Autophagy Analysis Using Object Spot Counting

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Abstract

A continuous supply of nutrients is required in a developing organism 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. Starvation, stress, or reduced availability of growth factors induce eukaryotic cells to adjust their metabolism in order to survive. One of the key responses to such a stress is autophagy. Autophagy is a highly conserved process by which cells break down their intracellular components, which among other things, aids in maintaining the amino acid pool during starvation. Autophagy is critical for the maintenance of cellular homeostasis. However, dysregulated autophagy can lead to death of healthy cells and survival of cancerous ones.

Traditional methods of autophagy analysis include electron microscopy and western blot analysis of autophagy associated proteins. Electron microscopy is limited by the necessity of specialized expertise, and open to interpretation when identifying autophagosome structures. Flow cytometry or western blot measurements of autophagy associated proteins, such as LC3-II, do not always correlate with formation of autophagosomes and do not give per-cell numbers of autophagosomes.

Spot counting analysis using manual methods is tedious, time consuming, and prone to error. In contrast, automated image analysis consistently and precisely determines the number of autophagy positive spots per nuclei across multiple samples. This rapid process saves time from manual counting and applies the same set of parameters to all of the samples, lessening the user bias which is inevitable in manual counting.

Here we present the use of an autophagy dye in combination with automated object-based spot counting to quantitatively assess the effects of known autophagy activators starvation and rapamycin on cellular autophagy. We perform automated object based spot counting analysis which allows for the determination of size and number of autophagosomes per cell.

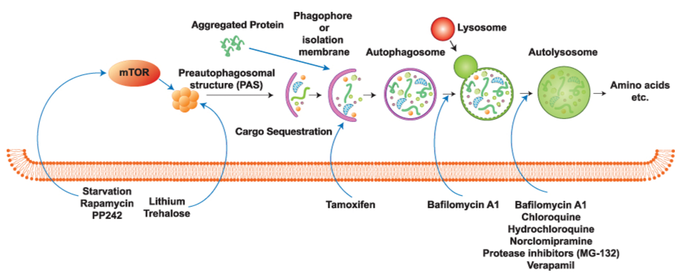

Figure 1. Schematic depiction of autophagy. Autophagy literally means "self eating". In this homeostatic process the cells break down their own components in the manner shown in the diagram. To start, the phagophore forms a cup shaped double membrane which elongates and engulfs portions of the cytoplasm including damaged organelles and misfolded proteins. This membrane then expands and fuses to form autophagosomes. The contents of the autophagosome become recycled when it fuses with the lysosome to form the autolysosome.

BioTek Instrumentation

Figure 2. Lionheart™ FX Automated Microscope with Augmented Microscopy™

All inclusive microscopy system: Optimized for live cell imaging with brightfield, color brightfield, phase contrast and fluorescence channels. Up to 100x air and oil immersion magnification.

Up to 20 frames per second (fps) image capture: Enables characterization of rapid cellular events and addition of reagents with uninterrupted monitoring of cellular response.

Integrated environmental control: Incubation up to 40 °C with CO_2 /O_2 and available humidity control provides optimal conditions for long-term imaging of live cells.

Powerful Gen5™ Microplate Reader and Imager Software: Automated image capture, processing, and analysis tools, including dual masking for cell counting and subpopulation analysis, object based spot counting, and annotation and movie maker functions.

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) 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 0.1 nM – 10 nM 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 this was removed and 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 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 pre-processing was used to ensure the best possible detection of nuclei and the best separation between individual autophagosomes. The GFP channel of all the images (Figure 3A) was pre-processed with a 0.5 µm rolling ball in order to obtain the best separation between individual spots (Figure 3B). Image pre-processing should be optimized on a per-experiment basis depending upon the size of particles being analyzed and how spread apart they are.

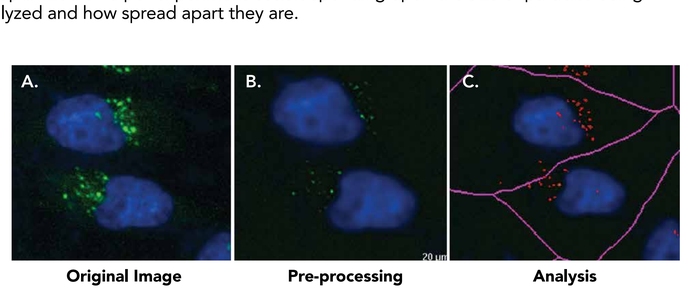

Figure 3. Autophagy spot counting workflow. (A) Original image with CYTO-ID dye in green and nuclei in blue. **(B)** Image after pre-processing in the GFP channel with a 0.5 µm rolling ball and image smoothing in the DAPI channel. **(C)** The results of the image analysis are shown with object masks highlighting the cell area in purple and spots for each individual cell which are shown in red.

Object smoothing of 5 cycles was applied to the DAPI channel to facilitate clean masking of the nuclei (Figure 3B). 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. A primary mask was set in the DAPI channel to highlight each individual nuclei. Next, a secondary mask was extended from each primary mask to highlight the area encompassing the autophagic spots, as shown in Figure 3C. In this case, the primary mask was extended by 30 µm. Then, the spot counting function of Gen5 was employed to highlight each individual spot, as shown by the red masks in Figure 3C. In this case, the size of the spots was from 0.5-3 µm and a threshold of 1200 was set to capture each individual spot.

