APPLICATION NOTE

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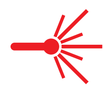

Single Cell Analysis of Autophagy

Data kindly provided by John F. Woolley of the University of Toronto, Department of Pharmacology and Toxicology, and by Leonardo Salmena of the Princess Margaret Cancer Center, Toronto.

Introduction

Mammalian cells regularly replace deteriorated organelles and dispose of misfolded proteins as part of normal housekeeping, preventing potentially dangerous components from building up and overwhelming the cell. Autophagy removes and recycles this waste by isolating the targeted materials within a double-membraned vesicle, called the autophagosome, which fuses with lysosomes to facilitate degradation via acidic lysosomal hydrolases. Not to be confused with apoptosis, which controls whole-cell turnover within an organism, autophagy occurs within a cell, while the cell remains viable. Although there are many stressors that sequentially elicit autophagy and apoptosis, within the same cell, the two tend to be mutually inhibitory.[1]

Objectives

- Discover the physical and chemical hallmarks of autophagy
- Learn how to stain autophagic vesicles for flow cytometric analysis
- Demonstrate a gating strategy for autophagic vesicle quantification

Autophagy has been identified as an important process in cancer, with impaired function linked to tumorigenesis, particularly in the early stages of development.[2] Compromised autophagy can lead to the accumulation of inactive mitochondria, reactive oxygen species, and protein aggregates, all of which may result in DNA damage, oncogene activation, and tumorigenesis.[3] Paradoxically, in established tumors, autophagy can act as a survival mechanism in response to stress signals during cancer progression, or in response to chemotherapy.[4] This occurs when tumor cells hijack the delivery of essential nutrients in support of the crowded, innermost tumor cells, preventing necrosis.[5]

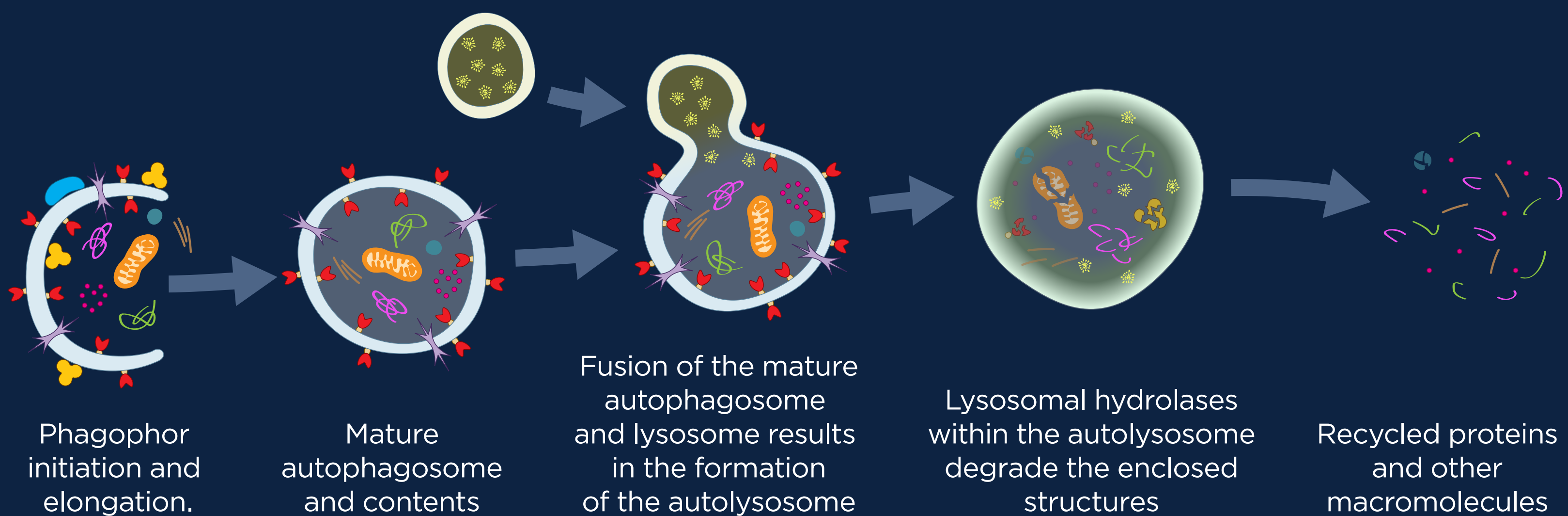

Figure 1. Autophagosome formation and identification. Autophagy is initiated in response to cellular signals. The phagophore is a double-walled membrane that extends to enclose cellular materials that are targeted for degradation and/or recycling. Once the contents are enclosed, the mature autophagosome is formed. The mature autophagosome then fuses with a lysosome, whose lysosomal hydrolases begin to degrade the enclosed cellular products. Various autophagy-related proteins are identified along with their temporal association with the formation of autophagosomes and their degradation and recycling of cellular debris.

