**Introduction**

Autophagy is an intracellular catabolic pathway which causes cellular protein and organelle turnover, and is associated with diverse diseases such as Alzheimer's disease, cancer, and Crohn's disease, in addition to aging. It is a tightly regulated process that plays a normal part in cell growth, development, and cellular homeostasis. Autophagy functions as a housekeeping mechanism through disposal of aging and dysfunctional proteins and organelles by sequestering and priming them for lysosomal degradation (Figure 1). Increasing evidence suggests that not only apoptosis, but also autophagy, can contribute to cell death and greatly influence general cell health. Malfunctions of autophagy can adversely impact longevity and the capability of cells to function at full capacity. In cancer cells, autophagy can compensate for hypoxic conditions and nutrient starvation; on the other hand, activation of cell death via autophagy can kill tumor cells. As a result, there is great interest in assays that can efficiently screen for activators and inhibitors of autophagy.
Types of cellular stress, such as nutrient limitation, hypoxia, oxidative stress, and DNA damage (genotoxic stress), can induce autophagy, often via inhibition of mTOR. Autophagy induction signaling prepares cells to construct a double membrane vesicle known as the autophagosome by catalyzing the scaffolding of Atg proteins (such as LC3) to the pre-autophagosome membrane, which engulfs aging organelles and recyclable proteins. In the final step of autophagy, the outer membrane of autophagosome fuses with the lysosome that provides the hydrolytic enzyme machinery and the contents are degraded and recycled.

Here we describe several new optimized assay kits specifically designed for autophagy evaluation by flow cytometry and/or imaging. Unlike current flow cytometry assays for autophagy, these assays can discriminate between cytosolic and autophagosome-associated LC3, thereby enabling robust, accurate evaluation of autophagy.

Two reagents make this discrimination possible as shown in this study. First, using a selective permeabilization solution to extract cytosolic LC3 and then flushing the extracted LC3 away during washing steps enables specific quantification of LC3 translocation using flow cytometry (Figure 2). Moreover, since autophagy is a constitutive cellular degradation process, we show that a second proprietary reagent to prevent the lysosomal degradation of LC3 allows for accurate quantification of LC3 fluorescence following its accumulation in the autophagosome. Using the selective permeabilization solution along with the lysosomal degradation inhibitor protects autophagosomal LC3 from both extraction and degradation, which allows its fluorescence to be measured by flow cytometry or imaging.

Three of the kits use a model reporter cell line format to evaluate the impact of compounds on LC3 translocation, while a fourth kit provides a proprietary, human anti-LC3-FITC antibody conjugate for specific evaluation of autophagy in primary cells and cell lines that express detectable LC3. The reporter cell lines express fusions with monomeric GFP or RFP to minimize dimer formation and aggregation and to facilitate translocation and flow cytometry analysis. The GFP or RFP used in the fusion proteins is attached on the 5’ end (N-terminal fusion), protecting the fluorescent tags from Atg4 cleavage, allowing their visualization within autophagosomes (Figure 3). The ability to track fluorescently labeled autophagosomes thus provides a tool for measuring autophagy in individual cells.

**Figure 2.**
Selective permeabilization aids in discriminating cytosolic from autophagic LC3. Discrimination between cytosolic GFP-LC3-I from autophagosome-associated GFP-LC3-II is achieved by disrupting the plasma membrane with a selective permeabilization solution. Selective permeabilization releases cytosolic LC3, which is then flushed away during washing steps. GFP-LC3-II trapped in the autophagosome remains intact and its fluorescence can be measured.
Our system utilizes cell lines stably expressing GFP- or RFP-LC3, transfected either into a Chinese hamster ovary (CHO) or human osteosarcoma (U20S) cell background. A stable GFP- or RFP-LC3 reporter system is ideal for screening of autophagy-modulating compounds. LC3 tagged at its N-terminus with GFP or RFP allows tracking the translocation of LC3 from the cytosol and into the autophagosome more clearly and definitively than monitoring endogenous protein by antibody-based methods. Especially when endogenous LC3 levels are below the limits of immunodetection, using exogenous LC3 constructs shows a clear benefit. Another advantage of using a stable GFP- or RFP-LC3 reporter system is the ability to analyze a larger number of cells, because nearly 100% of the population expresses tagged LC3. Analyzing more cells can yield greater statistical significance to experimental observations, for more productive research and better decision-making.

