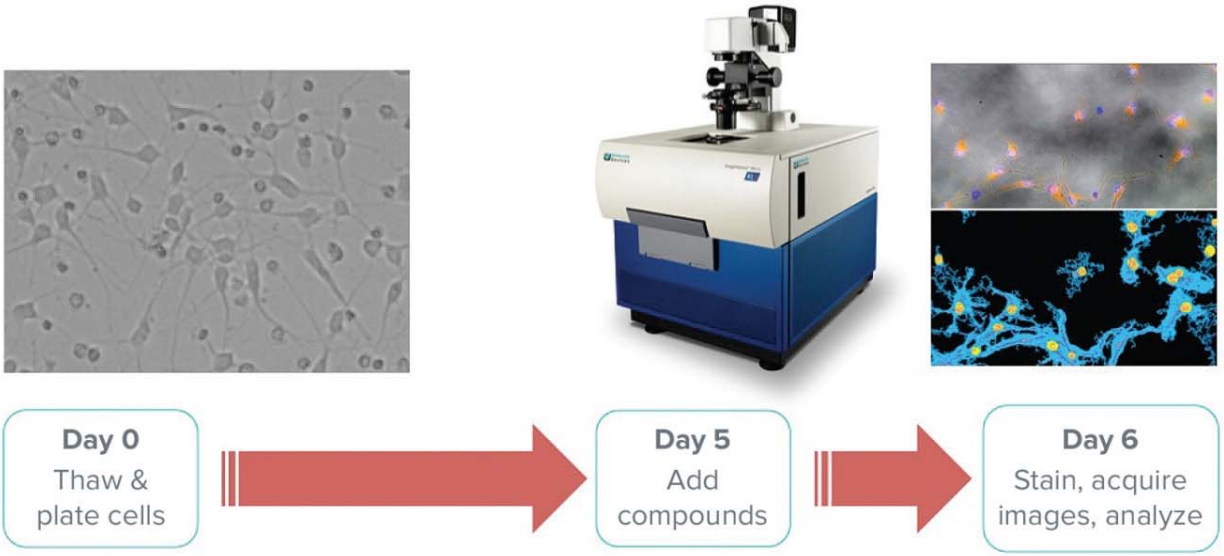

Figure 2: The straightforward and automated workflow is amenable to high-throughput screening with minimal hands-on time required even for an experiment that may take days from plating to end results.