Results

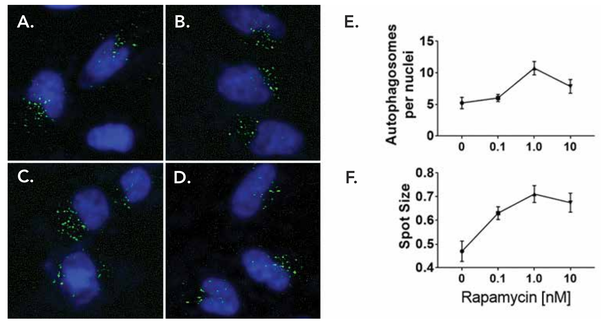

Figure 4. Autophagy positive spot counts and size increase after treatment with rapamycin. Panels A-D show images of HeLa cells treated with rapamycin. The autophagosomes are shown in green and the nuclei are shown in blue. **(A)** Control. **(B)** 0.1 nM rapamycin. **(C)** 1 nM rapamycin. **(D)** 10 nM rapamycin. **(E)** This graph shows the autophagosomes per nuclei increase with increasing concentrations of rapamycin. **(F)** This graph shows the autophagosomes spot diameter increases with increasing concentrations of rapamycin.

Results *(Cont.)*

HeLa cells were treated with 0.1 nM – 10 nM rapamycin for 18hr 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. Increased CYTO-ID fluorescence indicated autophagosomes formation in HeLa cells treated with rapamycin (Figure 4). There is an increase in both the size and number of autophagic vesicles per cell as a result of increasing rapamycin concentrations (Figure 4).

An accumulation of autophagosomes may be indicative of either an 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. Figure 5 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 (Figures 4 and 5).

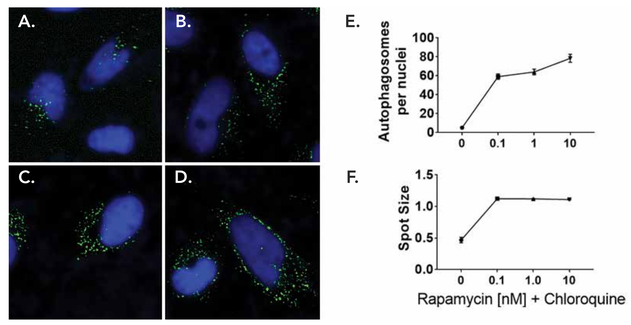

Figure 5. Autophagy positive spot counts and size increase after treatment with rapamycin + chloroquine. Panels A-D show images of HeLa cells treated with rapamycin in combination with 10 µM chloroquine. The autophagosomes are shown in green and the nuclei are shown in blue. **(A)** Control. **(B)** 0.1 nM rapamycin + 10 µM chloroquine. **(C)** 1 nM rapamycin + 10 µM chloroquine. **(D)** 10 nM rapamycin + 10 µM chloroquine. **(E)** This graph demonstrates that autophagosomes per nuclei increase with increasing concentrations of rapamycin and chloroquine. **(F)** This graph shows that autophagosomes spot diameter increases with increasing concentrations of rapamycin and chloroquine.

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

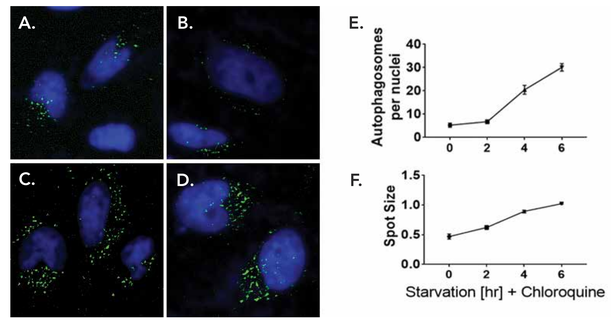

Figure 6. Autophagy positive spot counts and size increases with longer serum starvation. Panels A-D show images of HeLa cells treated with no serum media in combination with 10 µM chloroquine. The autophagosomes are shown in green and the nuclei are shown in blue. **(A)** Control. **(B)** 2hr serum starvation + 10 µM chloroquine. **(C)** 4hr serum starvation + 10 µM chloroquine. **(D)** 6hr serum starvation + 10 µM chloroquine. **(E)** The first graph shows the number of autophagosomes per nuclei increase according to time in serum-free media. **(F)** The second graph demonstrates that autophagosomes spot size increases in proportion to time in serum-free media.

Conclusions

1. 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.
2. Autophagosome number and size increase in response to known autophagy activator rapamycin.
3. Treatment with chloroquine causes an accumulation of autophagosomes up to seven times greater than rapamycin treatment alone.
4. Autophagosome size and number increase in response to serum starvation.
5. Use of CYTO-ID dye in combination with Gen5 analysis allows for consistent and precise measurement of object level data including spot count and spot size.