Trends in Biochemical Sciences

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REVIEWS

Review

Autophagy Assays for Biological Discovery and Therapeutic Development

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Autophagy is a lysosome-dependent intracellular degradation system required for various physiological processes and can be dysregulated in human disease. To understand its biological significance and underlying mechanisms, measuring autophagic activity (i.e., autophagic flux) is critical. However, navigating which assays to use, and when, is complicated and at times the results are often interpreted inappropriately. This review will summarize both advantages and disadvantages of currently available methods to monitor autophagy. In addition, we discuss how these assays should be used in high-throughput screens to identify autophagy-modulating drugs and genes and the general features needed for biomarkers to assess autophagy in humans.

Highlights

Autophagy is a fundamental process required for normal physiology and disruptions can cause disease; but measuring autophagy can be challenging.

By using specific flux assays and mechanistic readouts, it is possible to reliably interpret the status of autophagy in experimental systems.

High-throughput small molecule and genetic screens using autophagy assays have accelerated the discovery of basic mechanisms as well as potential drug targets.

Identifying autophagy biomarkers for use in clinical development represents a major opportunity for the future.

Importance of Accurate Autophagy Measurements

Autophagy is a process that facilitates lysosomal degradation of intracellular components [1,2]. There are several types of autophagy: **macroautophagy** (see Glossary) [1,2], microautophagy [3], and chaperone-mediated autophagy (CMA) (and a related process called RNautophagy/DNautophagy) [4,5] (Figure 1). Autophagy is important not only for constitutive turnover of intracellular components, but also for the active elimination of abnormal or potentially damaging materials and to access the by-products of degradation, such as amino acids, during starvation [1,2]. Due to its important role in homeostasis, defects in autophagy are linked to several human diseases [2]. Therefore, autophagy has been extensively studied across the biomedical field and the demand for accurate methods to measure autophagic activity has been increasing. However, measuring autophagy is still not easy or simple, especially in mammals [6–9]. This review summarizes various methods for measuring the activity of macroautophagy (used interchangeably with autophagy hereafter) in mammalian systems and the use of these assays to discover chemical probes and genes that modulate autophagy. Finally, for applied research and clinical development, it is also timely to discuss the need for autophagy biomarkers.

Overview of Autophagy Measurement

Autophagic flux is typically measured biochemically and is the amount of degradation of cytoplasm-derived material in lysosomes observed per unit time. In order to measure flux, it is necessary to directly quantify the amount of autophagy-dependent degradation of cellular components, or to use reporters capable of representing the cumulative amount of degradation. Historically, **radiometric long-lived protein degradation assays** were adapted to determine the rate of autophagy-dependent proteolysis. Although no longer in vogue, this remains one of the gold-standard approaches to definitively measure endogenous proteolysis, including autophagy. Electron microscopy has also been used to observe and characterize autophagic structures under different physio-pathological settings. However, as is often misunderstood, the number of autophagosomes does not necessarily indicate flux because autophagosome number could increase either by induction of autophagy or by the reduced consumption of autophagosomes by lysosomes. The latter can occur due to lysosomal dysfunction.

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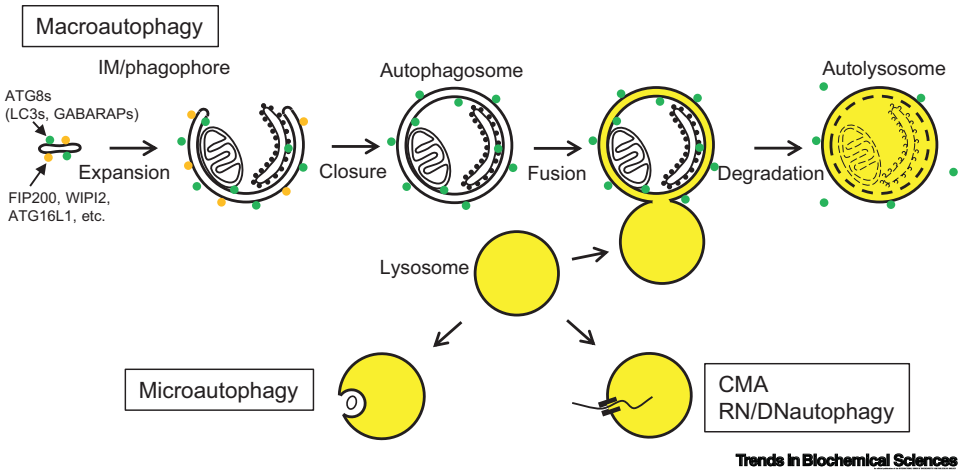


