

A Novel Image-Based Cytometry Method for Autophagy Detection in Living Cells

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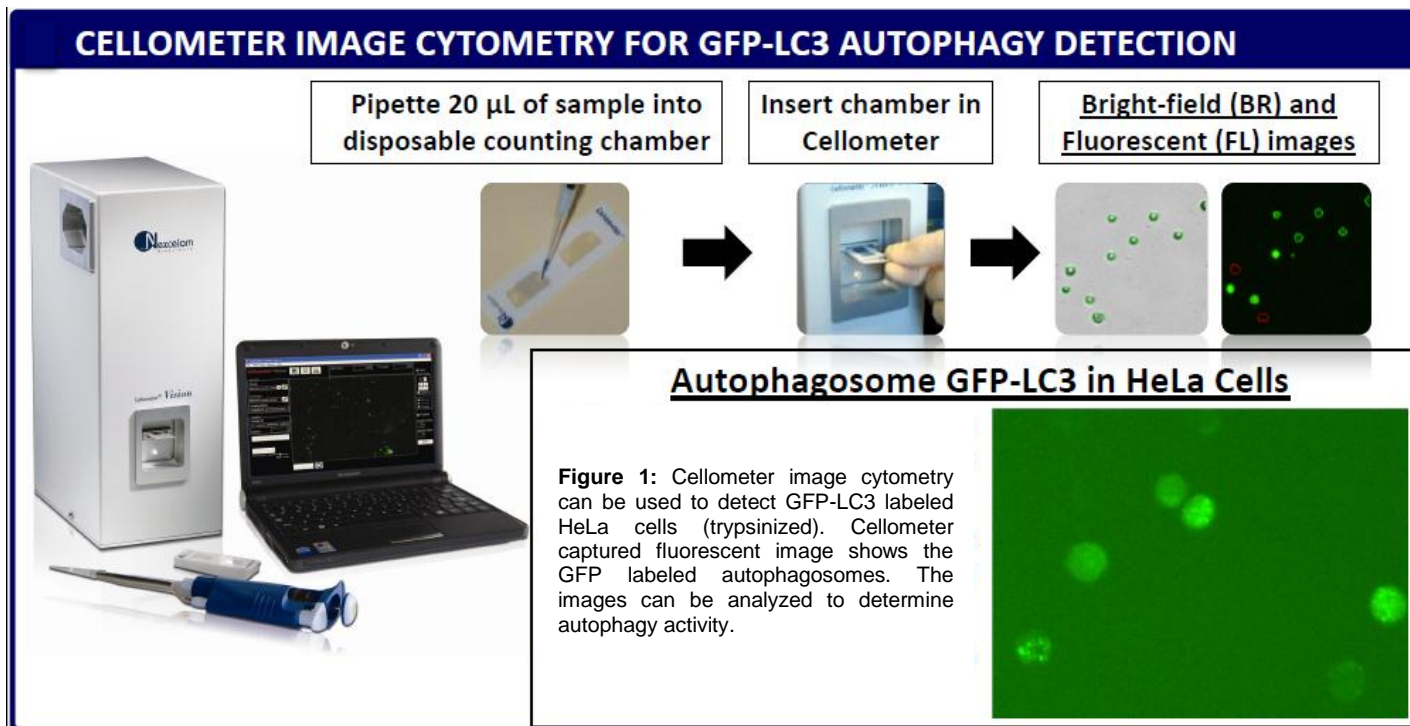
Cyto-ID® Autophagy Detection Kit (ENZ-51031)

