APPLICATION NOTE

Autophagy Analysis Using Object Spot Counting Using Gen5 to Analyze the Size and Number of Autophagosomes Per Nuclei

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CYTO-ID® AUTOPHAGY DETECTION KIT (ENZ-51031)

INTRODUCTION

A constant supply of nutrients is required during development to provide the energy necessary for growth, metabolism, and survival. Eukaryotic cells have evolved a variety of mechanisms to adjust their metabolic activities in response to changes in nutrient levels. Nutrient starvation, stress, or reduced availability of growth factors induces eukaryotic cells to adjust their metabolism in order to survive.[1] One of the key responses to such a stress is autophagy.

Autophagy or "self-eating" is a highly conserved process by which cells break down their intracellular components.[2] In a healthy cell under physiological conditions, autophagy is protective. In fact, autophagy plays a variety of important roles including maintenance of the amino acid pool during starvation, damaged protein and organelle turnover, prevention of neurodegeneration, tumor suppression, cellular differentiation, clearance of intracellular microbes, and regulation of innate and adaptive immunity.[3]

The first step of autophagy is formation of the phagophore, a cup-shaped double membrane. The edges of this membrane elongate and engulf portions of the cytoplasm, including intracellular material such as damaged organelles and misfolded proteins.[4] The isolation membrane expands and its open ends fuse to form a double-membrane structure called the autophagosome. Autophagosomes then fuse with lysosomes to form autolysosomes and the contents inside the autophagosome are degraded by lysosomal hydrolases. The intracellular material is then recycled back into the cytosol.[5]

One of the most well-known inducers of autophagy is starvation. Through autophagy, amino acids and other nutrients are recycled from long-lived proteins, organelles, and other components of the cytoplasm, providing an internal reserve of nutrients. Starvation rapidly induces autophagy, in part by inactivation of the mTOR (mammalian target of rapamycin) substrate S6K.[6] In a nutrient-rich environment, mTOR inhibitors such as rapamycin can induce autophagy.

Autophagy plays a role in both the pathogenesis and prevention of disease.[7] This is especially true in cancer, where elimination of damaged intracellular components through autophagy suppresses tissue injury and tumor initiation. However, in an established tumor, autophagy promotes cancer progression by providing substrates for metabolism, maintaining functional mitochondria, and fostering survival.[8]

Traditional methods of autophagy analysis include electron microscopy and western blot analysis of LC3- II. Electron microscopy is limited by the necessity of specialized expertise, and open when identifying an autophagosome structure.[9] Furthermore, flow cytometry or western blot measurements of LC3-II do not always correlate with formation of autophagosomes and do not give per-cell numbers of autophagosomes.[10] In this application note, we describe the process of using CYTO-ID® Autophagy Detection Dye combined with Gen5 3.03 software to analyze the effects of serum starvation and rapamycin on autophagosome number in HeLa cells. We perform analysis with Gen5 3.03 with object spot counting capability, which allows us to determine the number of autophagosomes per cell as well as their size.

MATERIALS AND METHODS

Cell Culture

HeLa cells were grown in Advanced Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY) with 10% FBS (Gibco) and 1x PennStrep- Glutamine (Cellgro, Manassas, VA). Cells were seeded into black-sided clear-bottom 96-well microplates (Corning, Corning, NY) at 20,000 cells per well.

CYTO-ID® Autophagy Detection Kit

The CYTO-ID Autophagy Detection Kit (donated by Enzo Life Sciences, Farmingdale, NY) was used to assess autophagy levels in HeLa cells. The probe is a cationic amphiphilic tracer (CAT) dye that rapidly partitions into cells. The dye is taken up by passive diffusion across the plasma membrane bilayer, and includes titratable moieties specific for selectively staining autophagic vesicles.

HeLa cells were grown in normal media, which was replaced with serum-free media containing 10 µM chloroquine for 2–6 hours to induce autophagy through serum-starvation. Alternatively, cells were treated with 0.0001 µM – 0.01 µM rapamycin with or without 10 µM chloroquine for 18 hours to induce autophagy through mTOR inhibition. Following treatment cells were washed 2x with 200 µL assay buffer (1x buffer + 5% FBS). Next, the assay buffer was replaced with 100 µL dual color detection solution (1 mL assay buffer + 1 uL Hoechst and 2 µL CYTO-ID Green Detection Reagent) for 30 minutes at 37 °C in the dark. Finally, the cells were washed with 2x 200 µL assay buffer and, after the buffer was removed, the sample was imaged in 100 µL assay buffer directly following the wash.