Figure 1. Scheme of Three Types of Autophagy. Macroautophagy represents an autophagosome-mediated type. A thin membrane cisterna termed the isolation membrane (IM) or phagophore encloses part of the cytoplasm and becomes the autophagosome. Upon fusion with lysosomes or the vacuole, cytoplasmic-derived material, including organelles, are degraded. The localization of ATG8 proteins (green dots) and IM/phagophore markers such as FIP200, WIPI2, ATG16L1 (orange dots) are indicated. By microautophagy, a portion of the cytoplasm is directly engulfed by the lysosomal or vacuolar membrane and degraded. Cytosolic proteins and RNA/DNA can also be directly delivered to the lysosomal lumen by the mechanism termed chaperone-mediated autophagy (CMA) and RNautophagy/DNautophagy, respectively.

During the early stages of autophagy, a membrane cisterna, termed the isolation membrane or phagophore, encloses a portion of the cytoplasm and eventually expands to become an autophagosome, which then fuses with lysosomes to degrade sequestered materials (Figure 1). A general strategy for measuring autophagic activity is shown in Figure 2. If experimental data suggest an accumulation of autophagosomes (Figure 2, Step 1), it is then essential to measure

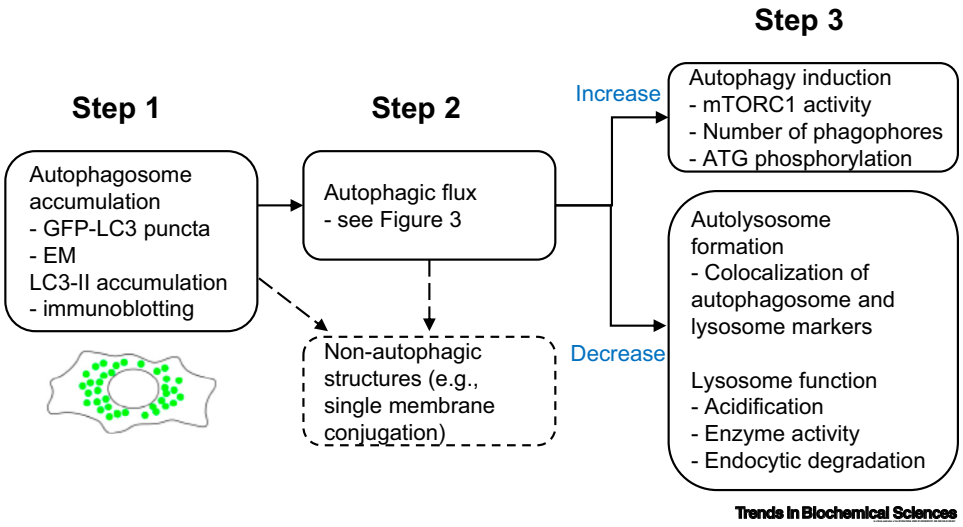


Figure 2. Flow Chart of the Procedures for Measuring Autophagic Activity. If experimental data (e.g., by GFP-LC3 puncta observation) suggest an accumulation of autophagosomes (Step 1), autophagic flux should be determined using methods shown in Figure 3 (Step 2). If autophagic flux increases, autophagy-inducing signals should be checked (Step 3, top). If autophagic flux decreases, downstream steps such as autophagosome–lysosome fusion and lysosomal function need to be checked (Step 3, bottom). In some cases, LC3 puncta represent non-autophagic structures such as single membrane endolysosomes where LC3 is conjugated, which should be carefully distinguished from true autophagy modulation.