Introduction

Autophagy is an important cellular catabolic process that plays a variety of important roles, including maintenance of the amino acid pool during starvation, recycling of damaged proteins and organelles, and clearance of intracellular microbes. Currently employed autophagy detection methods include fluorescence microscopy, biochemical measurement, SDS-PAGE, and Western blotting, but they are time-consuming, labor-intensive, and require much experience for accurate interpretation. More recently, development of novel fluorescent probes have allowed the investigation of autophagy via standard flow cytometry. However, flow cytometers remain relatively expensive and require a considerable amount of maintenance. Previously, image-based cytometry has been shown to perform automated fluorescence-based cellular analysis comparable to flow cytometry. In this study, we developed a novel method using the Cellometer image-based cytometer in combination with Cyto-ID® Green dye for autophagy detection in live cells. The method is compared to flow cytometry by measuring macroautophagy in nutrient-starved Jurkat cells. Results demonstrate similar trends of autophagic response, but different magnitude of fluorescence signal increases, which may arise from different analysis approaches characteristic of the two instrument platforms. The possibility of using this method for drug discovery applications is also demonstrated through the measurement of dose-response kinetics upon induction of autophagy with rapamycin and tamoxifen. The described image-based cytometry/fluorescent dye method should serve as a useful addition to the current arsenal of techniques available in support of autophagy-based drug discovery relating to various pathological disorders.

CURRENT METHODS FOR DETECTING AUTOPHAGY ASSOCIATED LC3		
Methods	Description	Known Issues
Western Blot	LC3 protein analysis	<ul style="list-style-type: none"> •Time-consuming and tedious process •Requires experienced user for accurate interpretation
Fluorescence Microscopy	Visualization of GFP-LC3 or FITC-LC3 labeled autophagosomes	<ul style="list-style-type: none"> •Qualitative observe instead of quantitative analysis •Not automated, low throughput
Flow Cytometry	<ul style="list-style-type: none"> •Quantitative flow-based cellular analysis of GFP-LC3 or FITC-LC3 •Automated analysis 	<ul style="list-style-type: none"> •Relatively expensive and high maintenance •Requires experienced user for proper operation •Cannot visually observe autophagosomes

Table 1.



CELLOMETER IMAGE CYTOMETRY AUTOPHAGY ACTIVITY ANALYSIS

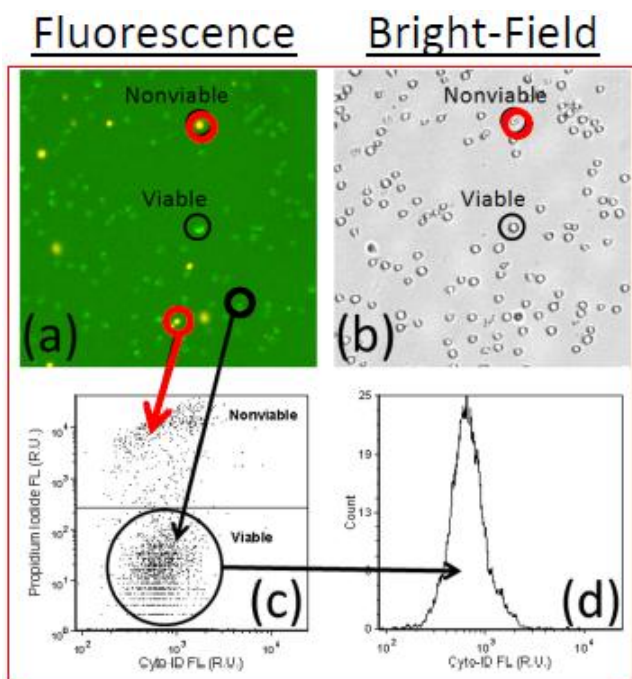


Figure 2 (left):

Image Analysis for Autophagy Activity Detection

Bright-field and fluorescent images of target cells are captured for image analysis at 4 locations. Fluorescence intensity is measured from each counted cell and exported into FCS Express 4 for fluorescence analysis. Mean Fluorescence Intensity (MFI) is measured directly.

