Measurement of Autophagy by Flow Cytometry

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ABSTRACT

In recent years, flow cytometry has been used to detect the presence of autophagy mainly by the fluorescent antibody labeling of the autophagy marker, the microtubule associated protein LC3-II. Here we describe the indirect antibody labeling of LC3-II in cells displaying drug-induced autophagy by the use of rapamycin and chloroquine, as well as cells undergoing serum starvation. Although the mechanism of action of LysoTracker dyes is not fully understood, lysosomal mass increases during the autophagic process to enable the cell to produce autolysosomes. Given that LC3-II and LysoTracker are measuring different biological events in the autophagic process, they surprisingly both up-regulated during autophagic process. This approach shows that although LysoTracker dyes do not specifically label lysosomes or autophagosomes within the cell, they allow the simultaneous measurement of an autophagy related process and other live cell functions, which is not possible with the standard LC3-II antibody technique. *Curr. Protoc. Cytom.* 68:9.45.1-9.45.10. © 2014 by John Wiley & Sons, Inc.

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INTRODUCTION

The biological detection and measurement of autophagy initially relied upon electron microscopy (Ashford and Porter, 1962; Deter and Duve, 1967) and more recently, time-consuming image analysis and immunoblot techniques (Kabeya et al., 2000; Barth et al., 2010; Hansen and Johansen, 2011). Only recently has flow cytometry been employed to study the autophagic processes, which not only centers upon the detection, but also the semi-quantification of the autophagic biological marker, microtubule-associated protein LC3-II located in the autophagosome (Thomas et al., 2011; Phadwal et al., 2012; Chikte et al., 2013). This double-membrane-bound vesicle, the autophagosome, is the precursor to the energy-producing autolysosome, which is formed by its fusion with lysosomes (Tooze and Yoshimori, 2010; Yang and Klionsky, 2010). This fusion process appears to result in the formation of more lysosomes during the autophagic process to enable the throughput of these autophagic associated structures the so called "autophagic flux."

Here we describe an antibody intracellular staining protocol for the semi-quantification of LC3-II during the process of autophagy by comparison of median fluorescent intensities (MFI) of autophagic cells induced by numerous inducers of autophagy, e.g., rapamycin, chloroquine and serum starvation,and compare this to that detected in untreated cells (Phadwal et al., 2012; Chikte et al., 2013). The generation of lysosomes during the autophagic process can be measured by the use of LysoTracker dyes, which detect lysosomal mass and whose fluorescent signal increases during autophagy (Boya et al., 2005; Rodriguez-Enriquez et al., 2006; Chikte et al., 2013). These two protocols are compared and their disadvantages are discussed. The two assays should be used in conjunction to gain more information about the autophagic cell, but the anti-LC3-II test must be used to determine the presence of autophagic cells.

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DETECTION OF AUTOPHAGY BY INTRACELLULAR LABELING WITH ANTI-LC3-II

LC3-II is the main biological marker for the detection of autophagy and is located within autophagosomes of cells undergoing the autophagic process. Indirect intracellular labeling with rabbit polyclonal anti-LC3-II, or rabbit immunoglobulin (isotype control), is carried out by incubation with fixed and permeabilized cells that have undergone autophagy. The fluorescent tag of goat anti-rabbit Alexa Fluor-647 is then incubated with these cells. The indirect labeling protocol has the advantage of enhancing the fluorescent signal from LC3-II, which is at relatively low amounts within cells thus enhancing the detection of LC3-II signal above that of the isotype control tube. The resulting cells are then analyzed on a flow cytometer with a red laser. Median Fluorescent Intensity (MFI) of LC3-II-AF-647 signal of the cells were then compared to determine relative increase in the LC3-II autophagic signal above resting control cells.

Materials

Jurkat T cells (European Collection of Cell Cultures)
K562 erythromyeloid cells (European Collection of Cell Cultures)
RPMI 1640 with glutamine (Life Technologies)
Fetal bovine serum (FBS; Life Technologies)
200 U/ml penicillin/200 μg/ml streptomycin solution
Chloroquine (CQ; see recipe)
Rapamycin (see recipe)
Phosphate-buffered saline (PBS; Life Technologies, cat. no. 14190-094)
Caltag Fix Solution A
5% (v/v) Triton X-100 solution (Sigma Aldrich; final concentration 0.25%)
Polyclonal LC3II rabbit antibody, 1 μg/μl (see recipe))
2 μg/μl rabbit immunoglobulin, (see recipe)
2 mg/μl goat-anti-rabbit Alexa Fluor-647 (see recipe)

75-cm^2 tissue culture flask
Tissue culture (TC) hood
50- and 15-ml conical tubes
Thermo Multifuge 3SR+ centrifuge
CO_2 incubator at 37°C
12 × 75–mm polystyrene tubes (Falcon)
Vortex mixer
Flow cytometer with red laser light source

1. Adjust Jurkat T cells and K562 cells to 2 and 1 × 10^6/ml in RPMI 1640 with glutamine and 10% FBS and penicillin and streptomycin.

