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Applications of flow cytometry for measurement of autophagy

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ABSTRACT

Autophagy is a dynamic catabolic process that plays a major role in sequestering and recycling cellular components in multiple physiological and pathophysiological conditions. Despite recent progress in our understanding of the autophagic process there is still a shortage of robust methods for monitoring autophagy in live cells. Flow cytometry, a reliable and unbiased method for quantitative collection of data in a high-throughput manner, was recently utilized to monitor autophagic activity in live and fixed mammalian cells. In this article we summarize the advantages and potential pitfalls of the use of flow cytometry to study autophagy.

1. Introduction

Autophagy is a dynamic intracellular trafficking pathway by which cells digest their own components [1]. Although constitutively active in mammalian cells it is significantly increased in response to nutrient starvation, thereby providing fuel for cellular metabolism [2]. Autophagy participates in diverse physiological and pathophysiological functions, such as programmed cell death, cancer, pathogen infection and degradation of ubiquitinated protein aggregates formed under pathological conditions [3,4].

The autophagic process starts with the sequestration of cytoplasmic constituents, including long-lived proteins and cytoplasmic organelles (mitochondria, peroxisomes), by a membrane sac known as the phagophore or isolation membrane. The phagophore matures through the activity of autophagy-related (ATG) proteins into double-membrane vesicles termed autophagosomes. More than 34 ATG proteins have been identified, many of which are conserved from yeast to humans [5–7].

First described more than 50 years ago as a system that degrades cytoplasmic components and cell organelles via the lysosomes [8,9], autophagy was considered to be a nonspecific bulk degradation process. However, with the growing body of evidence for and recognition of specific cargos, autophagy is now divided into two types: selective and nonselective [10]. Selectivity of autophagy is achieved by distinct interactions between adaptor proteins and their cargos, which are then recruited specifically to the autophagosomes.

Despite the recent advances in our understanding of the autophagic process there is still a shortage in experimental techniques for studying it, especially in live cells and whole organisms. There has been extensive discussion of autophagy-monitoring assays [11,12] among them electron microscopy, immunofluorescence, western-blot analysis of microtubule-associated protein (MAP) 1A/1B-light chain 3 (LC3) and ATG8 turnover, and flow cytometry.

Flow cytometry is a technology that simultaneously measures and analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The measured properties include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. Given these features, flow cytometry is well adapted for quantitative analysis in a high-throughput manner of individual cells or cell populations. In this review we focus on recent methods by which flow cytometric analysis is utilized to evaluate autophagic activity in live and in fixed mammalian cells, with specific emphases on the advantages and the potential pitfalls of these techniques.

Abbreviations: ATG, autophagy-related; LC3, microtubule-associated protein (MAP) 1A/1B-light chain 3; BafA, Bafilomycin A; BDI, bright detail intensity; BDS, bright detail similarity; mCherry, monomeric cherry; EGFP, enhanced GFP; FCS, fetal calf serum; MEF, mouse embryonic fibroblasts; TDI, time delay integration; PBMC, peripheral blood mononuclear cells; PE, phosphatidylethanolamine.

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2. Markers for monitoring of autophagic flux by flow cytometry

ATG8 proteins in mammals are divided according to their sequence similarities into three subfamilies: MAP1 LC3 (or LC3), γ-aminobutyric acid receptor-associated protein (GABARAP), and the Golgi-associated ATPase enhancer of 16 kDa (GATE-16) [13]. The three subfamilies have similar structures comprising an ubiquitin core and two additional α-helices at the N-terminus [14–16], and both the core and the α-helices are essential for autophagosome biogenesis [17]. The ATG8 proteins are processed at the C-terminus by ATG4, revealing a glycine residue that can then be conjugated to phosphatidylethanolamine found on the autophagosomal membrane. Thus, this lipid-conjugated form of the mammalian ATG8 protein (LC3-II, or GATE-II, or GABARAP-II) is widely used as a marker of autophagosomes in mammals [18,19]. Following fusion of autophagosomes with lysosomes to form autolysosomes, the lipid-conjugated pool of ATG8 proteins found on the inner autophagosomal membranes is delivered into the lysosomes and is consequentially degraded (Fig. 1A). The degradation of the ATG8 proteins can be utilized to measure the autophagic flux (i.e. the level of autophagic activity). Thus, a fluorophore-tagged version of the ATG8 proteins (GFP–LC3, for example) can be used to quantitatively measure autophagic flux by flow cytometry [20–23]. In addition to GFP–LC3 (or other ATG8 isoforms) that serve as structural markers for autophagic activity, the cargo receptors p62/SQSTM1 and NBR1 are widely used to measure autophagic flux [21,24]. However, not only the autophagic flux, but also the mere translocation of GFP–LC3 and its association with the autophagosomal membrane can serve as a readout in flow cytometric analysis [25,26]. In the next section we describe in more detail how these markers have been utilized to quantitatively evaluate autophagy by flow cytometry.