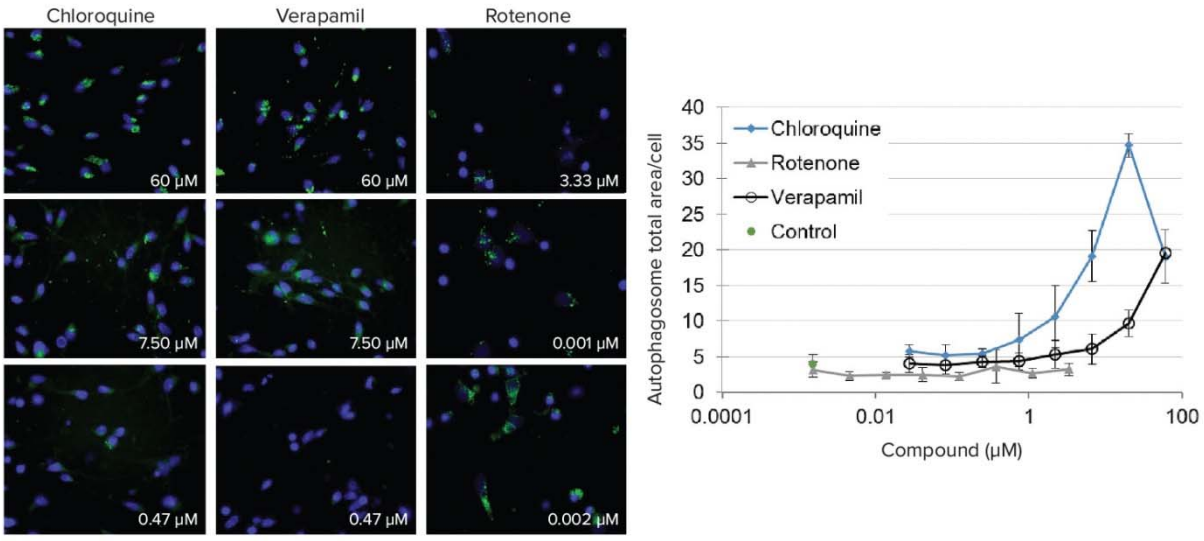

Figure 3: (Left) Representative 60X magnification images of human neurons following exposure to three different compounds at three different concentrations. Autophagosomes (green) respond to the cell treatment in a dose-dependent manner. **(Right)** Dose-response effect of chloroquine, rotenone, and verapamil on aggregate autophagosome area in human neurons. Autophagosomes may be inhibited or stimulated by compound exposure.

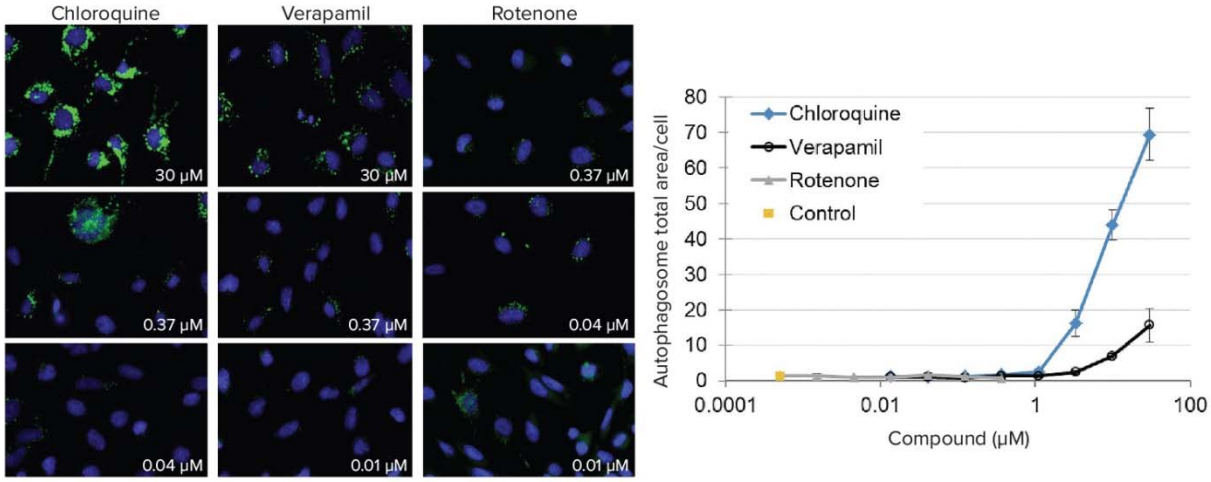

Figure 4: (Left) Representative 60X magnification images of rat PC-12 cells following exposure to three different compounds at three different concentrations. Autophagosomes (green) can be clearly visualized and measured. **(Right)** Dose-response effect of chloroquine, rotenone, and verapamil on aggregate autophagosome area in rat PC-12 cells. Autophagosomes may be inhibited or stimulated by compound exposure.

LYSOSOMAL RESPONSE INDICATES DISRUPTION IN THE AUTOPHAGIC PATHWAY

HUMAN NEURONS

The assay for lysosomes was conducted in the same wells and with the same procedure as that described previously but with the addition of LysoTracker Deep Red for detecting the lysosomes.

RAT PC-12 CELLS

The same assay for measuring multiple toxic events was conducted in PC-12 cells. Several parameters were measured to determine effect on intracellular lysosomes.