2. In a TC hood, transfer 25 ml of the cells into 3 × 75-cm^2 tissue culture flasks for controls (untreated cells), and then add 80 nM rapamycin or 50 μM CQ to the remaining 2 TC flasks.

3. Transfer 25 ml of the cells into a 50-ml conical tube. Centrifuge for 5 min at 300 × *g*, 4°C.

4. Pour off the supernatants in a TC hood and add 50 ml sterile PBS. Centrifuge for 5 min at 300 × *g*, 4°C.

5. Pour off the supernatants in a TC hood and add 25 ml sterile RPMI-1640 with glutamine only and transfer to a 75-cm^2 flask.

   This forms the serum starvation experiment.

6. Place all TC flasks into a CO_2 incubator at 37°C.

7. After 24 and 48 hr, remove 12.5 ml cells from each TC flask and transfer into 15-ml conical tubes.

   This experimenter therefore includes an untreated control, 80 nM rapamycin, 50 μM CQ, and serum starvation cell cultures.

8. Centrifuge the tubes for 5 min at 300 × *g*, 4°C, and pour off the supernatants.
9. Resuspend the pellet and split the contents equally into two 12 × 75–mm polystyrene tubes (Falcon).
10. Add 100 μl Caltag solution A, vortex for 2 sec, and incubate for 15 min at room temperature.
11. Add 5 ml PBS and centrifuge for 5 min at 300 × *g*, 4°C, and pour off the supernatants.
12. Add 0.25% (v/v) Triton X-100, vortex 2 sec, and incubate for 15 min at room temperature.
13. Add 5 ml PBS, centrifuge for 5 min at 300 × *g*, 4°C, and pour off the supernatants.
14. To each cell pellet add 0.25 μg anti-LC3-II or rabbit immunoglobulin to the two tubes for each of the cell treatments setups.
15. Vortex for 2 sec and incubate for 30 min at room temperature.
16. Centrifuge for 5 min at 300 × *g*, 4°C, and pour off the supernatants.
17. To each cell pellet add 0.125 μg goat anti-rabbit Alexa Fluor-647 to the two tubes for each of the cell treatment setups.
18. Vortex for 2 sec and incubate for 30 min at room temperature.
19. Centrifuge for 5 min at 300 × *g*, 4°C, and pour off the supernatants.
20. Adjust pellet to 250 to 500 μl with PBS.
21. Analyze 10,000 cells on a flow cytometer with excitation at or near 633 nm and emission collected at 660/20 nm.

    The LC3-II MFI of the 660/20 nm signal from test samples are used to compare autophagic cells to untreated cells, which have a small degree of detectable LC3-II.

ALTERNATE PROTOCOL

LysoTracker LABELING OF AUTOPHAGIC CELLS

During the autophagic process there is an autophagic flux of associated organelles starting with the formation of double-membrane-bound autophagosomes, which then fuse with lysosomes to form autolysosomes. In order for this autophagic flux to be maintained by the cell, more lysosomes are formed within the cell in order to fuel this process. LysoTracker dyes can therefore be used to measure this increase in lysosomal mass during the autophagic process within individual cells. LysoTracker Green is loaded into the cells at 50 ng/ml for 1 hr at 37°C, the cells are washed in PBS, and DAPI (200 ng/ml) is added to determine cell viability. The resulting cells are then analyzed on a flow cytometer with blue (488 nm) and violet lasers. The median fluorescent intensity (MFI) of the LysoTracker Green signal of the live cells is then compared to determine the relative increase in lysosome mass in the cell populations undergoing autophagy.

Additional Materials *(see Basic Protocol)*

LysoTracker Green (LTG; see recipe)

1. Follow steps 1 to 8 of the Basic Protocol.

2. Transfer cell pellet into a 12 × 75-mm polystyrene tubes.
3. Adjust the final volume to a ml after adding LysoTracker Green (LTG) at a final concentration at 50 nM.
4. Vortex for 2 sec and incubate for 1 hr at 37°C.
5. Add 5 ml PBS, centrifuge for 5 min at 300 × *g*, 4°C, and pour off the supernatants.
6. Adjust the cell pellet to 250 to 500 µl with PBS and add DAPI at a final concentration of 200 ng/ml.
7. Analyze 100,000 cells on a flow cytometer with excitation at 488 nm and 405 nm with LTG emission collected at 530/30 nm and DAPI collected at 440/40 nm, respectively.

   The MFI of the 530/30 nm signal is used to compare increasing LysoTracker Green (LTG) signal in cells undergoing autophagic treatments to untreated cells.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; *for suppliers, see* **SUPPLIERS APPENDIX**.