Autophagy Activity Factor

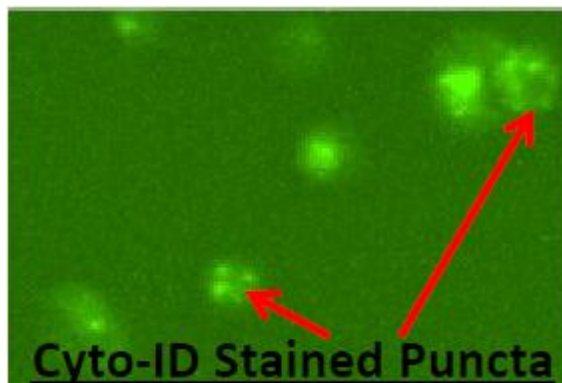
Autophagy Activity Factor (AAF) was used to measure the level of autophagic activity within a cell population. AAF is directly correlated to autophagy activity according to the following equation:

$$AAF = 100 \times \left(\frac{MFI_{treated} - MFI_{control}}{MFI_{treated}} \right)$$

SPECIFIC LABELING OF AUTOPHAGOSOMES USING NOVEL CYTO-ID® GREEN DYE

Figure 3 (right):

Cyto-ID Green autophagy dye has been used to specifically stain autophagosomes to investigate autophagy activity in live cells. Cyto-ID is a cationic amphiphilic tracer dye enters cell by passive diffusion. The dye is pH clamped so it can stain acidic particles within the cell. Functional moieties prevent accumulation within lysosomes, but enable labeling of the autophagosomes associated with autophagy. Cyto-ID-stained autophagosomes appears as puncta in the cell.



DETECTION OF CYTO-ID® GREEN DYE USING FLUORESCENCE MICROSCOPY

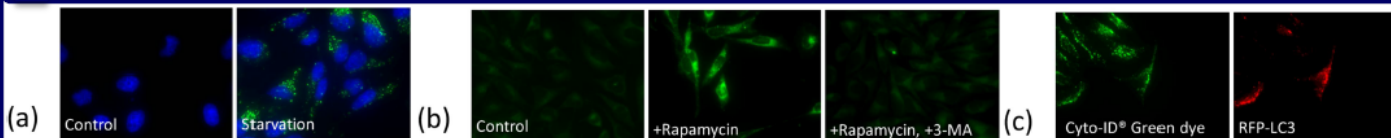


Figure 4: (a) HeLa cells were starved and stained with Cyto-ID, which showed increase in the number of autophagosomes. (b) HeLa cells were induced with Rapamycin, which also showed increase in the number of stained autophagosomes. (c) Autophagy-induced HeLa cells were stained with Cyto-ID Green dye and transfected with RFP-LC3. Co-localization was observed

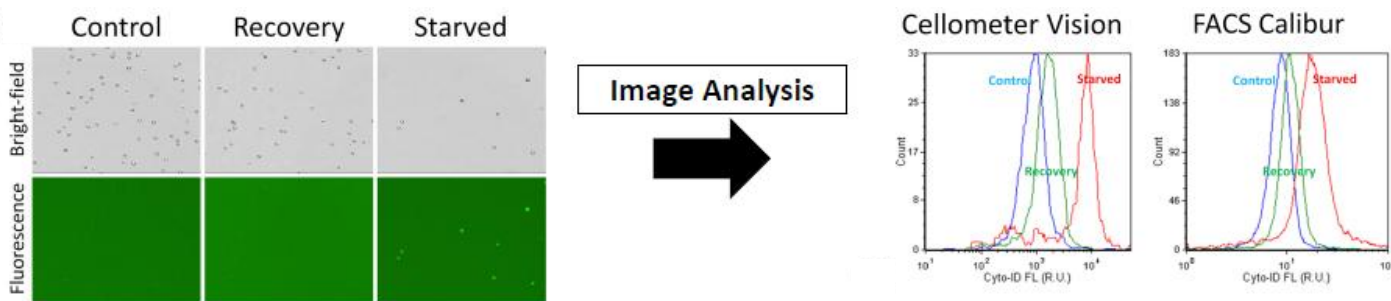


Figure 5: Jurkat cells were starved in Hank's Balance Saline Solution (HBSS) for 2 hours, and allowed to recover in media for 1 hour. The trend of cell population fluorescent signals was comparable between Cellometer and FACS Calibur. AAF values were calculated for Cellometer and FACS, which were shown to be comparable.