ACCURATE NEURON SEGMENTATION USING TRANSMITTED LIGHT IMAGES

It can be challenging to detect autophagosomes that are in the neurite outgrowths, away from the cell body. Obtaining a brightfield image of a nonstained cell body aids in segmenting cells with long processes. A Custom Module was constructed in order to accurately quantitate only stained organelles associated with a neuron. This assures the detection of only cellular objects-of-interest and not staining artifacts or debris (Figure 7).

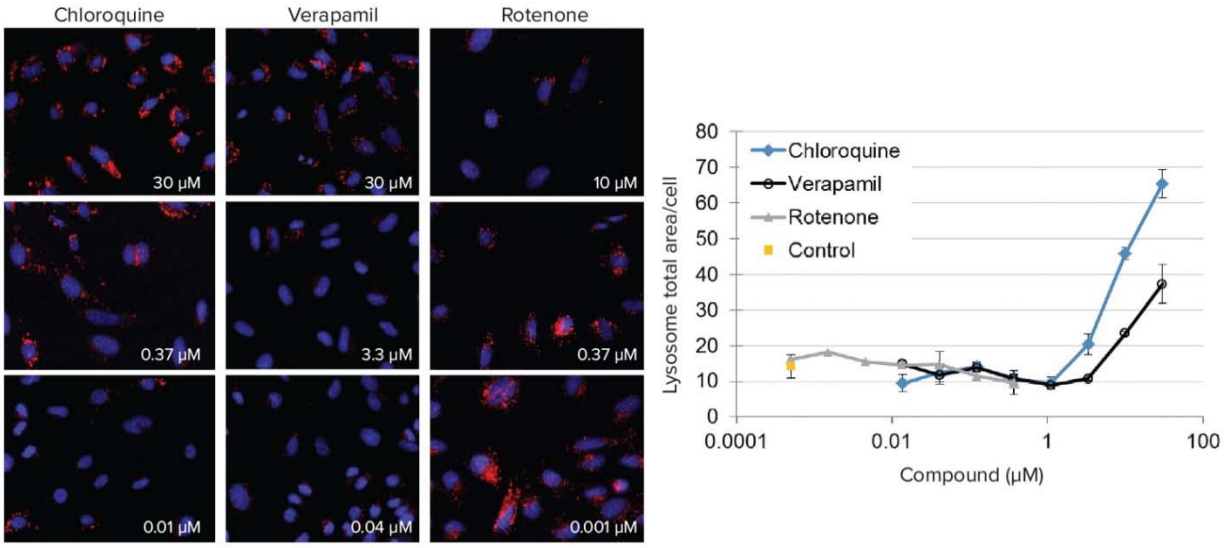

Figure 5: (Left) Representative images of iPSC derived neurons following exposure to three different compounds at three different concentrations. Lysosomes (red) can be visualized and measured to detect responses. **(Right)** Dose-response effect of chloroquine, rotenone, and verapamil on total lysosome area, a second measure of autophagy, in neurons. Lysosomal measurements can indicate disruption of the autophagosome pathway at the final step of fusion of the two vesicles.