Chloroquine (CQ)

Dissolve chloroquine powder (Sigma-Aldrich, cat. no. C6628) in phosphate-buffered saline (D-PBS; Life Technologies, cat. no. 14190-094) to make a 5 mM solution. Adjust the final concentration to 50 µM with PBS. Store up to 1 year at 4°C.

Goat anti-rabbit Alexa Fluor-647

Prepare a 0.5 ml stock solution of goat anti-rabbit Alexa Fluor-647 (Molecular Probes) at 2 mg/ml. Store up to 6 months at 4°C. Make a 1:40 dilution with PBS (store at 4°C) and add 5 µl or 0.125 µg per test.

LC3-II polyclonal antibody

The stock solution of LC3-II (B) polyclonal antibody (LC3B Antibody Kit for Autophagy, Molecular Probes, cat. no. L10382) is at a concentration of 1 µg/µl (total volume 55 µl). Store the stock solution up to 1 year at −20°C. Dilute the antibody in phosphate-buffered saline (PBS) to prepare a final volume of 1100 µl. Store up to 6 months at 4°C. Add 5 µl or 0.25 µg per test.

LysoTracker Green

Dilute a 50-µl aliquot (1 mM) of LysoTracker Green (LTG; Life Technologies, cat. no. L7526) in dimethyl sulfoxide (DMSO) to 2 µM. Store up to 1 year at −20°C. Add LTG at a final concentration of 50 nM for labeling cells.

Rabbit immunoglobulin

Dilute rabbit immunoglobulin (Sigma Aldrich) in phosphate-buffered saline (PBS) to 1 mg/ml. Store up to 1 year at −20°C. Dilute in PBS to a 1:20 dilution. Store at 4°C. The immunoglobulin is added at a final concentration of 0.25 µg.

Rapamycin

Adjust the concentration of rapamycin (Life Technologies, cat. no. PHZ1235) in dimethyl sulfoxide (DMSO) to 1 mM. Dilute 1:100 with DMSO to make 10 µM aliquots. Store up to 1 year at −20°C. Use at 8 µl/ml (of 10 µM aliquots) to give a final concentration of 80 nM.

COMMENTARY

Background Information

The term autophagy (Type II Apoptosis) is derived from the Greek roots "auto" (self) and "phagy" (eat) and was the term coined by De Duve in 1967 to epitomize this type of cell death (Ashford and Porter, 1962; Deter and Duve, 1967). Autophagy describes an intracellular bulk degradation system that channels malfunctioning components into the lysosomal machinery of the cell, and it is unclear whether the process protects or causes diseases, such as cancer and neurodegenerative disorders (Shintani and Klionsky, 2004; Rosello et al., 2012). Components degraded via autophagy may range from proteins to entire organelles (e.g., mitochondria) to invading microbes, and may be targeted specifically or nonspecifically (Kim et al., 2007; Xie and Klionsky, 2007; Komatsu and Ichimura, 2010; Hailey et al., 2010).

Autophagy is characterized by the formation of a double membrane around the protein aggregates, defective organelles and cytostolic components to be degraded, forming an autophagosome (Tooze and Yoshimori, 2010; Mehrpour et al., 2010) that then fuses with nearby lysosomes, giving rise to an autolysosome, where the intracellular components are degraded by hydrolytic enzymes to generate energy (Tooze and Yoshimori, 2010; Yang and Klionsky, 2010). The process of autophagy is a cell survival mechanism that occurs when the cell is under stress from external and internal environmental pressures, including the lack of nutrients.

Methods for monitoring autophagy were initiated by the discovery of the process by the use of electron microscopy showing the presence of double and single membrane structures termed the autophagosome and autolysosome or autophagolysosome, respectively (Ashford and Porter, 1962; Deter and Duve, 1967). Biochemical techniques, such as immunoblotting, can be used to quantitate the degree of autophagy in cells by measuring the autophagy marker protein, LC3I and LC3-II, which is normally located in the cytoplasm in the form of LC3I but when cleaved and lipidated by phosphatidylethanolamine is then incorporated into the autophagosome in the form of LC3-II (Kabeya et al., 2000; Barth et al., 2010; Hansen and Johansen, 2011). LC3-II can also be imaged and flow cytometrically analyzed with the addition of a fluorescent tag via transfections with GFP-RFP, with the benefit that GFP fluorescence is dissipated by the acidic conditions prevailing in autolysosomes, thus making LC3-II-GFP-RFP detection specific for autophagosomes and LC3-II-RFP only vesicles specific for autolysosomes (Kimura et al., 2007; Shvets et al., 2008; Wu et al., 2008; Barth et al., 2010). The employment of an antibody to LC3-II for image and flow cytometric analysis of puncta or median fluorescence intensity of labeled cells, respectively, allows the detection of autophagosomes and autolysosomes. The increase in the number of puncta labeled with anti-LC3-II can be quantitated by time-consuming image analysis, whereas increase in median fluorescent values of LC3-II antigen levels flow cytometrically makes the process significantly less burdensome (Chen et al., 2007; Kimura et al., 2007; Geng et al., 2010; Thomas et al., 2011).