Cell Imaging

Images were acquired using a 20x objective on the Lionheart FX (BioTek Instruments, Winooski, VT) configured with DAPI and GFP light cubes. The DAPI light cube is configured with a 377/50 excitation filter and a 447/60 emission filter. The GFP light cube uses a 469/35 excitation filter and a 525/39 emission filter.

Image Analysis

Image preprocessing was used to ensure the best possible detection of nuclei and the best separation between individual autophagosomes. Image preprocessing parameters are described in detail in Table 1. The GFP channel of all the images were preprocessed with a 0.5 µM rolling ball in order to obtain the best separation between individual spots (Figure 1). Image preprocessing should be optimized on a per-experiment basis depending upon the size of particles being analyzed and how spread apart they are.

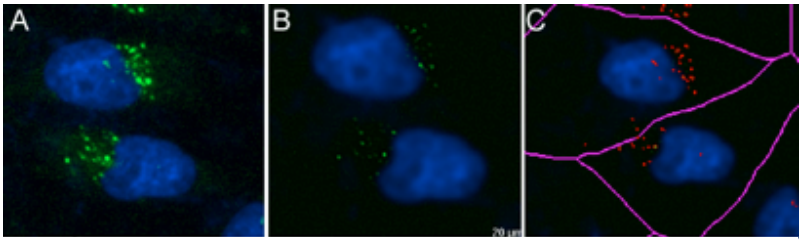

Figure 1. Autophagy spot counting workflow. (A) Original image. (B) Image after preprocessing. (C) Object Masks highlighting cell area in purple and spots in red.

Object smoothing of 5 cycles was applied to the DAPI channel to facilitate clean masking of the nuclei. Cell counting analysis was applied to the transformed DAPI channel to highlight each individual cell. Next, object spot counting was performed on the GFP channel to determine the size and number of autophagy positive spots per nuclei according to the parameters outlined in Table 1.

Imaging Preprocessing		
Image Set	**DAPI**	**GFP**
Background	Dark	Dark
Rolling Bar Diameter	Auto	0.5 µM
Priority	–	Fine results
Image Smoothing Strength	5	0
Cellular Analysis		
Detection Channel: Primary Mask and Count	Tsf[DAPI 377,447]	
Threshold	7000	
Secondary Mask	Tsf[GFP 469,525]	
Measure within a Secondary Mask	Checked	
Expand Primary Mask	30 µM	
Threshold	Unchecked	
Count Spots	Checked	
Size	0.5-3 µm	
Advanced Options	Count Spots Options	
Rolling-Ball Size	Default	
Threshold	1200	

Table 1. Gen5 image analysis software settings. Image analysis parameters for generating a cellular mask in the DAPI channel and an object mask in the GFP channel in order to count autophagic vesicles.

RESULTS

HeLa cells were treated with 0.1 nM – 10 nM rapamycin for 18 hr to determine the effect of increasing concentration of rapamycin on the number of autophagy-positive vesicles per cell. Rapamycin is an mTOR inhibitor that regulates cell growth and metabolism in response to environmental cues. Rapamycin induces autophagy due to the fact that inhibition of mTOR mimics cellular starvation by blocking signals required for cell growth and proliferation.[1] Increased punctate CYTO-ID fluorescence indicated autophagosome formation in HeLa cells treated with rapamycin **(Figure 2)**. There is an increase in both the size and the number of autophagic vesicles per cell as a result of increasing rapamycin concentrations **(Figure 2)**.

An accumulation of autophagosomes may be indicative of either the increased generation of autophagosomes or a block in autophagosome maturation and completion of the autophagic pathway. Chloroquine is a lysosomal inhibitor that increases the pH of the lysosome, thus preventing the activity of lysosomal acid proteases and causing autophagosomes to accumulate in the cell.[11] Figure 3 demonstrates an increase in the size and number of autophagy-positive spots-per-nuclei in response to combined rapamycin and chloroquine treatment. Notice an increase in both size and number of autophagosomes compared to rapamycin treatment alone **(Figure 2 and 3)**.