Glossary

Autophagic flux: the amount of autophagic degradation per unit time rather than the number of autophagosomes.
ER-phagy: a selective type of autophagy against ER subdomains.
Fluorescent Timer DsRed: a fluorescent protein that changes its fluorescence from green to red as it matures.
HaloTag: a versatile self-labeling protein tag. It can be conjugated to variable ligands, including fluorescent molecules, allowing pulse-chase experiments.
Macroautophagy: primary cellular process in which content is degraded by lysosomes and recycled. It occurs when a phagophore engulfs the content and forms a double membrane around it.
Mitophagy: a selective type of autophagy against mitochondria.
Positron emission tomography (PET): an imaging method that generates 3D images of an injected radioisotope-labeled small molecule in the body.
Radiometric long-lived protein degradation assays: a method to monitor isotope-labeled free amino acids that are derived from cellular proteins.
ULK1 complex: the most upstream protein kinase complex in the autophagy pathway. It is suppressed by mTORC1 during nutrient-rich conditions and activated during starvation.

autophagic flux to determine the rate of induction or if there is reduced consumption (Figure 2, Step 2). After measuring autophagic flux, it is highly recommended to probe specific autophagy-inducing signals to confirm the flux data (Figure 2, Step 3). Conversely, when a decrease in autophagic flux is suggested, the efficiency of autophagosome–lysosome fusion or lysosomal function should be checked, in addition to the autophagy-inducing signals.

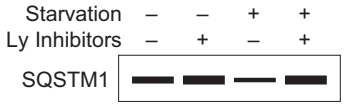
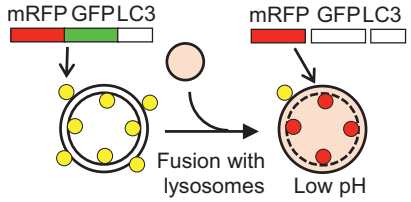
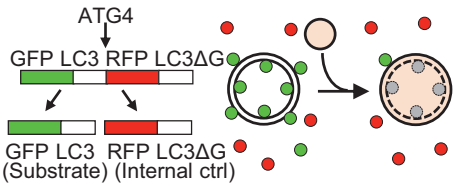
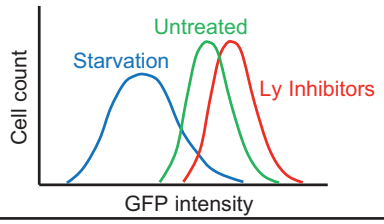
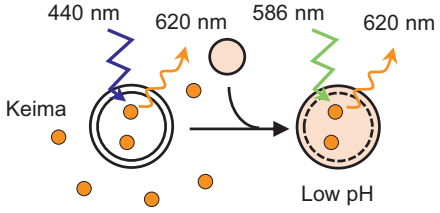
Measurement of Autophagic Flux

The most commonly used method to measure autophagic flux is to monitor the turnover rate of the autophagosomal protein ATG8 and its homologs such as LC3 and GABARAP family proteins (collectively referred to as ATG8s) that bind to the autophagosomal membrane (Figure 1) [10]. Cytosolic ATG8 (called ATG8-I) is covalently conjugated to phosphatidylethanolamine in the phagophore and autophagosomal membranes through its C terminal glycine residue and becomes a lipidated form termed ATG8-II. ATG8-II binds to both the outer and inner autophagosomal membranes, and the amount of ATG8-II roughly correlates with the number of autophagosomes. After fusion with lysosomes, ATG8-II on the outer membrane is gradually deconjugated and recycled, whereas ATG8-II on the inner membrane is degraded (Figure 1). Therefore, the activity of autophagy can be estimated by monitoring the amount of ATG8 degradation. It should be noted that ATG8 may not be strictly specific to macroautophagy; ATG8 and some of the autophagy adaptors such as sequestosome 1 (SQSTM1, also known as p62) and NDP52 can be degraded by endosomal microautophagy [11] and ATG8 can also be conjugated to single membranes (see later) [12] (Figure 2). Among ATG8s, LC3B was identified first and has been widely used (often referred to simply as LC3). Thus, we will generally use 'LC3' unless we have information on other homologs.

ATG8-Turnover Assay

When autophagy is induced, for example, by starvation, the number of autophagosomes and the amount of ATG8-II generally also increases (Figure 3A). However, to monitor autophagic flux, we should measure how much ATG8-II is actually degraded in lysosomes. This can be determined by comparing the amount of ATG8-II in cells treated with and without lysosomal inhibitors (Figure 3A). When autophagic flux is enhanced, the difference between these treatment groups will be high, but when flux or consumption is inhibited, the difference will be negligible [6]. For example, during nutrient starvation in most cell types, lysosome inhibition results in a significant increase in the ATG8-II levels, indicating high autophagic flux. By contrast, if lysosome inhibition does not change the ATG8-II level, it means that autophagic activity is low, even if the amount of ATG8-II is basally high.