Invitrogen's LysoTracker dyes label acidic spherical granules within cells and are not lysosome specific. The mechanism of retention within the granules is not established although its fluorescence is not reversed by weak basic compounds. The use of LysoTracker probes have also been used to investigate the degree of autophagy occurring by measurement of their fluorescence by microscopy and to a limited extent by flow cytometry (Boya et al., 2003, 2005; Rodriguez-Enriquez et al., 2006; Byun et al., 2009; Mellen et al., 2009; Chikte et al., 2013). The new autophagy dye from Enzo, Lyso-ID has also been similarly used to monitor autophagy being co-localized with LC3-II during the autophagic process by image cytometry (Phadwal et al., 2012).

These two protocols allow the researcher to compare changes in LysoTracker Green (LTG) signals or lysosomal mass to the main biological autophagy marker, anti-LC3-II, staining levels as measured by flow cytometry using two chemical inducers, rapamycin and CQ, as well as serum starvation using Jurkat T cell leukemia and K562 erythromyeloid leukemia cell lines. CQ not only induces autophagy, but then inhibits the process by blocking lysosomal fusion with the autophagosome by disruption of vesicular acidification, resulting in a build-up of autophagosomes and lysosomes within the cell (Boya et al., 2003, 2005; Shacka et al., 2006; Chikte et al., 2013). The employment of various reagents were chosen for specific purposes, in that CQ does not create an autophagic flux, and thus generates a large signal that can be more easily detected flow cytometrically. Serum starvation can cause apoptosis and cell

cycle arrest. Rapamycin inhibits the action of a nutrient-responsive serine-threonine kinase, mTOR, resulting in cell cycle arrest generating an autophagic flux or throughput of autophagosomes-autolysosomes. Thus, at any one time the autophagic machinery that can be detected is much lower than displayed by CQ, thus testing the sensitivity of these flow cytometric assays to detect autophagy (Paglin et al., 2005; Cao et al., 2006; Vega et al., 2006; Cho and Kwon, 2010; Chikte et al., 2013).

The autophagic process appears to involve the generation of more lysosomes that fuse with the autophagosomes, resulting in the formation of the autolysosomes, thus allowing the flow cytometric quantitation of the autophagic process indirectly in live cells. This also allows the simultaneous measurement of other cell functions, such as mitochondrial function, ER and mitochondrial autophagy (Boya et al., 2003, 2005; Shacka et al., 2006). The use of LTG dye was used to indirectly quantitate the degree of autophagy in two cell lines, Jurkat T cells and the erythromyeloid leukemia cell line K562. Increase in LysoTracker signal can be compared flow cytometrically to the level of LC3-II formation as detected by fluorescently tagged antibody in K562 and Jurkat cell lines. This comparison of the acidic granule and LC3-II levels is consequently not a direct comparison of the same processes involved in autophagy. The development of a cheap, reproducible and easy flow cytometric based method to determine the degree of autophagy in live cells will enhance the ability to study the cell death processes and mechanisms involved in autophagy in primary cells isolated from patients as well as cell lines. The use of LysoTracker dyes can be used in conjunction to measure other live cell functions (e.g., cell viability and mitochondrial function) with the definitive anti-LC3-II indirect intracellular labeling technique, as the increase in lysosomal mass during the autophagic process is not definitive as this may occur in other disease processes.

Critical Parameters

The quality of starting cell cultures is critical in order to achieve reproducible results. Thus, cell cultures must have the same cell numbers, and fresh culture medium should be used at the start of each experiment. The magnitude of the response to rapamycin, CQ, and serum-free growth conditions are all time- and cell-type dependent. The researcher should therefore perform a time and dye concentration experiment to determine optimum loading conditions for the cells in question.

Drugs used to induce autophagy are dose- and time-dependent depending upon the cell type used; therefore, these parameters should be tested to gain optimum results.

The polyclonal LC3-II antibody should be specific for this form of LC3, and the antibody used in this study has been previously demonstrated to be specific for LC3-II puncta (Campbell and Spector, 2011), as nonlipidated LC3 is located in the cytoplasm of the cell, so a pan-LC3 antibody would require the need for washing the labeled antibody from the cytoplasm to achieve a true reading of LC3-II.

The adequate loading of LTG is to some extent cell-type dependent. The manufacturer's product instructions state that the loading concentration is between 50 to 75 nM and the incubation time is 0.5 to 2 hr at 37°C. The researcher should therefore perform a time and dye concentration experiment to determine optimum loading conditions for the cells in question.

Troubleshooting

The researcher should use anti-LC3-II antibody that has been tested for use in immunohistochemistry only. The indirect intracellular labeling of LC3-II can fail or be incomplete when permeabilizing the cells with Triton X-100 (if added at the same time as the antibody). Therefore, with the Life Technologies polyclonal antibody, it is required to keep these two steps separate.

