Detection of Autophagic Vesicle Formation in Leukemia Cell Lines by Flow Cytometry

APPLICATION NOTE

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IN THIS PAPER YOU WILL LEARN

- To be introduced to autophagy
- How to stain autophagic vessicles for measurement by flow cytometry
- A gating strategy to quantify autophagic vessicles

Principal of the Technique

Background:

Autophagy is a cellular phenomenon that controls organelle and protein degradation essential for the regulation of cell survival, growth, development and homeostasis. Initially thought of as merely a mechanism whereby cells dispose of cellular junk, autophagy is now recognized as a mechanism by which the cell removes misfolded and long-lived proteins, damaged organelles, infectious microorganisms but also has diverse roles as an adaptive response to provide nutrients and energy upon activation by stress stimuli, and suggested involvement in cytokinesis. Autophagy is a dynamic process that is typically measured indirectly by the intracellular cleavage of the LC3 protein by western blot. Here we provide a simple and rapid assay to monitor autophagy dynamics in leukemia cells by flow cytometry, via the direct staining of autophagosomes with the fluorescent dye Cyto-ID.

Introduction:

Autophagy has been identified as an important process in cancer, with early studies suggesting impaired autophagy associated with tumorigenesis [1]. The question as to why a cellular process which is known to be essential for cell survival is also tumor suppressive. One potential explanation is that autophagy prevents death from necrosis in apoptosis-deficient cells [2]. Also, cells in which autophagy is impaired accumulate damaged mitochondria, reactive oxygen species (ROS) and protein aggregates that all may result in DNA damage, oncogene activation and tumorigenesis [3].

Thus the interest in autophagy regulation and its contribution to cellular homeostasis is disease is understandable. It is likely that the role of autophagy in individual cancers is extremely context-dependent. Thus being able to monitor autophagy on a single cell basis will allow for greater precision in elucidating the precise signaling dynamics of this fluid process in cells. The autophagic dye monodasylcadaverine (MDC) has previously been used in

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fluorescent microscopy but requires a 365 nm excitation source. The Cyto-ID dye is more convenient, being compatible with flow cytometers equipped with a blue laser (488nm). Cyto-ID has been shown to have excellent specificity for autophagosome staining, with only very weak staining of lysosomes.

Standard Procedure:

OCI/AML-3 cell lines were maintained at a culture density of $1x10^5$ – $1x10^6$ cell/mL in 10 mL of alpha-MEM medium supplemented with 10% fetal calf serum (FCS, *v/v*), 100 units of penicillin per mL and 100 µg of streptomycin per mL at 37°C and 5% CO_2.

The U937 cell line was maintained at a culture density of $1x10^5$ – $1x10^6$ cell/mL in 10mL of RPMI 1640 medium supplemented with 10% fetal calf serum (FCS, *v/v*), 100 units of penicillin per mL and 100 ug of streptomycin per mL at 37°C and 5 % CO_2.

1. Cell lines were cultured to a density of $5x10^5$ cells/mL and 2 mL of these cells were collected in 15 mL centrifuge tubes
2. Positive controls were generated by starving cells in culture medium with 0.5 % FBS for 6 hours, to induce autophagic vesicle formation
3. Cells were centrifuged at 400x g for 5 minutes
4. Supernatant medium was removed
5. Cells were washed with 1 mL of PBS
6. Cells were resuspended in 250 µL of PBS containing 5 % FBS
7. To this, 250 µL of Cyto-ID staining solution was added (see reagent list)
8. Cells were incubated for 60 minutes at 37°C
9. Cells were centrifuged at 400x g for 5 minutes
10. Cells were washed with 1 mL of PBS
11. Cells were resuspended in 250 µL of PBS, and placed on ice
12. Autophagic vesicle quantitation was measured via fluorescence on the FITC channel
13. Cells were analysed for fluorescence on a CytoFLEX flow cytometer (Beckman Coulter)
14. Cells were gated on FSC *vs*. SSC to identify the correct, viable cell population.
15. Gated cells were further gated on FSC-area vs. -height to discriminate singlet cells from doublet cells
16. Analysis of Cyto-ID fluorescence was done using overlay histograms of singlet cells

Reagents:

alpha-MEM medium (supplemented with 10% fetal calf serum (FCS, *v/v*), 100 units of penicillin per mL and 100 µg of streptomycin per mL). RPMI 1640 medium (supplemented with 10% fetal calf serum (FCS, *v/v*), 100 units of penicillin per mL and 100 µg of streptomycin per mL). Cyto-ID staining solution: 1 µL of Cyto-ID detection reagent in 1000 µL of 1X Assay buffer, supplemented with 5% FBS.