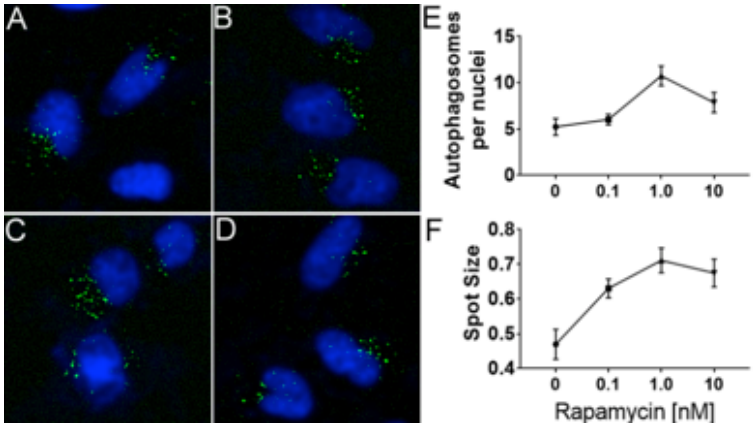

Figure 2. Autophagy positive spot counts and size increase after treatment with rapamycin. (A) Control (B) 0.1 nM rapamycim (C) 1 nM rapamycin (D) 10 nM rapamycin (E) Autophagosomes per nuclei increase with increasing concentration of rapamycin (F) Autophagosome spot diameter increases with increasing concentrations of rapamycin.

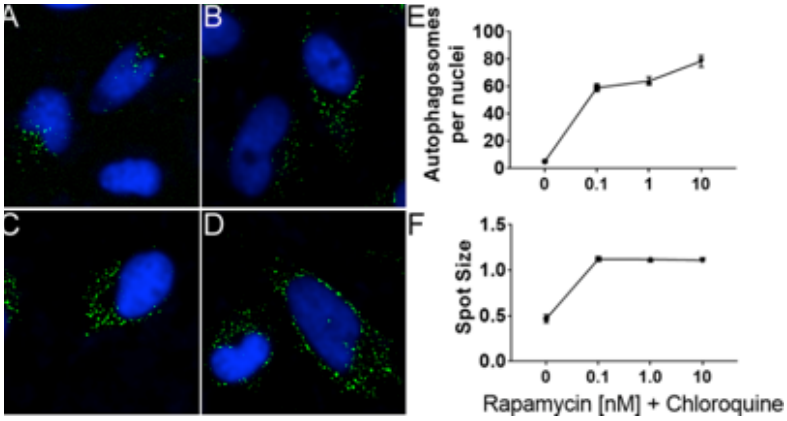

Figure 3. Autophagy positive spot counts and size increase after treatment with rapamycin + chloroquine (A) Control (B) 0.1 nM rapamycim + 10 µM chloroquine (C) 1 nM rapamycin + 10 µM chloroquine (D) 010 nM rapamycin + 10 µM chloroquine (E) Autophagosomes per nuclei increase with increasing concentration of rapamycin and chloroquine (F) Autophagy spot diameter increases with increasing concentrations of rapamycin and chloroquine.

Starvation is one of the most well-known inducers of autophagy. Here, we combined chloroquine treatment and serum starvation for 2-6 hrs. Figure 4 demonstrates that increased length of serum starvation results in increased size and number of autophagosomes per nuclei in HeLa cells.

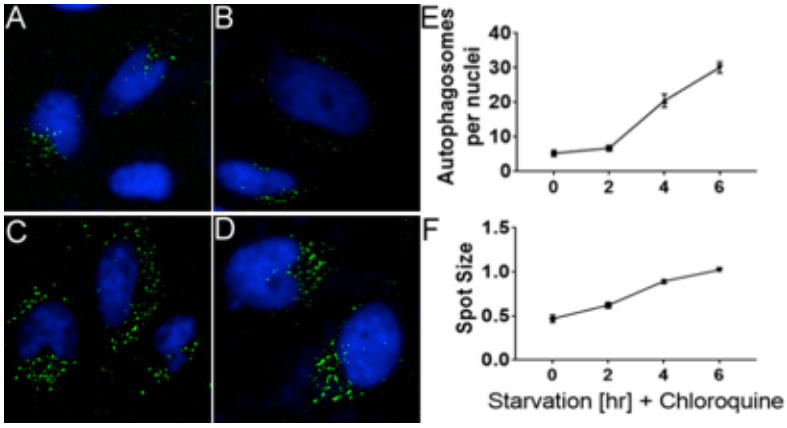

Figure 4. Autophagy spot count increases with longer serum starvation (A) Control (B) 2hr serum starvation + 10 µM chloroquine (C) 4hr serum starvation + 10 µM chloroquine (D) 6hr serum starvation + 10 µM chloroquine (E) The number of autophagosomes per nuclei increase according to time in serum-free media. (F) Autophagosome spot diameter increases with increasing time in serum-free media.

CONCLUSION

The ability to efficiently and rapidly analyze autophagy in living cells is critical for many applications such as screening for compounds that can potentially modify disease states. Here we demonstrate that autophagosome number and size increase in response to known autophagy activators rapamycin and serum starvation. Use of CYTO-ID Autophagy Detection Kit in combination with Gen5 analysis allows for consistent and precise measurement of object level data including spot count and spot size.

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