Having the ability to measure compound activity is crucial to screening and rank-ordering compounds in drug discovery campaigns. Here, we have demonstrated the utility of our above-described flow cytometry assay for measuring the autophagy-modulating activity of compounds. First, we show identification of an autophagy inducer (rapamycin) or an autophagy inhibitor (dynasore). Second, we describe the detailed compound analysis and rank-ordering of autophagy inducers (STF-62247 and PI-103) based on the mean fluorescence intensities generated by titration of these known autophagy inducing compounds. We show quantitative activity measurement for STF-62247 and PI-103 via dose response curves to derive EC_{50} values.

**Methods**

**Monitoring autophagosomes by flow cytometry**

To measure autophagy by LC3-II recruitment into the autophagosomes, we used a variety of adherent and suspension cell lines as well as cell lines stably expressing GFP-tagged LC3, transfected either into a CHO or a U20S cell background. Cells were harvested and placed into a 96-well assay plate, either in nutrient deprived/starved conditions or left in normal fed conditions as a control. Cells were then treated with a lysosomal degradation inhibitor for 2 hours. Cells were then washed with a selective permeabilization buffer at room temperature to extract all cytosolic LC3-I, followed by one wash with assay buffer to remove any residual permeabilization buffer from the cells. For the cell lines expressing only endogenous LC3, all cell samples were resuspended in 95 μL 1X Assay Buffer plus 5 μL of 20X optimized anti-LC3/FITC antibody for 30 minutes at room temperature in the dark, then washed once with assay buffer. Data were acquired using a guava easyCyte™ 8HT flow cytometer to measure the fluorescence signal from autophagosome-bound GFP-LC3-II.

**RFP-LC3 pH-stable reporter cell line for detecting autophagy within acidic cellular microenvironments**

As the pH environment of cell can vary, it can also be useful to evaluate LC3 by LC3-II recruitment into the autophagosomes, using a reporter cell line with a monomeric RFP fusion protein attached to LC3 at the 5’ end (N-terminal fusion). RFP is typically more pH-stable than GFP. This version of the LC3 is protected by RFP from Atg4 cleavage, which allows its visualization within the acidic compartment of the autophagosome. A monomeric RFP is preferred as a reporter to facilitate the translocation of the fusion protein, as other forms of RFP will form dimers and aggregate when over-expressed in the cells, which prevents their extraction from the cytoplasm and impairs the ability to measure translocation by flow cytometry.

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**Figure 3.**

N-terminally fused (but not C-terminally fused) GFP-LC3 is a valid marker for autophagy. According to Klionsky (2011), the location of GFP fusion to LC3 is critical for measurement of LC3 translocation to serve as a marker for autophagosomes. If GFP is fused to the C-Terminal (or 3' end), following Atg4 cleavage GFP is removed and subsequently GFP is now lost. But GFP fusion to the N-terminus (or 5’ end) will retain GFP, making this construct a suitable marker to track autophagic activity.
Identification of an autophagy inducer and inhibitor using the FlowCellect™ GFP-LC3 Reporter Autophagy Assay

In order to demonstrate that a GFP reporter-based system is a viable tool for compound hit identification by flow cytometry, GFP-LC3-expressing CHO cells were pretreated with either rapamycin (to induce autophagy) or dynasore (to inhibit autophagy) for 48 hours. Also, RFP-LC3-expressing U2OS cells were pretreated with 80 μM dynasore for 3 hours. A lysosomal degradation inhibitor was also added to the cells simultaneously (if treatment with dynasore) or 45 minutes after rapamycin addition (for a total of 48 hours incubation time) to prevent the autophagosome degradation by the lysosome.

After treatment, cells were then washed with a selective permeabilization buffer at room temperature to extract all cytosolic LC3-I, followed by one wash with assay buffer to remove any residual permeabilization buffer from the cells. Data were acquired using a guava easyCyte™ flow cytometer to measure the fluorescence signal from autophagosome-bound GFP-LC3-II.

