Flow Cytometric Analyses of Autophagic Activity

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[Abstract] Flow cytometry allows very sensitive and reliable high-throughput analysis of autophagic flux. This methodology permits to screen cells in flow and capture multi-component images. Using this technology autophagic flux may be analysed accurately in both suspension as well as adherent cells upon trypsinization independent of how heterogeneous the LC3 punctae content might be. The method is based on the fact that intra-cellularly expressed LC3-GFP serves as a potential autophagic substrate for degradation. Therefore changes in total intracellular LC3-GFP fluorescence intensity is used as an indicator of cellular autophagic activity in living cells. Increased autophagic flux is expected to result in a progressive delivery of LC3-GFP to autolysosome where this substrate undergoes degradation. Therefore, enhanced autophagic flux is detected as a decreased total cellular GFP signal. On the other hand an inhibition of autophagic flux independent of the stage (autophagosome formation, maturation or acidification) leads to accumulation of undegraded LC3-GFP and may be detected as an enhanced intracellular GFP signal (caution: This methodology is based on the assumption that LC3-GFP is expressed constitutively by the model system. Data from analysis of substances or conditions influencing cellular LC3-GFP expression should be interpreted with care).

Materials and Reagents

1. Cell lines of interest (HepG2, HUH7, CMK, K562 *etc.*) stably expressing LC3-GFP
   We recommend the following commercially available plasmids: pBABEpuro GFP-LC3 (plasmid 22405) and pBABE-puro mCherry-EGFP-LC3B (plasmid 22418) generated by Jayanta Debnath from Addgene to be inserted into retroviral constructs and used for cell transduction

2. Eagle's minimal essential medium (EMEM) (ATCC, catalog number: 30-2003) containing 10% fetal bovine serum (FBS) with 100 U/100 µg/ml penicillin/streptomycin (Life Technologies, Gibco®, catalog number: 15140-122)

3. RPMI 1640 with L-glutamine (Lonza, catalog number: BE12-702F) containing 10% FBS with 100 U/100 µg/ml penicillin/streptomycin

4. FBS (Biochrom, catalog number: S0615)
5. Dulbecco's Phosphate Buffered Saline (DPBS) (Biochrom, catalog number: L1825)
6. 1x 0.05% Trypsin-EDTA (phenol red) (Life Technologies, catalog number: 25300)
7. Hanks Balanced Salt Solution (HBSS) (Life Technologies, Gibco®, catalog number: 14025) containing 6 mM glucose (starvation medium)
8. Rapamycin from *Streptomyces hygroscopicus* (1-5 µmol/L) (Sigma-Aldrich, catalog number: R0395)
9. PP242 hydrate (1-5 µmol/L) (Sigma-Aldrich, catalog number: P0037)
10. 3-methyladenine (3-MA) (3-10 mmol/L) (Sigma-Aldrich, catalog number: M9281)
11. Wortmannin (30-100 nmol/L) (Sigma-Aldrich, catalog number: W3144)
12. LY294002 (7-20 µmol/L) (Sigma-Aldrich, catalog number: L9908)
13. Nocodazole (12-50 µmol/L) (Sigma-Aldrich, catalog number: M1404)
14. Vinblastine (12-50 µmol/L) (Sigma-Aldrich, catalog number: V1377)
15. Ammonium chloride (NH_4Cl) (10-20 mmol/L) (Sigma-Aldrich, catalog number: A0171)
16. Hydrohychloroquine sulphate (HCQ) (5-10 µmol/L) (Sigma-Aldrich, catalog number: H0915)
17. Chloroquine (CQ) (5-10 µmol/L) (Sigma-Aldrich, catalog number: C6628)
18. Dimethyl sulfoxide DMSO (Sigma-Aldrich, catalog number: D8418)

Equipment

1. 37 °C, 5% CO_2 humidified incubator
2. Centrifuge
3. FACS Calibur, LSR II (BD) or analogous equipment

Procedure

1. Maintain the cells under standard tissue culture conditions at 37 ºC, 5% CO_2 in a humidified incubator. Keep cell density below 1×10^6/ml and analyse at subconfluent stages.
   Caution: Prior to analysis cell should be kept for several hours (min 12 h) in fresh medium to avoid potential activation of autophagy due to nutrients exhaustion. Generally culture medium contains autophagy affecting substances: amino acids, glucose, growth factors, hormones etc. Take care when comparing autophagic flux under different conditions to normalize for all the necessary factors. Normalize also for the solvent used when analysing the effect of different substances on autophagy – for example DMSO, ethanol etc. might affect autopagic flux.