This method is highly versatile because it can be performed by immunoblot analysis against endogenous ATG8 proteins (e.g., LC3). However, there are some caveats. First, as is generally the case with immunoblotting, it is semiquantitative and the dynamic range of detection is narrow. Second, it is necessary to prepare and compare two samples with and without lysosome inhibitors. More importantly, lysosomal inhibition could suppress mTOR activity, which secondarily induces autophagy [13,14]. Third, care must be taken in selecting the type and concentration of lysosomal inhibitors. Commonly used lysosomal inhibitors are V-ATPase inhibitors bafilomycin A_1, lysosomotropic reagents such as chloroquine, and lysosomal enzyme inhibitors such as pepstatin and E64d. However, lysosomotropic reagents cause osmotic stress on lysosomes and promote ATG8-II formation on the single membrane of endolysosomes by a mechanism similar to LC3-associated phagocytosis, which is distinct from canonical autophagy [12] (Figure 2). For example, 100 μM chloroquine increases LC3-II in an autophagy-independent manner [12,15], significantly affecting autophagic flux measurement, and should be used at low concentrations (e.g., 25 μM) [15].

Methods	Mechanism	Pros	Cons
(A) ATG8 turnover 	Lysosomal turnover of autophagosomal ATG8 proteins (e.g., LC3)	• No transfection • Measures endogenous flux	• Narrow dynamic range • Semiquantitative • Lysosomal inhibitor required
(B) Substrate turnover 	Degradation of autophagy substrates	• No transfection • Measures endogenous flux	• Semiquantitative • Affected by transcription • Lysosomal inhibitor required
(C) RFP-GFP-LC3 (tfLC3) reporter 	Quenching of the GFP but not RFP fluorescence in lysosomes	• Can monitor individual structures • Not reliant on lysosomal inhibitors • Can be used for selective substrates	• Puncta counting required • High background (due to RFP accumulation in lysosomes) • Transfection required
(D) GFP-LC3-RFP(-LC3ΔG) reporter 	Quenching of the GFP in lysosomes normalized with cytosolic RFP control	• Easy quantification • Internal control for reporter expression • Applicable for various types of equipment (e.g., flow cytometer, microplate reader) • Wide dynamic range • Not reliant on lysosomal inhibitors	• Low sensitivity • Low time resolution • Transfection required
(E) GFP-LC3 flow cytometry 	Quenching of the GFP in lysosomes	• Easy quantification • Wide dynamic range	• Low sensitivity • Low time resolution • Transfection required • No internal control
(F) Keima 	pH-dependent change in Stokes shift	• ATG8-independent (not specific to macroautophagy) • Can be used for selective substrates • Not reliant on lysosomal inhibitors	• Transfection required • Uncommon fluorescence filter sets • Live cell only

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(See figure legend at the bottom of the next page.)

Degradation of ATG8-Binding Substrates

Autophagic flux can also be evaluated by monitoring the levels of selective autophagy substrates (Figure 3B). Such substrates are generally recognized and physically bound by autophagosomal ATG8 proteins and, similar to the ATG8 flux assay, substrate degradation can be measured using lysosomal inhibitors. Among known substrates, SQSTM1/p62 is frequently used. It should be emphasized that the expression of this protein is also highly regulated at the transcriptional level. For example, accumulation of SQSTM1 may indicate inhibition of autophagy, but in some cases such as oxidative stress, it may simply represent overproduction of SQSTM1 protein [16]. Therefore, when the amount of an ATG8-binding substrate is used as an index of autophagic activity, its mRNA levels should be measured to confirm that the change in protein is not due to transcriptional induction. Alternatively, this problem can be avoided by using pulse-labeling with p62 tagged with **HaloTag** [17].