Detectable results are achieved by the amplification step of indirect labeling and using a highly quantum efficient fluorochrome, e.g., Alexa Fluor-647. All detectable signals can be increased by the use of lysosomal inhibitors, which stop the autophagic flux.

Even though the antibody used was polyclonal, the use of antibody blocking reagents was shown previously not to be required.

The use of an indirect labeled isotype control aids in the demonstration that the cells in question contain higher levels of LC3-II than untreated cells, which are also slightly positive for LC3-II.

Live cells should be gated upon by the use of DAPI (200 ng/ml), as dead cells have significantly fewer lysosomes than live cells. The use of DAPI also allows this to be done with LTG without the need for compensation.

Anticipated Results

Flow cytometry can be employed to detect the primary autophagy marker, LC3-II, when

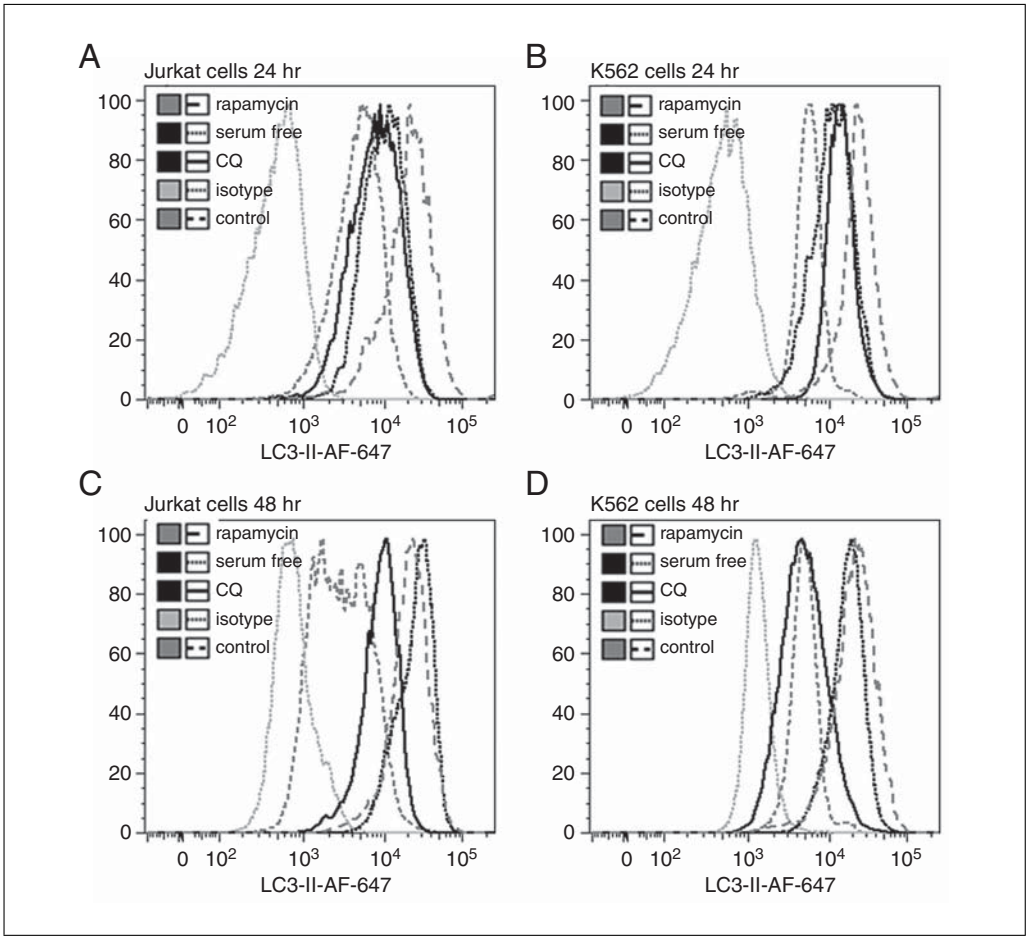

Figure 9.45.1 Jurkat and K562 cells were untreated, treated with 50 μM CQ respectively, grown in serum-free conditions, and treated with 80 nM rapamycin for 24 and 48 hr. All cells were then fixed and permeabilized and labeled with anti-LC3-II-AF-647 as described in the Basic Protocol. (**A**) Jurkat cells after 24 hr were analyzed for LC3-II MFI values with controls (4,933), isotype (495), 50 μM CQ (7,958), serum-free (10,223), and 80 nM rapamycin (22,318). (**B**) K562 cells after 24 hr were analyzed for LC3-II MFI values with controls (5,658), isotype (514), 50 μM CQ (14,642), serum-free (11,835), and 80 nM rapamycin (24,163). (**C**) Jurkat cells after 48 hr were analyzed for LC3-II MFI values with controls (2,849), isotype (730), 50 μM CQ (9,323), serum-free (27,576), and 80 nM rapamycin (22,382). (**D**) K562 cells after 48 hr were analyzed for LC3-II MFI values with controls (4,973), isotype (1,282), 50 μM CQ (4,761), serum-free (18,448), and 80 nM rapamycin (22,545).