Early on, autophagy was successfully monitored using Western blotting and immunohistochemistry to analyze the location and relative quantity of intracellular autophagy-associated proteins, including LC3, ATG5, ATG12, and ATG16, but flow cytometry has become the method of choice for in vitro applications.[6] Flow cytometry has made it possible to study autophagy on a single-cell scale, faster and more quantitatively than previously possible. Flow cytometry can analyze a cell's size (via forward-scatter analysis), complexity (via side-scatter analysis), and constituent molecules (via fluorescently tagged antibodies or fluorescent proteins).

In this application note, you will find a simple and rapid assay to monitor autophagy in single cells, using the CytoFLEX Flow Cytometer. Cyto-ID dye is a cationic amphiphilic tracer that provides high specificity for autophagosome staining by specifically accumulating in autophagic vesicles. Due to the reagent's design, Cyto-ID is excluded from lysosomes, enabling easy discrimination of autophagic vesicles from lysosomes. Flow cytometric analysis identified differing degrees of autophagy in two cell lines, demonstrating that autophagy, as a response to serum starvation, is governed by cell-specific process that vary from cell line to cell line, and that the CytoFLEX Flow Cytometer has the sensitivity to accurately and reliably analyze intracellular autophagosome formation.

Materials

- Mammalian cancer cell lines harvested at log phase
  - Cell lines used in this demonstration: OCI/AML3 and U937 human cancer cell lines
- Culture media supplemented with 100 U/100 µg/mL penicillin/streptomycin
  - Culture media used in this demonstration: Alpha-minimum essential medium (Alpha-MEM) and RPMI 1640
- Fetal bovine serum (FBS)
- Phosphate-buffered saline (PBS)
- Cyto-ID® Autophagy Detection Kit (Enzo Life Sciences, Farmingdale, NY, Cat# ENZ-51031)
- CytoFLEX Flow Cytometer (Beckman Coulter, Brea, CA)

Protocol

1. Cell lines were cultured to a density of $5x10^5$ cells/mL and harvested in log phase.

2. Set up two cultures of each cell line as shown in Table 1.

Table 1. Culture conditions

OCI/AML3 Cell Line		U937 Cell Line	
Control	Serum-starved	Control	Serum-starved
Alpha-MEM media	Alpha-MEM media	RPMI 1640 media	RPMI 1640 media
10% FBS	0.5% FBS	10% FBS	0.5% FBS

To evaluate nutrient starvation as a trigger for autophagy in two leukemia cell lines, cells were subjected to low-FBS serum, which is known to induce autophagy

3. Prepare two aliquots of each media (both alpha-MEM and RPMI1640), one with 10% FBS, and the other with 0.5% FBS.

4. Change the media in the 'control' samples by centrifuging, aspirating the spent media, and replacing it with 10% FBS-containing media. Incubate for 6 hours.

5. Change the media in the 'starved' samples by centrifuging, aspirating the spent media, and replacing it with 0.5% FBS-containing media. Incubate for 6 hours.

6. Add 2 mL of each culture condition (four total) into separate 15 mL conical centrifuge tubes.

7. Centrifuge at 400 x g for 5 min, then carefully aspirate the supernatant without disturbing the pellet.

8. Wash cells with 1 mL of 1X PBS.

9. Centrifuge at 400 x g for 5 min, and carefully aspirate the supernatant.

10. Resuspend the cell pellet in 250 µL of 1X PBS containing 5 % FBS.

11. Add 250 µL of Cyto-ID Green Detection Reagent, and gently flick the tube to mix.

12. Incubate the cells for 60 minutes at 37 °C in the dark.

13. Centrifuge at 400 x g for 5 min, and carefully aspirate the supernatant.

14. Wash cells with 1 mL of 1X PBS.

15. Centrifuge at 400 x g for 5 min, and carefully aspirate the supernatant.

16. Completely resuspend the pelleted cells in 250 µL of 1X PBS, and then place on ice.

17. Quantify autophagy via flow cytometry with the CytoFLEX Flow Cytometer using the FITC channel. Cyto-ID has an excitation peak at 463 nm and an emission peak at 534 nm. Refer to the gating strategy detailed in Figure 2.