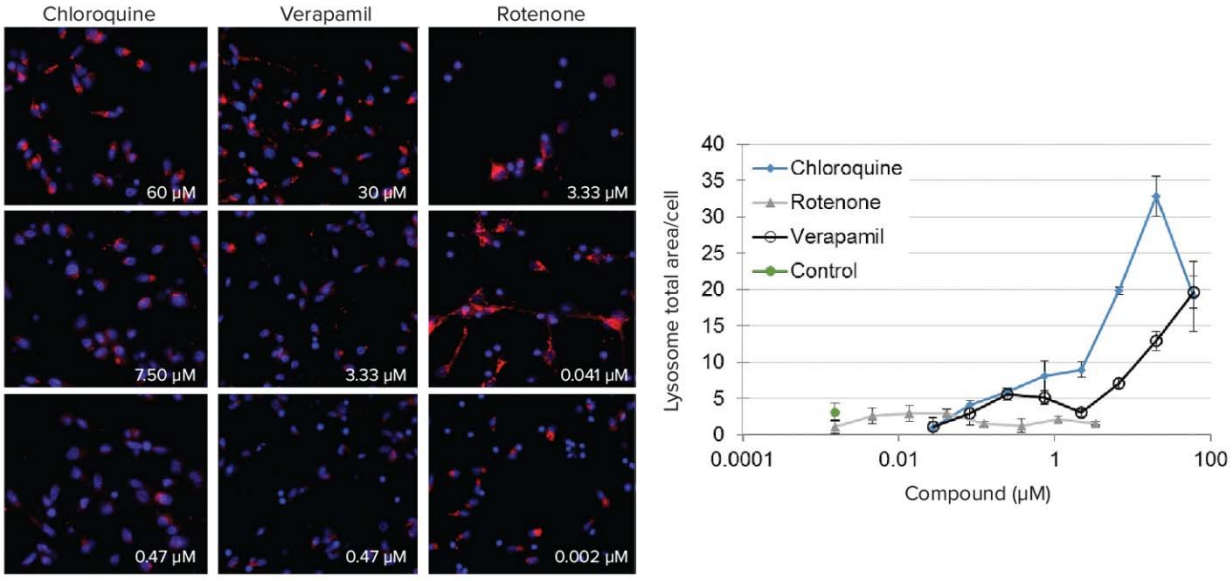

Figure 6: (Left) Representative images of rat PC-12 cells following exposure to three different compounds at three different concentrations. Above images were acquired using a 60X PF objective. Lysosomes visualized by LysoTracker Deep Red stain are shown in red. Quantitating the decrease or increase in intracellular lysosomes gives clues to the compound's mechanism of toxicity. **(Right)** Dose-response effect of chloroquine, rotenone, and verapamil on aggregate lysosome area, a second measure of autophagy, in rat PC-12 cells. Lysosomes may be inhibited or stimulated by compound exposure.

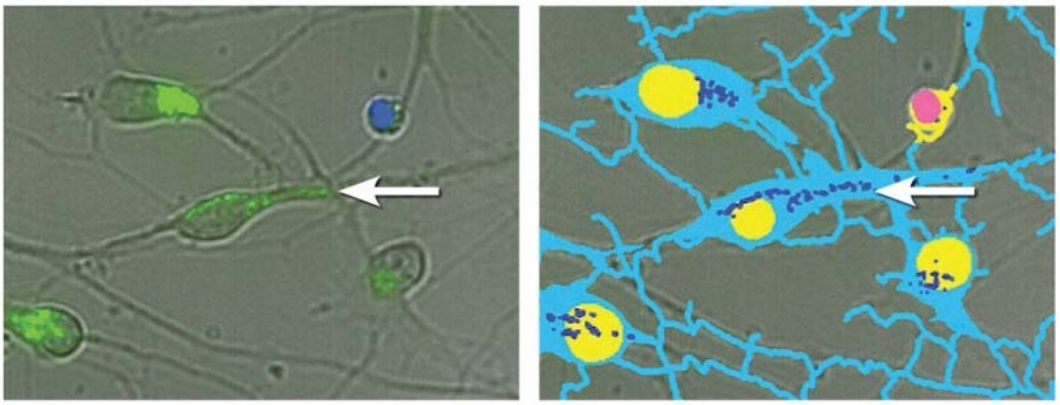

Figure 7: (Left) Overlay of zoomed 40X magnification images of transmitted light (grey), Hoechst stained nuclei (blue), and Cyto-ID stained autophagosomes (green). **(Right)** Segmentation mask with cell bodies and outgrowths identified in teal from the transmitted light image, healthy nuclei identified as yellow, apoptotic nuclei identified as pink, and autophagosomes identified by royal blue. Autophagosomes can be counted even if located far from the cell body (as pointed out by the arrows).