2.1. Use of GFP–LC3 to monitor autophagic flux by flow cytometry

2.1.1. Rationale

In a number of reported studies, a fluorescent version of LC3 has been used to measure autophagy by flow cytometry. These techniques exploit the fact that LC3 protein, once associated with the inner autophagosomal membrane, is delivered into the lysosome and degraded there. Importantly, the GFP-tagged (but not the RFP-tagged) version is quenched prior to its degradation, owing to the sensitivity of this fluorescent protein to the acidic lysosomal environment. Accordingly, the reduction in GFP intensity in autophagy-induced samples relative to untreated samples serves as a readout for autophagic activity (Fig. 1).

2.1.2. Studies utilizing flow cytometry to measure turnover of tagged LC3

A report by Shvets et al. [23] provided the first description of the use of flow cytometry to measure autophagic activity in live cells. In that work, CHO and HeLa cells stably expressing GFP–LC3 were used to quantify the turnover of autophagosomes. Autophagy was induced by several means, including the use of rapamycin or amino-acid starvation, and flow cytometry was utilized to measure the fluorescence intensity of GFP–LC3. Induction of autophagy was followed by a marked reduction in the fluorescence intensity of GFP–LC3 but not of GFP–LC3G120A, a mutant unable to undergo lipidation and the consequent degradation. Furthermore, the reduction was blocked by autophagic or lysosomal inhibitors, confirming its selectivity for the autolysosomal pathway. Thus, the changes in GFP–LC3 intensity was shown to be a reliable and specific readout that can be measured by flow cytometry to study the autophagic activity. This technique was further used to determine the specific amino acids required for suppression of autophagic activity.

Advantages. Measurement of autophagic activity by flow cytometric analysis has many advantages, both of a general nature (relating to the technique itself) and more specifically (for autophagy). In general, this quantitative technique is simple to apply and can be used for high-content analyses with simultaneous collection of numerous parameters. Notably, as living cells are used for analysis by this method, flow cytometry may be also utilized to sort specific subpopulations for further characterization. Most importantly, this assay provides a functional readout of autophagic activity (as explained in Fig. 1A), because the reduction in GFP intensity is an outcome of the proper formation of autophagosomes, and their targeting to and fusion with the lysosomes. Therefore, conditions that affect any step in the autophagic pathway will result in changes in the GFP-signal readout. Since GFP is rapidly quenched in the acidic lysosomal lumen, this method is more sensitive than a biochemical approach as it measures the delivery and fusion of autophagosomes with lysosomes rather than proteolytic degradation inside lysosomes.

Disadvantages. A major disadvantage of this technique is that it requires the ectopic expression of fusion proteins, and this is usually an overexpression relative to endogenous levels. Although the technique can be performed with transiently transfected cells, problems arise from artifacts of nonspecific aggregation and from the heterogeneity of protein expression. To improve sensitivity and reproducibility, it is therefore advisable to develop stable isogenic cell lines. However, even when protein is stably expressed, ectopic expression may result in different kinetics of GFP reduction.

As this assay is restricted mostly to living cells, samples cannot be stored or combined with immunolabeling of intracellular markers. Importantly, since it relies on reduction in GFP intensity, care must be taken to exclude the possibility of nonspecific leakage or quenching of GFP, or artificial effects on general protein synthesis. Similarly, any condition affecting lysosomal function or acidification will result in blockage of GFP reduction, which might be misinterpreted as blockage of autophagosome formation.

Another disadvantage is that this assay detects the total level of LC3 protein and does not differentiate between LC3-I and LC3-II. Finally, most methods measuring autophagy by flow cytometry suffer from the limitation that they focus mainly on one ATG8 protein, namely LC3B. The mammalian family of ATG8 proteins, however, contains three homologues, each having several isoforms that should also be tested, as they might be responsible for different subtypes of autophagy [27].

Sheen et al. [22] also utilized flow cytometry to monitor autophagic activity after amino-acid deprivation. In their study, however, a double-tagged version of LC3, namely DsRed–LC3–GFP, was used to monitor autophagy. Since the GFP tag was located C-terminally to the ATG4 recognition site, it was cleaved upon activation of autophagy, and this loss in GFP fluorescence was monitored by flow cytometry. The authors introduced an autophagic index to normalize the GFP fluorescence level to that of the DsRed–LC3, which was relatively stable. This index accounted for possible changes in the synthesis level of the reporter after different treatments. As a control they used mutated DsRed–LC3ΔGFP, in which the ATG4-recognition sequence was deleted. This autophagic index was further utilized in that study to examine the effect of leucine deprivation in human melanoma cells with hyper-activated RAS-MEK pathway (Mel-ST cells).

Advantages. The advantage of this method is that the location of the GFP tag downstream of the ATG4 recognition site enables selective measurement of ATG4 activity.

Disadvantages. Tagging of the C-terminus of LC3 has certain disadvantages for the evaluation of autophagic activity (i.e. auto-