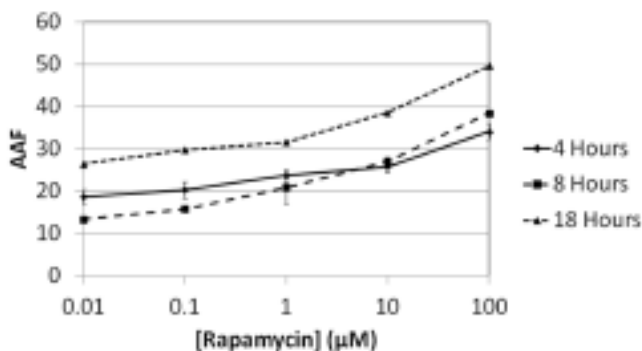


Figure 6: Rapamycin Time-Dependent Dose Response. Jurkat cells were treated with Rapamycin, an autophagy inducing chemical for 4, 8, and 18 hours. Rapamycin [0.01, 0.1, 1, 10, and 100 μM] were tested for dose response. Results showed increasing AAF values for Rapamycin at various concentrations as incubation time increased

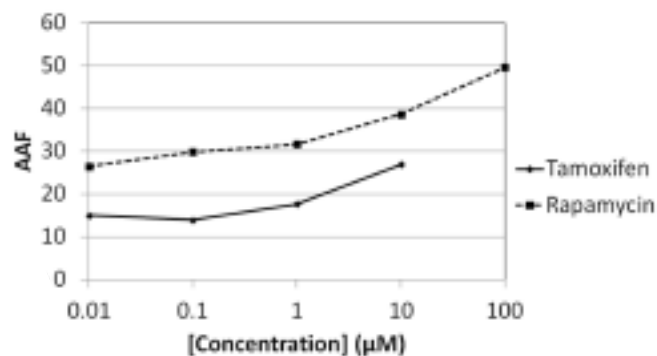
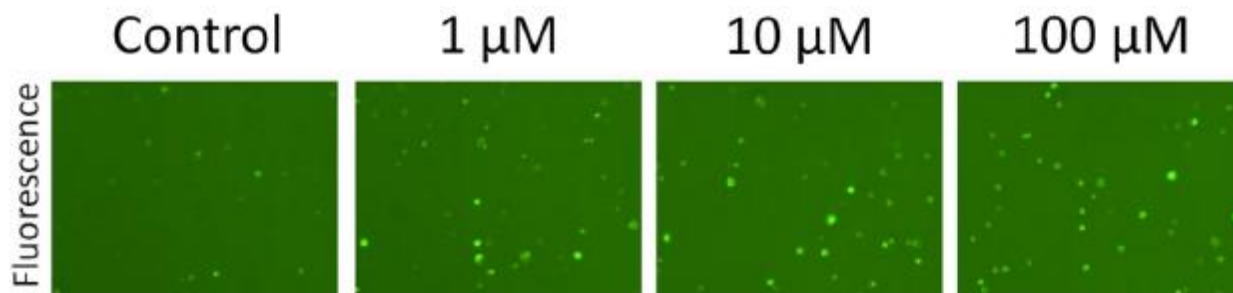


Figure 7: Comparison of Rapamycin and Tamoxifen. Jurkat cells were treated with Rapamycin and Tamoxifen, both were autophagy inducing chemicals for 18 hours. Results showed increasing AAF values for both chemicals at various concentrations (0.01, 0.1, 1, 10, and 100 μM). Rapamycin showed higher AAF values comparing to Tamoxifen, which indicated that Rapamycin can more readily induce autophagy in Jurkat cells.

AUTOPHAGY DETECTION OF TRYPSINIZED ADHERENT PC3 CELLS



[Rapamycin]	Control	1 μM	10 μM	100 μM
Mean FL Intensity (R.U.)	3543.53	4093.25	5536.15	6042.04
AAF Values	0.00	13.43	35.99	41.35

Figure 8: PC3 prostate cancer cells were used to show the capability of detecting autophagic activities in adherent cell lines. PC3 cells were treated with Rapamycin, as the concentration increased, more puncta were observed in the fluorescent images. Correspondingly, cell populations with higher mean fluorescence intensity are shown. The calculated AAF values also increased as the Rapamycin concentration increased.



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