Laser	405nm					488nm					638nm		
Fluor	Krome Orange	Pacific Blue	V610	V660	V780	FITC	PE	ECD	PC5.5	PC7	APC	APC AF700	APC AF750
Marker						Cyto-ID							

Results

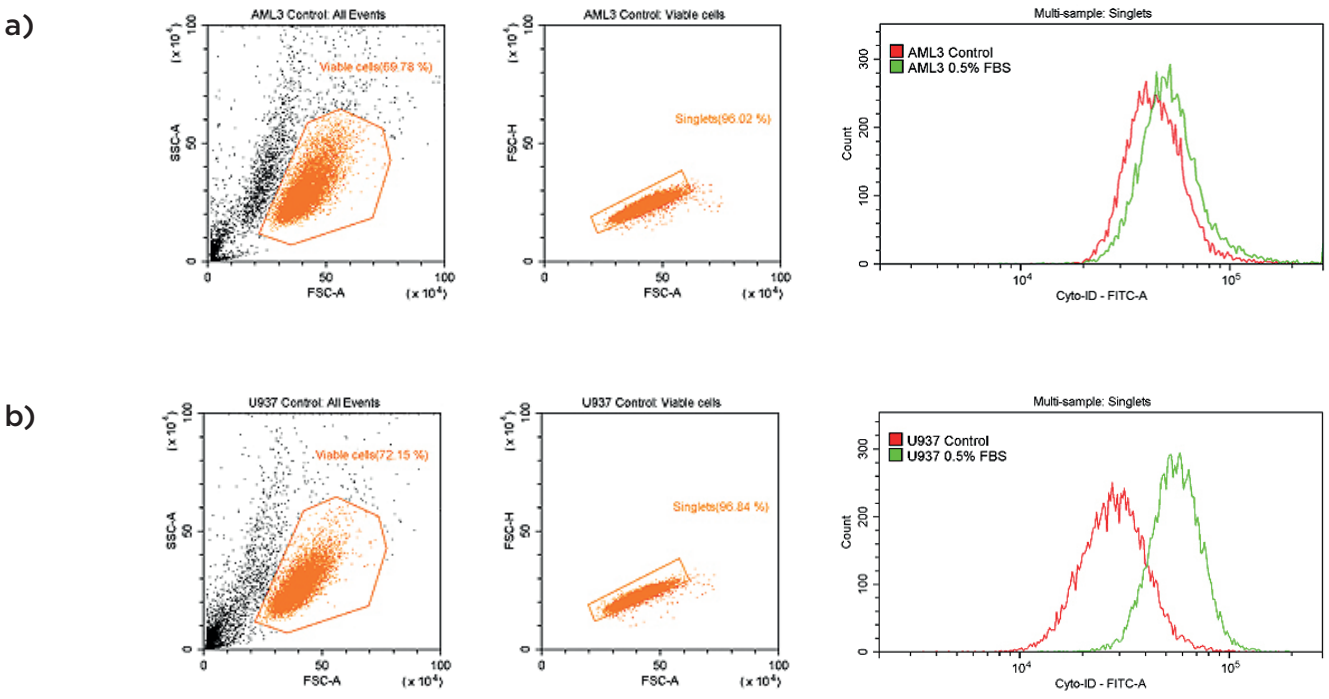

Figure 1: Detection of Autophagic vesicle formation in Leukemia cell lines by Flow Cytometry.

a) Gating strategy for OCI/AML-3 cells viable cell population on a FSC *vs.* SSC dot-plot (upper left). Viable cells were further gated to remove possible doublet cells on a dot-plot for FSC-area *vs.* –height (upper right). Autophagic vesicles were quantified via Cyto-ID fluorescence in the FITC-A channel and plotted as overlayed histograms. Autophagy levels in OCI/AML-3 cells cultured for 6 hours, in the presence of 10 % FBS (red histogram) or 0.5% FBS (green histogram) is shown (bottom).

b) Gating strategy for U937 cells viable cell population on a FSC *vs.* SSC dot-plot (upper left). Viable cells were further gated to remove possible doublet cells on a dot-plot for FSC-area *vs.* –height (upper right). Autophagic vesicles were quantified via Cyto-ID fluorescence in the FITC-A channel and plotted as overlayed histograms. Autophagy levels in U937 cells cultured for 6 hours, in the presence of 10 % FBS (red histogram) or 0.5 % FBS (green histogram) is shown (bottom).

Autophagic vesicle formation is measured by fluorescence in the FITC channel on the Cytoflex. The Cyto-ID reagent has an excitation peak of 463 nm and an emission peak of 534 nm. We used the dye here to examine the effect of starvation on the induction of autophagy in two leukemia cell lines, namely OCI/AML-3 and U937. Cells were starved for 6 hours, by serum withdrawal and compared to control cells grown in full serum. In both cell lines we observed small amounts of apoptosis, as seen by an anti-clockwise shift in cells on the FSC vs. SSC plot. However, the bulk of cells remained in the main population. These cells were gated for singlet cells, FSC-area vs. –height and then plotted for Cyto-ID fluorescence on histogram overlays. From this we can see that starvation in both cell lines increases the fluorescent signal in the FITC channel in the order of a half-log shift to the right. Interestingly, we see that the basal autophagic level of U937 is lower than that of OCI/AML-3. Also, after treatment of both cell lines with serum-depleted medium resulted in a much greater increase in fluorescence in U937 cells. This may be due to their lower baseline levels of autophagic vesicles to begin with. This shows that we can quantify levels of autophagomes with cells, as well as the dynamic increases in these vesicles in cell culture.

References

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Reagent Details

Reagent	Supplier	Order Details
Cyto-ID Autophagy Detection Kit	Enzo Life Sciences	ENZ-51031

For Research Use Only. Not for use in diagnostic procedures.

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