RFP-GFP-LC3 (tfLC3) and RFP-GFP-p62 Reporters

As mentioned earlier, simple detection of autophagic structures using fluorescent protein-based reporters such as GFP-LC3 is not sufficient to determine autophagic flux. To overcome this issue, tandem fluorescent protein-tagged LC3 (tfLC3), which has both RFP (or another related red fluorescent protein such as mCherry) and GFP at the N terminus of LC3 (or any other ATG8 family protein) was developed [18,19] (Figure 3C). This reporter emits both red and green fluorescence such that autophagosomes will appear yellow when images are merged. However, within the acidic environment of the autolysosome, the GFP fluorescence is immediately quenched, leaving only the red fluorescent signal, which is unaffected by the low pH. Following induction of autophagy, there is an increase in yellow (indicating more autophagosomes) and red puncta (indicating more autolysosomes). However, when autophagy flux is reduced due to lysosomal inhibition, yellow ATG8 puncta dominate, which indicates a reduction in autophagosome–lysosome fusion and/or degradation within the autolysosome. When autophagy induction is suppressed, both yellow and red structures are reduced. Instead of LC3, selective substrates such as SQSTM1/p62 can also be used [18] and this complements the tfLC3 system because SQSTM1 is not (or only weakly) recruited to single membranes [12,20]. Flux determination using the tandem fluorescent reporters does not require lysosome inhibition and the tandem fluorescent (tf) module can be adopted to determine whether individual autophagic structures are degraded after fusion with lysosomes. Furthermore, this method can also be used to monitor the total cellular autophagic flux [21–23]. However, the combination of GFP and RFP/mCherry in tandem reporters may not be optimal because, as fluorescence resonance energy transfer (FRET) occurs from GFP to RFP/mCherry, the RFP/mCherry (a FRET acceptor) signal becomes weaker after degradation of GFP (a FRET donor). This issue was recently solved by conjugating the lysosome-resistant FRET donor TOLLES with the lysosome-sensitive FRET acceptor YPet to produce a novel tandem construct named signal-retaining autophagy indicator (SRAI) (Box 1) [24].

GFP-LC3-RFP(-LC3ΔG) Reporter

The GFP-LC3-RFP-LC3ΔG reporter is a second-generation tf assay that provides an alternative approach to measure the flux. The GFP-LC3-RFP-LC3ΔG fusion protein expressed in cells is cotranslationally processed by the ATG4 endopeptidase to generate GFP-LC3, for measuring autophagy activity, and 'free' RFP-LC3ΔG, which lacks the C terminal glycine needed for conjugation to membrane and serves as a control for reporter expression and cellular health (Figure 3D)

Figure 3. Summary of Representative Autophagic Flux Assays. Representative methods to measure autophagic flux are listed with their underlying mechanisms, advantages, and disadvantages. See text for details of each method. Modified versions are also available: mTagRFP-mWasabi-LC3 [117] and pHluorin-mKate2-LC3 [118], mCherry-GFP-p62 [18] for method (C), and pHluorin-LC3-mCherry [119] for method (D). Abbreviations: Ly, lysosomal.

Box 1. Methods for Monitoring Flux of Selective Autophagy

Specific proteins, organelles, and intracellular pathogens can be selectively recognized and degraded by autophagy [100], but their steady-state levels within the cell is a factor of synthesis and degradation. Some representative flux reporters for selective autophagy of mitochondria (mitophagy) and the endoplasmic reticulum (**ER-phagy**) are listed later, but these methods are applicable to all substrates.

Mitophagy

The delivery of mitochondria to lysosomes can be monitored by using mt-Keima (Keima with a mitochondria-targeting sequence) [27,101–104]. However, Keima can only be used in live cells with lysosomal acidification intact. To overcome this problem, the mito-QC reporter was used [48,105]. This is a mCherry-GFP tandem reporter that is fused to the mitochondria-targeting sequence from FIS1. Like the tfLC3 reporter, the GFP signal of mito-QC is quenched when mitochondria are delivered to lysosomes by autophagy. This reporter has the advantage that it can be used in fixed cell and tissue samples. Another mitophagy reporter that can be used in fixed samples is mito-SRAI [24]. This is also a tandem fluorescent protein reporter but gives better fluorescent signals in lysosomes. To assess total mitochondrial mass, the amount of mitochondrial DNA or proteins in the inner membrane or matrix should be measured. It is not recommended to use outer membrane proteins as an indicator of the mitochondrial mass because these proteins can be selectively degraded by the proteasome in a ubiquitin- and p97-dependent manner [106–108].

ER-phagy

Given that the total mass of the ER is large and its consumption by autophagy is relatively small, it is difficult to monitor ER-phagy by detecting changes in the levels of endogenous ER proteins. To this aim, it is important to use specific ER-phagy flux reporters such as the ER luminal reporter (ssGFP-RFP-KDEL [109]) and ER membrane reporters (mCherry-GFP-RAMP4 [110,111] and mCherry-GFP-REEP5 [112]). ER-phagy adaptors can also be used as reporters by tagging them with tandem fluorescent proteins [113] or Keima [29], but as these adaptors bind to ATG8, they can be degraded more efficiently than the ER itself. A HaloTag-Sec62 has been used to characterize the normalization of ER volume following ER stress [114]. Lysosomal delivery of ER-phagy reporters can be detected by either fluorescence microscopy or immunoblotting (i.e., measuring the proteolytic 'cleavage' of the reporter). ER-phagy can also be evaluated by immunoblotting to evaluate cleavage of mCherry-RAMP4 [110] in mammalian cells and Sec63-mCherry (general ER marker), Hmg1-GFP (perinuclear ER), and Rtn1-GFP (cortical ER) in yeast cells [115]. It should be noted that the ER is not a homogeneous structure; autophagic degradation of specific regions can be differentiated by using region-specific reporters derived from the different ER phagy adaptors [116].

[22]. Thus, GFP-LC3 and RFP-LC3ΔG act as an autophagy substrate and internal control, respectively. Moreover, the ratio of GFP-LC3 relative to RFP-LC3ΔG inversely correlates with cumulative autophagic degradation activity. Because this method does not require microscopy-based imaging of ATG8 puncta, it can be readily used with a flow cytometer or a fluorescence microplate reader. The internal control portion of this reporter does not necessarily have to be RFP-LC3ΔG as RFP alone (i.e., GFP-LC3-RFP) can behave similarly [22].

While direct measurement of the reporter expression using the internal control is ideal, a simple method to determine flux and consumption of autophagosomes is to measure the amount of reduction in only GFP-LC3 by flow cytometry (Figure 3E) [25,26]. In this case, additional control experiments are needed to ensure that changes in cell health are not responsible for the reduction in reporter signal.

Keima

Keima is a unique fluorescent protein that has two excitation peaks at 440 nm and 586 nm under neutral and acidic conditions, respectively, and a single emission peak at 620 nm (Figure 3F) [27]. This property allows us to monitor the delivery of Keima from the cytosol to lysosomes. As this is an ATG8-independent method, Keima is suitable to monitor bulk (nonselective) autophagy as well as microautophagy. However, if Keima is fused to ATG8 or other organellar makers, lysosomal delivery of these components can also be monitored [27–29]. A limitation of this method is that Keima relies on continuous lysosomal acidification by ATP-dependent V-ATPase activity. Therefore, Keima cannot be used with fixed samples, which makes it difficult to use antibodies for other markers or to use this reporter in animals (see later).

Evaluation of Specific Steps during Autophagy

Induction of Macroautophagy

If results from flux assays suggest autophagy induction, the next step is to check whether relevant signaling events are modulated in a similar direction (Figure 2, Step 3). One of the most important regulatory mechanisms to consider is the repression of mechanistic target of rapamycin complex 1 (mTORC1)-dependent suppression of the **ULK1 complex**, which can be monitored by measuring ULK1-Ser757 phosphorylation [30]. However, the ULK complex can be regulated independently of the mTORC1 pathway, so a reduction in mTOR activity (e.g., indicated by dephosphorylation of S6 kinase) does not necessarily have to accompany autophagy induction. When autophagy is initiated, ULK substrates should also be phosphorylated and this can be monitored by measuring the phosphorylation of ATG14-Ser29 [31] and ATG16L1-Ser278 [32], which are direct ULK1 substrates.

Induction of autophagy can also be evaluated using fluorescence microscopy to measure phagophore numbers because this is regulated by the rate of autophagosome biogenesis and not the rate of consumption. Other than ATG8, most ATG proteins such as FIP200 [33], WIPI2 [34], and ATG16L1 [32] are primarily present on the phagophore membrane but not on mature autophagosomes (Figure 1). Thus, their localization can be monitored and used to infer phagophore numbers.

Autophagosome–Lysosome Fusion and Lysosomal Activity

If flux data suggest a defect at a downstream step in the autophagy pathway, then autophagosome–lysosome fusion and lysosomal activity need to be checked (Figure 2, Step 3). The efficiency of autophagosome–lysosome fusion can be estimated by determining the colocalization between autophagosomal markers (e.g., ATG8, syntaxin 17) and lysosomal markers [e.g., (lysosomal-associated membrane protein 1) LAMP1, LysoTracker] [35]. Lysosomal activity can be evaluated by monitoring lysosomal acidification (e.g., LysoTracker), degradation capacity [e.g., epidermal growth factor (EGF) receptor degradation assay], overall lysosomal integrity (e.g., lysosomal-METRIQ [36]), membrane damage (e.g., galectin-3 [37]), and cathepsin enzyme activity (e.g., MagicRed).

Autophagy Measurement in Animal Models

Measuring autophagic flux is more difficult in organisms such as mice than in cultured cells. In previous reports, ATG8 turnover assays have been carried out in mice using lysosomal inhibitors such as chloroquine [38,39], leupeptin [40,41], and colchicine [42,43]. However, as the exposure and efficacy of these lysosomal inhibitors may vary among tissues, optimal conditions need to be carefully determined. Furthermore, in addition to mTORC1 suppression as discussed earlier, lysosomal inhibitors could have broad adverse effects *in vivo*, for example, in metabolism [44]. Thus, results should be interpreted with caution.

Transgenic mice expressing tfLC3 [45,46] and mice transfected with mCherry-GFP-LC3 by intraventricular injection of adeno-associated viruses [47] have been produced, allowing autophagic activity to be measured *in vivo* without the use of lysosomal inhibitors. Transgenic mice expressing the GFP-LC3-RFP-LC3ΔG reporter have also been generated, but the currently available line can be used only for analysis of skeletal muscle autophagy due to biased expression in that tissue [22]. To monitor mitophagic flux *in vivo*, mt-Keima mice [28], mito-QC mice [48], and mito-SRAI transfected mice [24] were used (Box 1). These autophagy-monitoring mouse models are useful for further elucidation of the physiological and pathological significance of autophagy *in vivo*.

Autophagy Assays for Quantitative Biology at Scale

Discovery of novel pathway perturbagens, whether genetic or pharmacological, requires a cell-based assay: (i) that demonstrates an autophagy phenotype of interest, (ii) that can be miniaturized,

(iii) that has an acceptable signal/noise ratio, and (iv) that is highly reproducible across experiments. Having such an assay allows for unbiased testing of thousands of perturbagens through high throughput (HT) screening and applying robust statistical parameters [49]. The most common approach to evaluating autophagy in HT settings has been to use imaging devices to track the status of LC3 in cells (i.e., total levels as well as puncta numbers). Recently, machine learning methods have been developed to aid in the analysis of autophagy and lysosomal-related features within images [50,51]. In addition to rudimentary counting of objects such as LC3 puncta, these approaches can also detect more nuanced aspects such as size, shape, texture, and position, which could help classify modifiers that regulate distinct steps in autophagy.

Over the last several years, significant progress has been made in adapting the LC3 and autophagy cargo adaptor assays for HT quantitative approaches. In general, before any cell-based screen can be initiated, it is highly advantageous to carefully select and characterize cell lines or individual clones for autophagy responsiveness using pharmacological and genetic tools. This is especially critical in the case of LC3 because its subcellular distribution can show heterogeneous behavior at the population level and biological uniformity is a major factor influencing the success of large-scale functional screens. However, as discussed earlier, LC3 levels and puncta counting are not reliable measures of flux. More recently, the steady-state levels of autophagy cargo adaptors such as SQSTM1/p62 have been used in genomic screens to discover new pathway components. The strengths and weaknesses of these two HT assay approaches are discussed later.