two cell lines are undergoing autophagic flux (small signal) after treatment with three different inducers of autophagy (these included chloroquine, rapamycin, and serum starvation; see Fig. 9.45.1). LysoTracker dyes also allowed the detection of increased LTG signal or lysosomal mass in cells undergoing the autophagic process by chloroquine, rapamycin, and serum starvation (Fig. 9.45.2). The different treatments employed show that not only can autophagy be detected when there was a buildup of LC3-II and LTG signals when cells are not undergoing autophagic flux as in the case of chloroquine, but also when the cells are undergoing autophagic flux. This demonstrates the sensitivity of these autophagic flow cytometric assays, without the need for expensive lysosomal inhibitors (Phadwal et al., 2012). The use of two cell types also shows that the level of the autophagic response can be different with the same inducers of autophagy. Although the apparent buildup of acidic granules, as detected by increased LTG signals during the autophagic process this is not a direct measure of autophagy and hence was not expected to match LC3-II levels in an identical fashion. However, this study has shown that this LTG up-regulation appears biologically to be highly significant in autophagy.

Figure 9.45.2 shows the LC3-II MFI response of Jurkat T cells and K562 cells to the treatments employed in this protocol. The

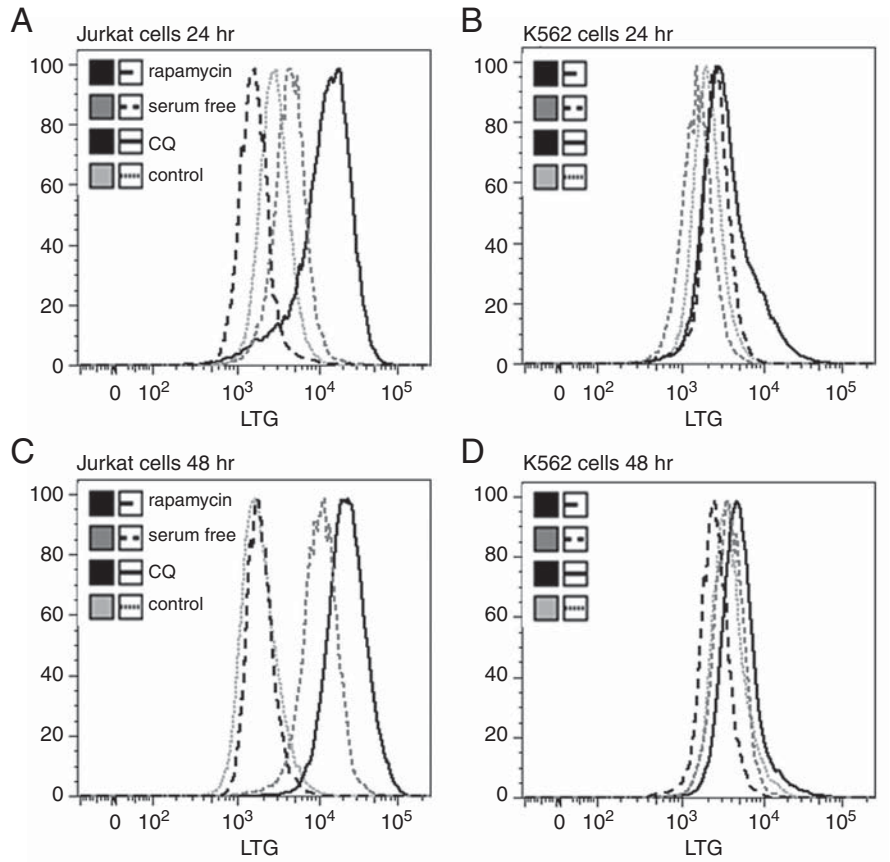

Figure 9.45.2 Jurkat and K562 cells were untreated, treated with 50 μM CQ respectively, grown in serum-free conditions, and treated with 80 nM rapamycin for 24 and 48 hr. All cells were then loaded with 50 nM LTG and DAPI as described in the Alternate Protocol. (**A**) Jurkat cells after 24 hr were analyzed for LTG MFI values with controls (2,806), 50 μM CQ (13,344), serum-free (4,433), and 80 nM rapamycin (1,563). (**B**) K562 cells after 24 hr were analyzed for LTG MFI values with controls (1,976), 50 μM CQ (3,119), serum-free (1,496), and 80 nM rapamycin (2,550). (**C**) Jurkat cells after 48 hr were analyzed for LTG MFI values with controls (1,742), 50 μM CQ (22,815), serum-free (10,704), and 80 nM rapamycin (1,835). (**D**) K562 cells after 48 hr were analyzed for LTG MFI values with controls (3,570), 50 μM CQ (4,796), serum-free (3,711), and 80 nM rapamycin (2,458).

magnitude of the responses at 24 hr in the different cell types to the different treatments is broadly similar. Rapamycin gives the largest response, with serum starvation generating half the level of LC3-II signal. CQ gives a larger LC3-II response than serum starvation in K562 cells at 24 hr, with CQ treatment of Jurkat cells generating the lowest amount of detectable LC3-II of the treatments at 24 hr. After 48 hr the rapamycin response is maintained for both cell types used in this study; 48 hr of serum starvation results in an increase in LC3-II compared to that observed at 24 hr. The CQ autophagic response is maintained by Jurkat cells at 48 hr; K562 cells show no autophagic response to CQ above control levels at 48 hr.

Figure 9.45.2 shows the LTG MFI response of Jurkat T cells and K562 cells to the treatments employed in this protocol. The magnitude of the LTG responses at 24 hr in the different cell types to the different treatments is different to that observed with LC3-II levels found in the Basic Protocol. Rapamycin now gives the lowest LTG response, with serum starvation generating a detectable autophagic LTG response in Jurkat cells but not K562 cells at 24 hr. CQ gives the largest LTG autophagic response in both cell types at 24 hr. After 48 hr the lack of a response to rapamycin is maintained for both cell lines; 48 hr of serum starvation and CQ treatment results in an increase in LTG compared to levels at 24 hr.

Time Considerations

LC3-II labeling (Basic Protocol)

The indirect labeling of LC3-II will take 20 to 30 min each for the fixing and permeablization and washing steps. The process can be stopped at the fixation step with samples stored at 4°C if the researcher does not have the required time to complete the procedure. The two antibody labeling steps both take 35 min each with the whole procedure taking just over 2 hr to complete, depending on the number of samples in each run.

LTG labeling (Alternate Protocol)

The loading of harvested cells with LTG takes 1 hr with 5 min for washing.

Literature Cited

Ashford, T.P. and Porter, K. 1962. Cytoplasmic components in hepatic cell lysosomes. *J. Cell Biol.* 12:198-202.

Barth, S., Glick, D., and Macleod, K. Autophagy: Assays and artifacts. 2010. *J. Pathol.* 221:117-124.

Boya, P., Gonzalez-Polo, R.A, Poncet, D., Andreau, K., Vieira, H.L.A., Roumier, T., Perfettini, J.L., and Kroemer G. 2003. Mitochondrial membrane permeabilization is a critical step of lysosome-initiated apoptosis induced by hydroxychloroquine. *Oncogene* 22:3927-3936.

Boya, P., Gonzalez-Polo, R.A., Casares, N., Perfettini, J.L., Dessen, P., Larochette, N., Metivier, D., Meley, D., Souquere, S., Yoshimori, T., Pierron, G., Codogno, P., and Kroemer, G. 2005. Inhibition of macroautophagy triggers apoptosis. *Mol. Cell. Biol.* 25:1025-1040.

Byun, J.Y., Yoon, C.H., An, S., Park, I.C., Kang, C.M., Kim, M.J., and Lee, S.J. 2009. The Rac1/MKK7/JNK pathway signals upregulation of Atg5 and subsequent autophagic cell death in response to oncogenic Ras. *Carcinogenesis* 30:1880-1888.

Campbell, G.R. and Spector, S.A. 2011. Hormonally active vitamin D3 (1α, 25-dihydroxycholecalciferol) triggers autophagy in human macrophages that inhibits HIV-1 infection. *J. Biol. Chem.* 286:18890-18902.

Cao, C., Subhawong, T., Albert, J.M., Kim, K.W., Geng, L., Sekhar, K.R., Gi, Y.J., and Lu, B. 2006. Inhibition of mammalian target of rapamycin or apoptotic pathway induces autophagy and radiosensitizes PTEN null prostate cancer cells. *Cancer Res.* 66:10040-10047.

Chen, Y., McMillan-Ward, E., Kong, J., Israels, S.J, and Gibson, S. 2007. Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. *Cell Death Different.* 15:171-182.

Chikte, S., Panchal, N., and Warnes, G. 2013. Use of LysoTracker dyes: A flow cytometric study of autophagy. *Cytometry A* doi:10.1002/cyto.a.22333.

Cho, Y.S. and Kwon, H. 2010. Control of autophagy with small molecules. *Arch. Pharmacol. Res.* 33:1881-1889.

Deter, R.L. and Duve, C.D. 1967. Influence of glucagon, an inducer of cellular autophagy, on some physical properties of rat liver lysosomes. *J. Cell Biol.* 33:437-449.

Geng, Y., Kohil, L., Klocke, B.J., and Roth K.A. 2010. Chloroquine-induced autophagic vacuole accumulation and cell death in glioma cells is p53 independent. *Neuro-Oncology* 12: 473-481.

Hailey, D.W., Rambold, A.S., Satpute-Krishnan, P., Mitra, K., Sougrat, R., Kim, P.K., and Lippencott-Schwartz, J. 2010. Mitochondria supply membranes for autophagosome biogenesis during starvation. *Cell* 141:656-667.

Hansen, T.E. and Johansen, T. 2011. Following autophagy step by step. *BMC Biol.* 9:1-4.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., and Yoshimori, T. 2000. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J.* 19:5720-5728.

Kim, I., Rodriguez-Enriquez, S., and Lemasters, J. 2007. Selective degradation of mitochondria by mitophagy. *Arch. Biochem. Biophys.* 462:245-253.

Kimura, S., Noda, T., and Yoshimori, T. 2007. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. *Autophagy* 3: 452-460.

Komatsu, M. and Ichimura, Y. 2010. MBSJ MCC Young Scientist Award 2009: Selective autophagy regulates various cellular functions. *Genes Cells* 15:923-933.

Mehrpour, M., Esclatine, A., Beau, I., and Codogno, P. 2010. Overview of macroautophagy regulation in mammalian cells. *Cell Res.* 20:748-762.

Mellen, M.A., de la Rosa, E.J., and Boya P. 2009. Autophagy is not universally required for phosphatidyl-serine exposure and apoptotic cell engulfment during neural development. *Autophagy* 5:964-972.

Paglin, S., Lee, N.Y., Nakar, C., Fitzgerlad, M., Plotkin, J., Deuel, B., Hackett, N., McMahill, M., Sphicas, E., Lampden, N., and Yahalom, J. 2005. Rapamycin-sensitive pathway regulates mitochondrial membrane potential, autophagy, and survival in irradiated MCF-7 cells. *Cancer Res.* 65:11061-11070.

Phadwal, K., Alegre-Abarrategui, J., Watson, A.S., Pike, L., Anbalagan, S., Hammond, E.M., Wade-Martins., R., McMichael, A., Klenerman, P., and Simon, A.K. 2012. A novel method for autophagy detection in primary cells: Impaired levels of macroautophagy in immunosenescent T cells. *Autophagy* 8:677-689.

Rodriguez-Enriquez, S., Kim, I., Currin, R.T., and Lemasters, J.L. 2006. Tracker dyes to probe mitochondrial autophagy (mitophagy) in rat hepatocytes. *Autophagy* 2:39-47.

Rosello, A., Warnes, G., and Meier, U.C. 2012. Cell death pathways and autophagy in the central nervous system and its involvement in neurodegeneration, immunity and CNS infection: To die or not to die—that is the question. *Clin. Exp. Immunol.* 168:52-57.

Shacka, J.J., Klocke, B.J., Shibata, M., Uchiyama, Y., Datta, G., Schmidt, R.E., and Roth, K.A. 2006. Bafilomycin A1 inhibits chloroquine-Iinduced death of cerebellar granule neurons. *Mol. Pharmacol.* 69:1125-1136.

Shintani, T. and Klionsky, D. 2004. Autophagy in health and disease: A double-edged sword. *Science* 306:990-995.

Shvets, E., Fass, E., and Elazar, Z. 2008. Utilizing flow cytometry to monitor autophagy in living mammalian cells. *Autophagy* 4: 621-628.

Thomas, S., Thurn, K.T., Biçaku, E., Marchion, D.C., and Münster, N. 2011. Addition of a histone deacetylase inhibitor redirects tamoxifen-treated breast cancer cells into apoptosis, which is opposed by the induction of autophagy. *Breast Cancer Res. Treatment* 130: 437-447.

Tooze, S. and Yoshimori, T. 2010. The origin of the autophagosomal membrane. *Nat. Cell Biol.* 12:831-835.

Vega, F., Medeiros, L.J., Levetaki, V., Atwell, C., Cho-Vega, J.H., Tian, L., Claret, F.X., and Rassidakis, G.Z. 2006. Activation of mammalian target of rapamycin signaling pathway contributes to tumor cell survival in anaplastic lymphoma kinase-positive anaplastic large cell lymphoma. *Cancer Res.* 66:6589-6597.

Wu, Y.T., Tan, H.L., Huang, Q., Kim, Y.S., Pan, N., Ong, W.Y., Liu, Z.G., and Shen, H.M. 2008. Autophagy plays a protective role during zVAD-induced necrotic cell death. *Autophagy* 4:457-466.

Xie, Z. and Klionsky, D. 2007. Autophagosomes formation: Core machinery and adaptations. *Nat. Cell Biol.* 9:1102-1109.

Yang, Z. and Klionsky, D. 2010. Eaten alive: A history of macroautophagy. *Nat. Cell Biol.* 12:814-822.