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

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1. ABSTRACT

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.

2. INTRODUCTION

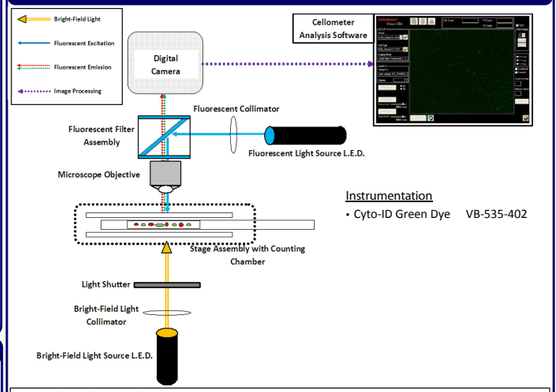
Autophagy is an important evolutionarily conserved cellular catabolic process characterized by a set of complex and highly regulated events that lead to the engulfment of misfolded proteins, protein complexes, and entire organelles in double-membrane sequestering vesicles referred to as autophagosomes. Recently, a novel fluorescent probe, Cyto-ID® Green autophagy dye, has been developed to facilitate the investigation of the autophagic process. In this study, a novel method using the Cellometer image cytometry in combination with Cyto-ID® Green autophagy dye is presented for detecting autophagy in live cells. The image-based cytometry/fluorescent dye workflow provides useful advantages compared with other commonly implemented techniques used in the field and should find application in performance of autophagy-based drug discovery studies relating to a variety of pathological disorders.

3. CELLOMETER® VISION IMAGE CYTOMETRY

Cellometer Vision Operation

- Target cell samples are pipetted into a disposable counting chamber
- The chamber is inserted into the Cellometer Vision
- The fluorescence and bright-field images are captured at 4 locations
- Dynamic concentration range is 10^5 – 10^7 cells/ml
- Image analysis data is exported into FCS Express 4 for fluorescence analysis
- Process of image acquisition and analysis takes less than 2 min, which can be dependent on the fluorescence exposure time

4. MATERIALS AND METHODS



Instrumentation

- Cyto-ID Green Dye VB-535-402

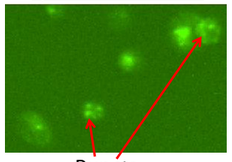
Cyto-ID Green Dye Staining Protocol

- Cyto-ID Green dye (8 µl) is mixed with 4 ml 1X buffer
- Cell sample is centrifuged and resuspended into 200 µl of prepared Cyto-ID Green dye
- Cell sample is incubated for 30 min at 37 °C before image or flow cytometric analysis

Autophagy Detection & Image Analysis

- Bright-field and fluorescent images are captured for image analysis
- Cells are automatically counted in bright-field
- Fluorescence intensity is measured from each counted cell
- The mean fluorescence intensity is measured from autophagic cell population

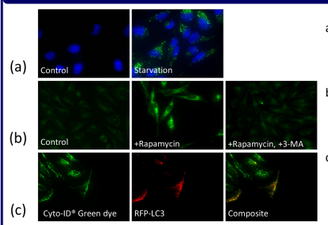
Cyto-ID Stained PC3 Cells



Puncta

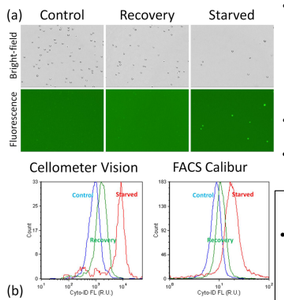
- Puncta are Cyto-ID Green dye-stained autophagosomes that appears when cells become autophagic

5. VALIDATION OF CYTO-ID® AUTOPHAGY GREEN DYE



a) HeLa cells were starved and stained with Cyto-ID Green dye, 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

6. COMPARISON OF IMAGE AND FLOW CYTOMETRY



- Jurkat cells were starved in Hank's Balance Saline Solution (HBSS) for 2 hours, and allowed to recover in media for 1 hour

  Fluorescent signals were compared between Cellometer and FACS Calibur, which showed high and low signals for starved and recovery, respectively
- AAF values were calculated by comparing starved Jurkat cells to the control sample
- Trends in AAF values were comparable between the Cellometer and FACS Calibur

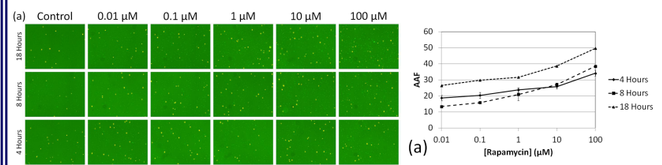
Autophagy Activity Factor

- Autophagy Activity Factor (AAF) was used to measure the level of autophagic activity within a cell population

$$(1)\ AAF = 100 \times \left(\frac{MFI_{treated} - MFI_{control}}{MFI_{treated}}\right)$$

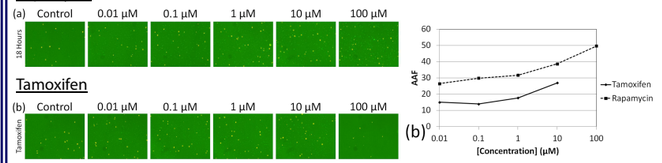
7. DETCTION OF CHEMICAL-INDUCED AUTOPHAGY ACTIVITY IN JURKAT CELLS

Rapamycin Time-Dependent Dose Response



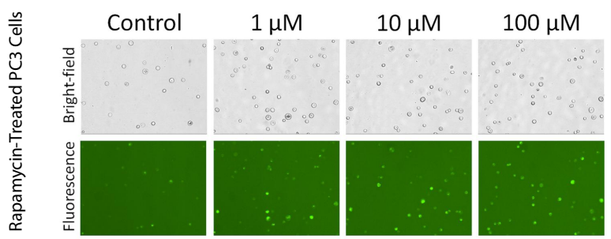
- Jurkat cells were treated with Rapamycin , an autophagy inducing chemicals for 4, 8, and 18 hours
- Various concentrations were tested to study the dose response of Rapamycin (0.01, 0.1, 1, 10, and 100 µM)
- Results showed increasing AAF values for Rapamycin at various concentrations as incubation time increased
- The 18 hour time point showed the highest AAF values compared to 4 and 8 hours.

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

8. 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

- Adherent cells were commonly studied in the autophagy research field, thus PC3 prostate cancer cells were used to show the capability of Cellometer image cytometry for detecting autophagic activities in adherent cell lines
- PC3 cells were treated with Rapamycin at various concentrations, as the concentration increased, more puncta were observed in the fluorescent images
- Correspondingly, cell populations with higher mean fluorescence intensity are shown in the table above
- The calculated AAF values also showed increase as the Rapamycin concentration increased

9. CONCLUSION

We have demonstrated a novel method for autophagy detection in live cells. The described combination of automated fluorescence imaging and data processing allows rapid analysis of autophagic flux and addresses some of the limitations encountered using other detection methods. The image-based cytometry/fluorescent dye workflow provides useful advantages compared with other commonly implemented techniques used in the field and should find application in performance of autophagy-based drug discovery studies relating to a variety of pathological disorders.