2. Incubate the cells for the desired time and under the conditions of interest.

   Caution: When analysing prolonged periods of time under conditions potentially affecting cell numbers or viability, differences in nutrient consumption and therefore abundance might occur and influence your results as autophagic activity is highly related to the nutritional status.

3. Positive controls [rapamycin (1-5 µmol/L), PP242 (1-5 µmol/L), Hanks' Balanced Salt Solution containing 6 mmol/L glucose (starvation medium)] and negative controls [3-methyladenine (3-MA) (3–10 mmol/L), wortmannin (30–100 nmol/L), LY294002 (7–20 µmol/L), nocodazole (12–50 µmol/L), vinblastine (12–50 µmol/L), ammonium chloride (NH$_4$Cl) (10–20 mmol/L), hydrohychloroquine (HCQ) or chloroquine (CQ) (5–10 µmol/L)] may be also included. Positive controls activate autophagy and should result in a decreased total cellular LC3-GFP signal. Negative controls inhibit different stages of autophagic process and generally should lead to an increase in total cellular LC3-GFP signal.

   We also recommend analysing the effect of a new substance not only on basic, but also on rapamycin, PP242 or starvation activated autophagic flux.

4. At the end of incubation period transfer the cells to FACS tubes, put them on ice and analyse by flow cytometry plotting the data of cell counts as GFP/FITC fluorescence intensity measured on FL1 channel. For adherent cells, wash once with DPBS, trypsinize, wash again with DPBS, resuspend in DPBS (2% FBS), put on ice and analyse.

   Caution: Generally pharmacological inhibitors of autophagy lack specificity and therefore some cell type specific effects might occur.

   Table 1 may be used to facilitate the interpretation of the results.

Table 1. Analysis of unknown substance X

X plus	Medium	Activator
I	→	↓↓
II	↓↓	↓↓↓
III	↑↑	↓ or ↑

Notes:

a. *→↑↓: Change in LC3-GFP mean fluorescence intensity (MFI).*

b. *(Activator): autophagy induction [rapamycin (1-5 µmol/L), PP242 (1-5 µmol/L) or starvation medium]. Suggested incubation time 4 to 6 h.*

c. *Analysis of unknown substance X should include co incubation with one or more activators. Table 1 illustrates three possible scenarios. a) Scenario (I) the unknown*

substance X does not impact cellular autophagic activity; b) Scenario (II) X is an activator; c) Scenario (III) X is an inhibitor of autophagy.

d. *Caution: Although considered the most accurate sensitive and reliable method for analysis of autophygic flux, flow cytometry determination of LC3-GFP turnover should be combined with alternative methods with non overlapping limitations such as electron microscopy (EM) fluorescence microscopy (FM), western blotting (WB) etc.*

e. *Example data:*
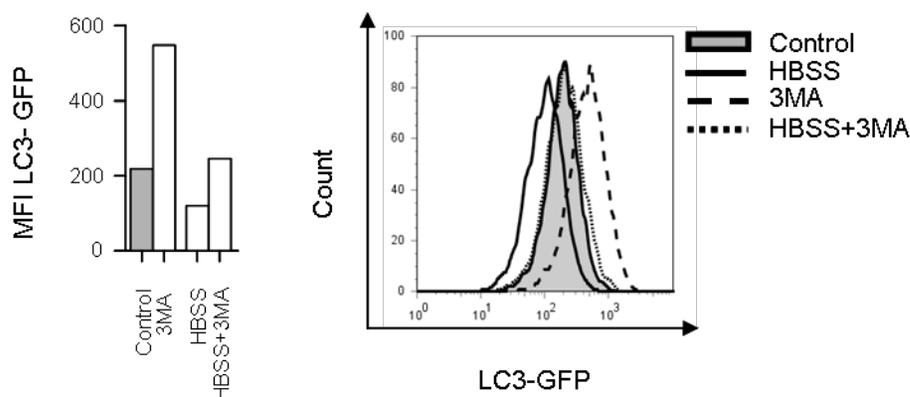
Figure 1. Flow cytometry analysis of autophagic flux in K562 cells expressing LC3-GFP fusion protein incubated with autophagy inhibitor 3MA (10 mM) in the presence or absence of autophagy activation through starvation (HBSS) (left) with representative histograms (right)

Acknowledgments

References

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