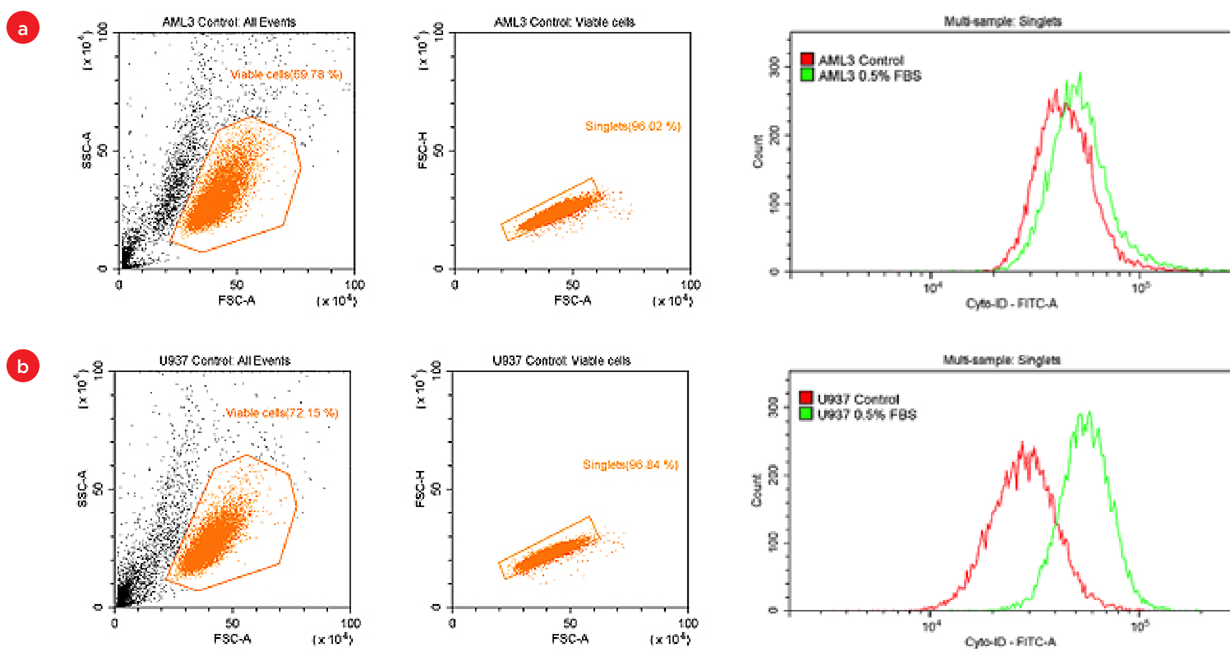

Figure 2. Analysis of Autophagy Induction in Cancer Cells with the CytoFLEX Flow Cytometer. Each column (1-3, left to right) demonstrates the sequential gating strategy for detecting the presence of autophagic vesicles; OCI/AML3 cells are shown in Row 'a' and U937 cells in Row 'b'. Column 1 depicts the gate for viable cells, excluding debris, on a FSC X SSC dot-plot. Cells were then gated on a FSC AREA X HEIGHT dot-plot to exclude apoptotic cells (Column 2). Using Cyto-ID fluorescence in the FITC-A channel, autophagic vesicles were quantified and plotted as cell counts in superimposed histograms (Column 3). Cell lines were cultured 6 hours in the presence of 10% FBS (red histogram) or 0.5% FBS (green histogram).

Results and Discussion

Assessment of cell viability by flow cytometry revealed that the OCI/AML3 'control' culture was 60 % viable, as compared to 72% viability in the U937 'control' culture (Figure 2; Column 1). This disparity in viability reflects the normal variability seen in cultured cell lines. Cell viability is the first attribute that flow cytometers typically gate on, to exclude any dead and dying cells.

Both cell lines showed a low level (< 4%) of apoptosis, as seen by a tight, linear cluster on the FSC AREA x HEIGHT dot plot (Figure 2; Column 2). The cells that remained outside of the gate were considered to be undergoing apoptosis, although the possibility remains that a proportion of cells within the gate were exhibiting only the earliest signs of apoptosis and were, therefore, not detectable by Forward Scatter alone.

Serum starvation of U937 cells increased the fluorescent signal in the FITC channel by one half log, while only a modest increase was seen for OCI/AML3 cells (Figure 2; Column 3). This indicates that the proportion of autophagic vesicles in U937 cultured cells increased following serum starvation, while the proportion of autophagic vesicles in OCI/AML3 cells remained relatively constant; every cell line responds to serum starvation differently, as demonstrated here. While the 'control' OCI/AML3 cells demonstrated lower overall viability than the 'control' U937 cells, viability was more closely correlated to increased autophagy in response to starvation, as described in the literature.[4]

The presence of autophagic vesicles was measured by fluorescence in the FITC channel by the CytoFLEX Flow Cytometer. Cyto-ID is effective for the detection of autophagy, as it results in only minimal lysosomal staining. Using the above gating strategy (Figure 2), one can reliably identify cells containing autophagosomes from cells without autophagosomes, based on cell health and autophagic staining.

Tips for success

- Carefully read cell-thawing specifications prior to experimentation and ensure that culture growth phase is controlled in the experimental conditions.
- Keep the Cyto-ID reagent away from light; carry out all Cyto-ID incubation steps in the dark

Conclusions

In summary, it is possible to identify cells containing autophagosomes using Cyto-ID Autophagy Detection Kit and the CytoFLEX Flow Cytometer. The protocol for rapid detection of Cyto-ID-stained autophagic vesicles in serum-starved and control leukemia cells could easily be extended to other cell lines and cell types. Using this cost-effective, compact laboratory solution, researchers are able to identify the graded and variable appearance of autophagosomes in cell culture.

Notes

The results shown here represent data generated on the Beckman Coulter CytoFLEX Flow Cytometer. Due to differences in the performance of makes and models of flow cytometers, the authors cannot guarantee similar results with the use of other flow cytometers. CytoFLEX is for Research Use Only (RUO), not intended or validated for use in the diagnosis of disease or other conditions.

References

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