Small molecule structure-activity relationship (SAR) evaluation using the FlowCellect™ GFP-LC3 Reporter Autophagy Assay

The utility of the GFP-LC3 reporter cell line as a screening tool was further illustrated by measuring the dose-dependent activity of specific small molecule autophagy inducers, STF-62247 and PI-103. STF-62247 and PI-103 were titrated in a 12-point, half-log serial dilution and incubated for 8 hours at 37°C. A lysosomal degradation inhibitor was also added to the cells approximately 7 hours into the incubation period for 45 minutes to prevent autophagosome degradation by the lysosome. Following treatment, cells were then washed with a selective permeabilization buffer at room temperature to extract all cytosolic LC3-I, followed by one wash with assay buffer to remove any residual permeabilization buffer from the cells. Data were acquired using a guava easyCyte™ flow cytometer to measure the fluorescence signal from autophagosome-bound GFP-LC3-II. The mean fluorescence values, or MFI, were then determined and plotted using a curve-fitting algorithm built into the InCyte™ software module to construct EC_{50} dose response curves.
**Results**

By implementing a selective permeabilization step plus the addition of a lysosomal degradation inhibitor, we were able to measure autophagy via LC3 translocation and subsequent lipidation into autophagosomes by flow cytometry. Combining selective permeabilization with the lysosomal degradation inhibitor was important for developing a suitable platform for screening of molecules for induction or inhibition of autophagy. Figure 4A shows that, without selective permeabilization, the fluorescence signal of the cell population does not change upon autophagy induction, even when a lysosomal degradation inhibitor was added. In contrast, Figure 4B shows that, with selective permeabilization, nutrient deprivation results in an increased localization of LC3 to autophagosome membranes, resulting in a rightward shift in the peak. Nutrient deprivation, together with lysosome inhibition, resulted in an even more dramatic change in the LC3-II signal. Next, we used an RFP-LC3 reporter cell line to measure autophagy within acidic cellular environments (Figure 5). LC3 translocation to the autophagosomes was detected only upon starvation and addition of a lysosome inhibitor.

*Figure 5.*

RFP-LC3 pH-stable reporter cell line for detecting autophagy. RFP-LC3 level remains high in autophagosomes when starved in the presence of lysosome inhibitor (yellow). All the cytosolic RFP-LC3 is washed away if no autophagy is induced by starvation (gray).
Flow cytometry detection of endogenous LC3 translocation to the autophagosome using anti-LC3-FITC conjugate

In this experiment, flow cytometry was used to detect LC3 translocation under starved and non-starved conditions for a variety of adherent and suspension human cell lines. After selective permeabilization and treatment with lysosomal degradation inhibitor, these cell lines were treated with anti-LC3-FITC antibody and analyzed using flow cytometry (Figure 6). Without selective permeabilization (Figure 6A), no shift of LC3 level is detected using flow cytometry before and after starvation to induce autophagy. The position of the histograms indicates low endogenous LC3 expression and constitutive degradation. With selective permeabilization (Figure 6B), the shift of LC3 to the right indicates an increased level of LC3, and is observed during autophagy only in the presence of the lysosome inhibitor (green), which delays degradation of LC3 in the autophagosomes and prolongs the signal.

Small Molecule Analysis #1

Demonstrating the utility of the FlowCellect™ GFP-LC3 Reporter Autophagy Assay for drug screening and structure-activity relationship studies

We evaluated the effects of the autophagy inducer, rapamycin, and the autophagy inhibitor, dynasore, on GFP-LC3 translocation using the FlowCellect™ GFPLC3 Reporter Autophagy Assay. Rapamycin is an mTOR inhibitor and induced autophagy in our assay (Figure 7). This observation was consistent with the known role of mTOR in regulating autophagy. mTOR is a member of the PI3-kinase family and is a central modulator of cell growth in response to environmental signals. It plays a critical role in transducing proliferative signals by activating downstream protein kinases that are required for both ribosomal biosynthesis and translations. 2000 Nobel Laureate Paul Greengard demonstrated that a small molecule enhancer of rapamycin, SMER28, decreased levels of amyloid-β(Aβ) peptide, a hallmark of Alzheimer’s disease. Autophagy is one major cellular pathway leading to the removal of such proteins, further suggesting that modulating autophagy can have therapeutic value for Alzheimer’s disease. By targeting mTOR, rapamycin mimics the cellular starvation response by inhibiting signals required for cell cycle progression, cell growth, and proliferation and leads to the activation of autophagy.

Dynasore is a cell-permeable inhibitor of dynamin. Dynamin is essential for clathrin-dependent coated vesicle formation. Dynasore acts as a potent inhibitor of endocytic pathways known to depend on dynamin by rapidly blocking coated vesicle formation within seconds of dynasore addition. As a result, dynasore can prevent autophagosome formation, in turn inhibiting autophagy. Consistent with this model, dynasore inhibited LC3 translocation to autophagosomes in both GFP-LC3 and RFP-LC3-based assays (Figure 8).
To further investigate the effect of small molecule activity on autophagy, we titrated two well-known autophagy inducers, STF-62247 and PI-103 on our GFP-LC3 reporter cell line, demonstrating that the FlowCellect™ GFP-LC3 Reporter Autophagy Assay was a viable tool for advancing drug candidates. To achieve a quantitative structure-activity relationship study, we quantified the level of autophagosome-associated LC3-II by flow cytometry by calculating the mean fluorescence intensity of the compound-treated cells relative to the baseline negative control. From these values, using the InCyte™ software module, we were able to derive EC_{50} values showing that PI-103 was a more potent inducer of autophagy than STF-62247 (Figure 9).

**Discussion**

Data from EMD Millipore’s optimized FlowCellect™ kits for autophagy provide quantitative assessments of autophagy by flow cytometry and/or imaging. By measuring the fluorescence signal of translocated LC3 by a stably expressed GFP- or RFP-LC3 fusion protein, we could detect and measure the rate of cellular autophagy using flow cytometry. This was achieved by using a proprietary selective permeabilization buffer to remove the cytosolic LC3 from cells. Selective permeabilization enabled the discrimination between cytosolic and lipidated LC3, which was sequestered into the autophagosomes. By the addition of a lysosomal degradation inhibitor, we achieved prolonged fluorescence signals, yielding more accurate and reliable data. Because RFP is a more pH-stable fluorescent tag, the RFP-LC3-expressing cell line proved useful in providing robust signals in acidic environments.

We also demonstrated that by using the FlowCellect™ GFP-LC3 Reporter Autophagy Assay, we could successfully characterize compounds that induced autophagy (rapamycin) or inhibited it (dynasore). This novel flow cytometry assay is therefore ideal for screening small molecules for autophagy modulating effects and amenable to high-throughput environments. Moreover, we were also able to successfully implement this method for making SAR determinations by generating EC_{50} curves and rank-ordering compounds. This was demonstrated by the dose response curves for selective autophagy inducers, STF-62247 and PI-103. By implementing this assay, autophagy-modulating compounds can be rank-ordered to help complement any SAR campaign during drug development, improving and accelerating decision-making during the process of advancing lead compounds further in the development process.

Ultimately, having the ability to accurately measure autophagosome activity via LC3 translocation (which is a hallmark of the autophagic process), as well as perform deep-dive analysis of small molecule activity by deriving dose response curves, can greatly enhance studies of cancer, neurodegeneration, and other diseases affected by autophagy.
Key Aspects of FlowCellect™
Autophagy Kits for Flow Cytometry

EMD Millipore’s FlowCellect™ GFP-LC3 Reporter Autophagy Assay Kits provide a quantitative solution for studying autophagy and measuring the potency of autophagy inducers using flow cytometry. These kits have four unique features to aid in the detailed evaluation of autophagy by flow cytometry:

- **Selective permeabilization solution** discriminates between cytosolic LC3 from autophagic LC3 by extracting the soluble cytosolic proteins, while protecting LC3 which has been sequestered into the autophagosome.
- **Monomeric GFP or RFP** is used as a reporter to facilitate the translocation of the fusion protein. Other forms of GFP or RFP form dimers and aggregate when overexpressed in the cells, making it difficult to extract from the cytoplasm and impossible to measure translocation by flow cytometry.
- **Included autophagy detection reagent** prevents lysosomal degradation of LC3, allowing its quantification by flow cytometry and prolonging the signaling event for robust measurement.
- The monomeric GFP or RFP used in our LC3 fusion protein is attached on the N’ end (N-terminal fusion), protecting the GFP or RFP from Atg 4 cleavage, allowing its visualization within the autophagosomes.

Figure 9.
PI-103 is a more potent inducer of autophagy than STF-62247. Comprehensive analysis of STF-62247 (A) and PI-103 (B) activity via dose response curves and EC₅₀ determination by flow cytometry using the InCyte™ Software Module illustrated wide dynamic range of the reporter cell line and validated the effective use of the assay for rank-ordering compounds based on activity.

Compound Activity: PI-103 > STF-62247

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**96-well Plate Heat Map**
(12 pt, dose response assay)

**A. STF-62247 Dose Response Curve**

**B. PI-103 Dose Response Assay**

**Dose Response Curve**

**EC₅₀: 2.7 µM**  
**EC₅₀: 1.9 µM**

**EC₅₀: 2.7 µM**  
**EC₅₀: 1.9 µM**
References


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