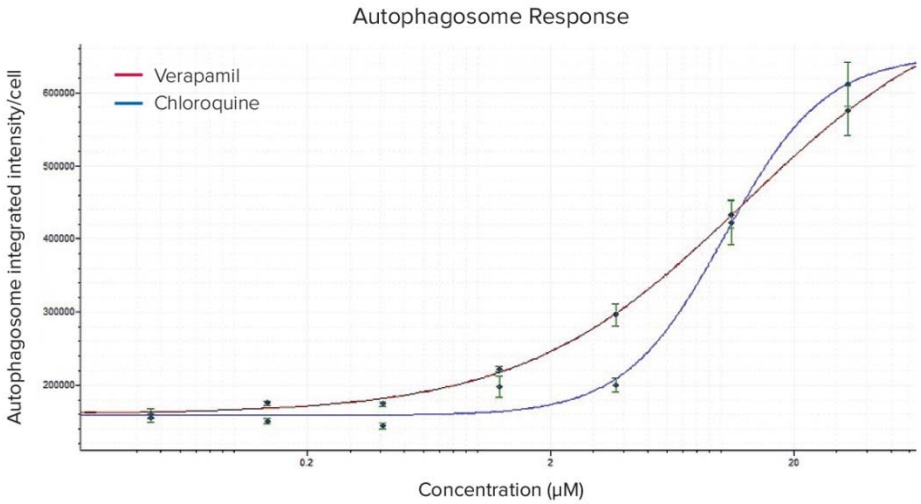

Figure 8: Dose response curves of autophagosome formation for Verapamil and Chloroquine. Images were analyzed using a Custom Module as defined in Figure 7 and the measurements generated were used to plot the curves in AcuityXpress™ Analysis Software.

EC_{50}	Autophagosomes		Lysosomes	
Compound	PC-12	Neuron	PC-12	Neuron
Chloroquine	5.5 µM	5.8 µM	4.4 µM	2.0 µM
Verapamil	21 µM	>60 µM	18.8 µM	9.5 µM
Rotenone	Flat, no fit	Flat, no fit	73 nM	Flat, no fit

Figure 9: IC50 or EC50 values are calculated for compounds that either inhibit or stimulate autophagy by fitting the dose response curves to a non-linear model. It is not unexpected for different cell types to display different sensitivities to drug exposure.

SUMMARY

Scientific interest in the quantitation of neuronal autophagy is driven, in part, by the potential value of the cellular process of autophagy as a target for therapeutic intervention against neurodegenerative diseases, such as Parkinson's, Alzheimer's and Huntington's disease. Off-the-shelf reagents exist for specific fluorescence microscopy visualization of autophagosomes, lysosomes, and other cell components involved in autophagy. The ImageXpress Micro XLS System offers a set of features, including widefield high-resolution brightfield and multiplewavelength fluorescence microscopy, that enable the capture of images of human and rat neuronal cells cultured in 384-well microplates. MetaXpress software analyzes these images and charts the relationships between experimental conditions and the occurrence of autophagy, as quantified by the measured aggregate areas for autophagosomes, lysosomes, and other visualized cellular components. The ability to assess autophagy in specific neuronal cell cultures relative to experimental conditions offers researchers the opportunity to examine dose-response effects of experimental compounds on neuronal autophagy or to screen libraries of compounds that may affect autophagy and therefore neuronal physiological mechanisms relevant to neurodegenerative diseases.[2]

REFERENCES

1. Mizushima, Yoshimori and Levine (2010) Methods in Mammalian Autophagy Research. Cell 140:313-326.
2. Jin H. Son, et. al. (2012) Neuronal autophagy and neurodegenerative diseases. Experimental and Molecular Medicine 44, 2:89-98.

In collaboration with: