Chapter 14

Quantification of Autophagy During Senescence

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Abstract

Autophagy constitutes an evolutionarily conserved catabolic process that contributes to the clearance of damaged cellular components in response to a variety of stress conditions. Additionally, it plays a variety of physiological and pathophysiological roles in maintaining cell homeostasis. Recently, the critical role of autophagy during cellular senescence has been supported by evidences demonstrating the reversal of senescence by the reestablishment of autophagy. As considerable attention has been directed toward understanding the molecular mechanisms underlying senescence and autophagy, a method to accurately quantify autophagy during senescence is critical to understand its role in senescence and senescence-related diseases. In this chapter, we describe the use of CYTO-ID® green dye and DQ™ Red BSA to monitor the autophagic flux as an accurate method to quantify autophagic activity. This technique relies on the specificity of CYTO-ID® green dye in staining autophagosome and the cleavage of the self-quenched DQ™ Red BSA protease substrates in an acidic compartment. In particular, herein we describe protocols to quantify autophagy during senescence.

Key words Autophagy, Senescence, CYTO-ID® green dye, DQ™ Red BSA, Autophagic flux

1 Introduction

Autophagy constitutes a highly dynamic and multistep process required for the lysosomal degradation of cytoplasmic proteins and organelles [1]. It utilizes a double-membraned vesicle known as an autophagosome, in which intracellular substrates are enwrapped as cargo (Fig. 1) [2]. The autophagosome fuses with a lysosome to form an autolysosome, which allows for the turnover of cytoplasmic components in bulk (cytosol with any content present in that area) or selectively (e.g., mitochondria, ribosomes, lipids, peroxisomes, or endoplasmic reticulum) (Fig. 1) [2]. Autophagy plays a variety of physiological and pathophysiological roles including starvation adaptation, clearance of damaged cellular components, and regulation of cellular differentiation [3, 4].

Cellular senescence, defined as a state in which normal somatic cells lose their replicative capacity [5], can be triggered in response to diverse forms of cellular stress including as a consequence of

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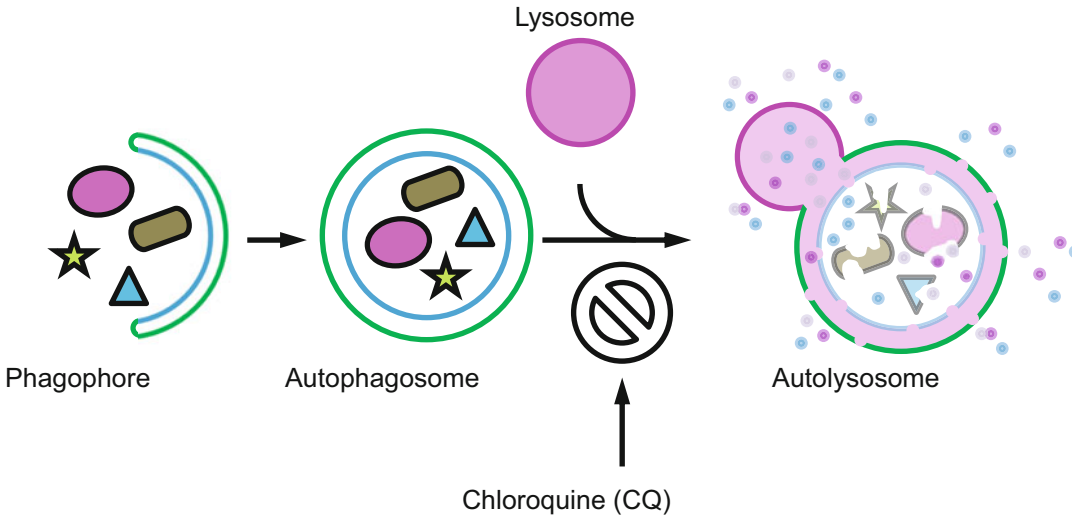

Fig. 1 The process of autophagy and its dynamic regulation. A portion of cytoplasm, including organelles, is enclosed by a phagophore to form an autophagosome. The outer membrane of the autophagosome fuses with a lysosome to form an autolysosome, and the enclosed materials are degraded in the resulting autolysosome. As an autophagy inhibitor, CQ, which blocks the fusion of autophagosomes with lysosomes, is depicted in the diagram

oxidative stress, telomere erosion, or DNA damage [6]. Autophagy also plays an important role during senescence through the elimination of old or unneeded materials [7]. However, autophagic activity has been known to decrease, resulting in the accumulation of indigestible material in senescent cells [8]. This phenomenon is further supported by the recent finding that the inhibition of autophagic activity induces premature senescence in reactive oxygen species (ROS)- and p53-dependent manner [9]. Furthermore, the knockdown of several autophagy-related genes accelerates aging-related pathologies, consequently resulting in the reduction of life span [10]. Similarly, loss of function in Beclin-1 (also known as Atg6) leads to the early onset of aging and age-associated diseases [11].

As considerable attention has been directed toward understanding the molecular mechanisms underlying senescence and autophagy, numerous new techniques have been developed to quantify autophagy during senescence [12]. In particular, a method to accurately quantify autophagy is critical to understand its role in senescence and senescence-dependent diseases. However, a common misconception is the notion that increased numbers of autophagosomes correspond to increased autophagic activity in cells. For example, autophagosome accumulation may represent either the increased generation of autophagosomes and/or a block in autolysosomal maturation [13]. As the number of autophagosomes is a function of the balance between their generation and conversion into autolysosomes, the simple determination of autophagosome numbers is therefore insufficient for quantifying autophagic

activity. In contrast, autophagic flux is defined as a measure of autophagic degradation activity and can be determined by comparing the numbers of autophagosomes in the absence and presence of lysosomal inhibitors (e.g., chloroquine, verapamil, norclomipramine, and hydroxychloroquine) [14, 15]. It provides a meaningful way in which the balance of autophagosome generation and clearance can be measured [14, 15]. This chapter summarizes a manageable, widely applicable approach for the quantification of autophagy, principally focused on the measurement of autophagic flux. Specially, this chapter is based on the introduction of CYTO-ID® green dyes and DQ™ Red BSA to quantify autophagic flux (Subheadings 3.2 and 3.3).

2 Materials

1. CYTO-ID® Green dye (ENZ-51031-K200; Enzo Life Sciences).
2. 10× Assay Buffer (ENZ-51031-K200; Enzo Life Sciences).
3. LysoTracker® Deep Red (L12492; Life Technologies).
4. Chloroquine (C6628; Sigma).
5. DQ™ Red BSA (D12051; Life Technologies).
6. Total growth medium suitable for each cell type.
7. Fetal bovine serum.
8. Penicillin/streptomycin.
9. Trypsin–EDTA (0.05%).
10. MycoAlert Mycoplasma Detection kit.
11. Deionized water.
12. Tubes appropriate for holding cells for the flow cytometer.
13. Flow cytometer (BD LSR II flow cytometer, BD biosciences).
14. Analysis software (FlowJo 7.6.1 software, Tree Star Inc.)

3 Methods

The methods described below outline the establishment of senescent fibroblast cells (Subheading 3.1) and the measurement of autophagic flux by labeling with CYTO-ID® green dye (Subheading 3.2) and DQ™ Red BSA (Subheading 3.3).

3.1 Establishment of Senescent Fibroblast Cells

1. Cells are cultured in the complete culture medium under standard tissue culture conditions at 37 °C, 5% CO_2 in a humidified incubator. The selection of the medium depends on the cell types to be cultured.

2. Cells are serially passaged at 1:4 dilution during early passages and at 1:2 during late passages. When the population doubling time (PD) of the cells is less than 2 days or over 14 days, the cells are considered young or senescent, respectively.
3. Cells are tested for mycoplasma contamination every other week by using the MycoAlert Mycoplasma Detection kit.

3.2 Measurement of Autophagic Flux by Labeling with CYTO-ID® Green Dye

A conventional fluorescent probe, monodansylcadaverine (MDC), has served as a useful fluorescent marker for lysosomal and autophagic vacuoles [16]. However, this probe is known to generate high background and weak fluorescent signal. In comparison, the recently developed 488 nm-excitable CYTO-ID® green dye becomes brightly fluorescent in vesicles produced during the autophagy pathway, generating low background and high fluorescent signal (Fig. 2) [17, 18]. Unlike the lysomotrophic dyes (e.g., MDC, LysoTracker® Red, and Acridine Orange), which are able to penetrate the lysosomes, the CYTO-ID® green dye is a cationic amphiphilic tracer that rapidly partitions into cells in a similar way as many cationic drugs (Fig. 2) [19]. This dye weakly stains lysosomes, albeit strongly staining autolysosomes and earlier autophagic compartments [20–22]. Moreover, it has been optimized for the detection of autophagy in live cells by flow cytometry [19].

Autophagic flux can be determined by comparing the levels/numbers of autophagosomes with or without chloroquine (CQ) treatment (Fig. 2) [14, 15]. CQ neutralizes the acidic pH required for optimal lysosomal enzyme activity, which blocks the fusion of autophagosomes with lysosomes, resulting in inhibition of autolysosomal and lysosomal degradation [23–25].

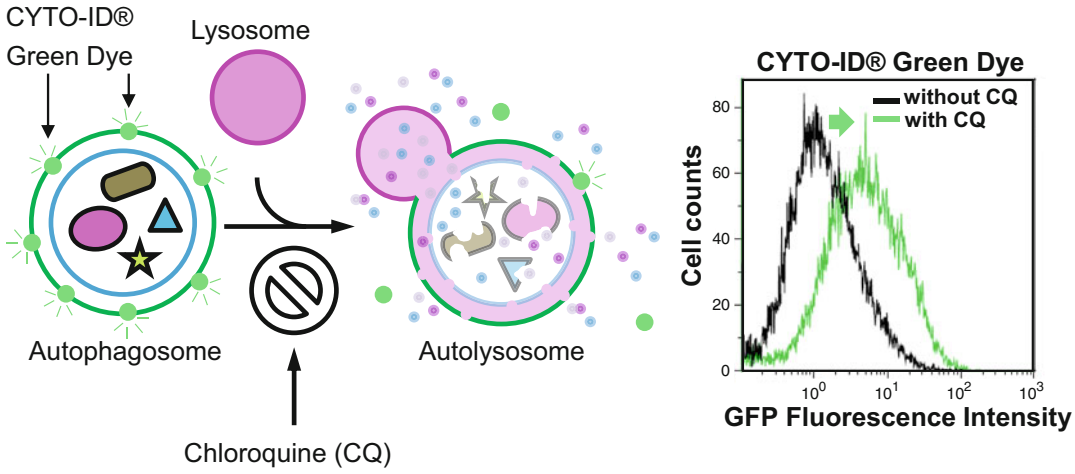

Fig. 2 Schematic representation of CYTO-ID® green dye staining (Left) and flow cytometry analysis of autophagic flux in senescent fibroblast cells incubated with or without CQ (30 μM) for 2 h, followed by further staining with a CYTO-ID® staining solution for 30 min (Right). Histogram plot presentation of the GFP fluorescence intensity vs. cell counts (Right)

The protocol for labeling autophagosomes with CYTO-ID® green dye and assessing autophagic flux by flow-cytometric analysis is as follows:

1. Maintain cells under standard tissue culture conditions in a 5% CO_2 incubator at 37 °C (*see* **Note 1**). Maintain cell density at 65–85% confluence.
2. Cells are incubated in complete culture medium, with or without 30 μM CQ, which is used to block autophagic degradation, for 2 h at 37 °C.
3. Prepare a CYTO-ID® staining solution by diluting 1 μL CYTO-ID® green dye to 1 mL complete culture medium (*see* **Note 2**). To measure lysosomal mass, dilute LysoTracker® Deep Red (LTDR) to 50 nM in a CYTO-ID® staining solution.
4. Prepare 1× Assay Buffer by diluting 1 mL of the 10× Assay Buffer with 9 mL of deionized water (*see* **Note 3**).
5. At 2 h after incubation with or without CQ, cells are further stained with a CYTO-ID® staining solution and 50 nM LTDR for 30 min at 37 °C. To measure background autofluorescence, cells are also incubated in complete culture medium without any dyes.
6. After incubation, trypsinize cells with trypsin–EDTA (0.05%) solution.
7. Centrifuge at 200 × *g* for 5 min to pellet the cells. Wash the cells by resuspending the cell pellet in 1× Assay Buffer and collect the cells by centrifugation.
8. Resuspend the cell pellets in 1× Assay Buffer. Samples should contain 1×10^5 to 1×10^6 cells per mL. It is important to achieve a monodispersed cell suspension at this step by gently pipetting up and down repeatedly.
9. Analyze the samples in the green (530/30 nm bandpass filters with excitation at 488 nm) and red (690/50 nm band-pass filters with excitation at 647 nm) channel of a flow cytometer.
10. Subtract background intensity. Autophagic flux is calculated using the following equation: ΔMFI (mean fluorescence intensity) = [MFI CYTO-ID® (+CQ)/MFI LTDR (+CQ)] − [MFI CYTO-ID® (−CQ)/MFI LTDR (−CQ)].

3.3 Measurement of Autophagic Flux with DQ™ Red BSA

Another method to quantify autophagy is to incubate cells with DQ™ Red BSA that is labeled to a high degree with red fluorescent BODIPY® TR-X dye [26, 27]. DQ™ Red BSA will accumulate in autophagosomes and will be combined with functional lysosomes to generate autolysosomes (Fig. 3) [26, 27]. Then, DQ™ Red BSA will be cleaved by proteases in the autolysosomes (Fig. 3) [26, 27]. The proteolysis of this conjugate can be easily monitored because digestion results in de-quenching and releases brightly fluorescent fragments (Fig. 3). Thus, the use of DQ™ Red BSA is

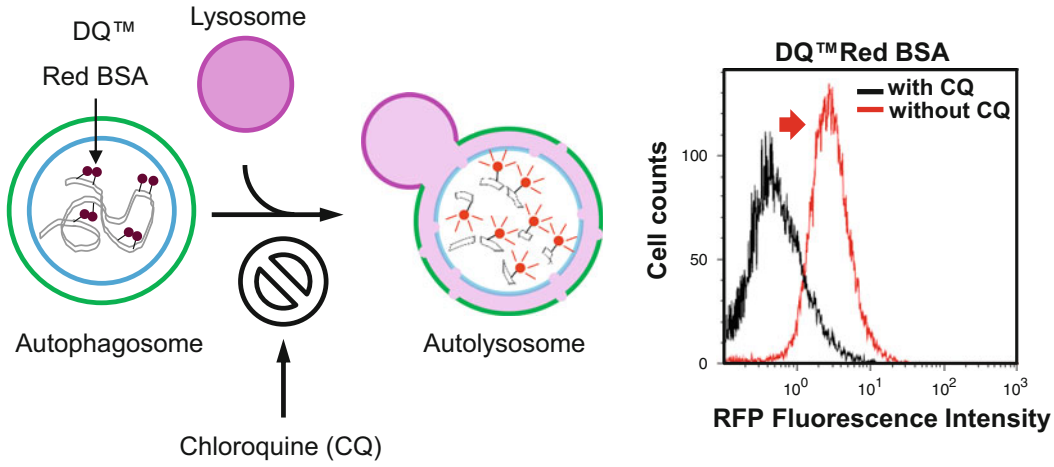

Fig. 3 Schematic representation of self-quenched DQ™ Red BSA upon cleavage by proteases in acidic compartments (Left). Proteolysis results in fragment formation with fluorescence dequenching, which is observed as an increase in fluorescence intensity. Flow cytometry analysis of autophagic flux in senescent fibroblast cells incubated with DQ™ Red BSA (100 μg/mL) in complete culture medium, with or without 10 μM CQ for 2 days (Right). Histogram plot presentation of the RFP fluorescence intensity vs. cell counts (Right)

effective for detecting intracellular proteolytic activity as a measure of a functional autolysosomes. DQ™ Red BSA labeling can be combined with or without CQ to assess the autophagic flux (Fig. 3). A cation to interpret the labeling results with DQ™ Red BSA is that this dye can be dequenched in late endosomes [28, 29]. Given that CYTO-ID® green dye can stain autolysosomes and earlier autophagic compartments, DQ™ Red BSA labeling can be combined with CYTO-ID® green dye to monitor the convergence of autophagosomes with functional degradative compartments.

The protocol for detecting intracellular proteolytic activity as a measure of a functional autolysosome with DQ™ Red BSA and assessing autophagic flux by flow-cytometric analysis is as follows:

1. Maintain cells under standard tissue culture conditions in a 5% CO_2 incubator at 37 °C (*see* **Note 1**). Maintain cell density at 65–85% confluence.
2. Cells are incubated for 2 days at 37 °C with DQ™ Red BSA (100 μg/mL) in complete culture medium, with or without 10 μM CQ, which blocks the autophagic degradation of endocytosed DQ™ Red BSA (*see* **Note 4**).
3. After incubation with DQ™ Red BSA, replace medium with a CYTO-ID® staining solution (diluting 1 μL CYTO-ID® green dye to 1 mL complete culture medium, with or without 10 μM CQ) (*see* **Note 2**). Cells are incubated in a CYTO-ID® staining solution for 30 min at 37 °C. To measure background autofluorescence, cells are also incubated in complete culture medium without any dyes.

4. After incubation, trypsinize cells with trypsin–EDTA (0.05%) solution.
5. Centrifuge at 200 × *g* for 5 min to pellet the cells. Wash the cells by resuspending the cell pellet in 1× Assay Buffer (*see* **Note 3**) and collect the cells by centrifugation.
6. Resuspend each live cell pellet in 1× Assay Buffer. Samples should contain 1×10^5 to 1×10^6 cells per mL. It is important to achieve a monodispersed cell suspension at this step by gently pipetting up and down repeatedly.
7. Analyze the samples in the red (630/30 nm bandpass filters with excitation at 590 nm) and green (530/30 nm bandpass filters with excitation at 488 nm) channel of a flow cytometer.
8. Fluorescence from DQ™ Red BSA is normalized with fluorescence from CYTO-ID® green dyes, following subtraction of background autofluorescence.
9. Autophagic flux is calculated using the following equation: ΔMFI (mean fluorescence intensity) = [MFI DQ™ Red BSA (−CQ)/MFI CYTO-ID® (−CQ)] − [(MFI DQ™ Red BSA (+CQ)/MFI CYTO-ID® (+CQ)].

4 Notes

1. Prior to analysis cells should be kept in fresh medium for at least 12 h to avoid potential activation of autophagy owing to nutrient exhaustion.
2. The concentration of the CYTO-ID® green dye in complete culture medium necessary to obtain optimal staining will depend on the cell types.
3. Allow the 10× Assay Buffer to warm to room temperature. Make sure that the reagent is free of any crystallization before dilution.
4. To avoid inefficient localization of DQ™ Red BSA, cells should be incubated for 2 days with DQ™ Red BSA. However, optimal durations should be determined according to cell type.

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Competing financial interests statement

The authors declare no competing financial interests.

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