Small Molecule Screens

Unbiased screens to discover small molecules that activate or inhibit autophagy have been performed by several groups. For example, modulators of LC3B cellular distribution have been identified through the use of HT imaging [52–56] and in some cases the molecular targets of these compounds have been identified [57–59]. However, aside from exerting direct, autophagy-specific effects, small molecules that emerge from LC3 screens could also impact cell and organellar stress, which can promote LC3 conjugation to single membranes (Figure 2) [15]. Such complications necessitate the need for intensive mechanistic downstream analysis before pharmacological agents identified from cell-based screens are referred to as autophagy activators or inhibitors. Moreover, some small molecules have been reported to activate autophagy, yet have many intracellular targets, or have been inadequately profiled for specificity, leading to a high potential for polypharmacological activity. This creates significant challenges when concluding that a biological phenotype linked to such agents is due to autophagy modulation [60]. In fact, some of the autophagy activators identified through the use of the LC3 puncta formation assay later turned out to be autophagy inhibitors at downstream steps [22]. While cell-based autophagy screens have the advantage of discovering cell-active chemical probes to aid basic research, rigorous downstream work is therefore needed to validate whether these represent bona fide autophagy pathway modulators.

How can the discovery of small molecule modulators of autophagy be streamlined? The use of autophagy flux reporters that quantitate total cellular levels of LC3 and which also control for pathway specificity and cellular stress have been used successfully in screens [22,58]. The use of the HaloTag-LC3 assay helped resolve distinct steps involved in autophagosome biogenesis [61] and using this in HT screens might allow identification of a unique set of chemical probes compared with screens where 'total' LC3 behavior has been measured. It is important to note that flux reporters based on LC3 homologs such as GABARAP proteins have not yet been used in HT screens and they could help uncover a distinct class of autophagy modifiers. The **Fluorescent Timer DsRed** protein can be used to follow protein and organelle maturation with time and this

has recently been used to discover small molecules that regulate **mitophagy** [62]. Fluorescent Timer is a mutant form of DsRed that emits green fluorescence when newly translated, but transitions over time to red fluorescence as the protein matures, and is insensitive to pH [63]. This probe could be used to develop tagged versions of autophagy components to enable discovery of small molecules that alter the steady-state expression of individual autophagy proteins or substrates.

One of the challenges in performing HT imaging screens is the need for significant data storage capabilities and, to this end, alternatives such as luciferase-based autophagy assays offer significant advantages and are much faster to screen [58,64]. Future HT assays could be devised that measure specific steps or nodes within the autophagy pathway and that are not reliant on imaging. Homogeneous assay formats that use luciferase could be developed to focus on abundance of autophagy adaptors and substrates and key regulatory nodes within the pathway could be screened using FRET technology, as has been reported for the Beclin 1/Bcl-2 complex [65]. As much as possible, screening assays should be developed using physiologically relevant cell lines and models to aid in the discovery of high value autophagy-modulating small molecules.

Forward Genetic Screening to Identify Autophagy Regulators

Not surprisingly, the success of forward genetic approaches to map the autophagy pathway in *Saccharomyces cerevisiae* [66–68] has inspired the use of genetic screening in mammalian cells to better understand autophagy in higher eukaryotes. Initially this manifested in the completion of several arrayed RNAi high content screens for the discovery of new regulators of LC3, SQSTM1/p62, and selective autophagy substrates [69–75] as well as 'pooled' short hairpin (sh) RNA screens for genes that regulate SQSTM1/p62 levels [76]. However, the advent of genome-wide CRISPR-mediated gene knockout technology (using CRISPR Cas9 nuclease or CRISPRn) demonstrated that this approach has a lower false negative rate compared with RNAi and is a robust approach to interrogate the autophagy pathway in mammalian cells [76]. Similar conclusions were made by comparing CRISPRn with RNAi loss of function for cancer cell line lethality [77,78]. Using CRISPRn, multiple laboratories have reported new mechanistic insights into the regulation of LC3, SQSTM1/p62, NDP52, NBR1, TAX1BP1, and PARKIN [23,76,79–86].

An important step when validating hits from screens that use exogenous autophagy reporters is to confirm function against endogenous pathway markers. This is critical because overexpressed epitope-tagged reporters could exert cell stress and/or have aberrant subcellular distribution (however the later phenotype should negate the screen being performed in the first place). Alternatively, validation approaches could include the use of endogenous tagging using the CRISPR technology as a useful way to measure endogenous proteins and structures [87,88]. Validating hits can also be accomplished at-scale by performing a secondary screen using a mini-library of hit single guide (sg)RNAs [76] and, in a recent study, the authors were able to identify sgRNAs that were responsible for downregulating the GFP-SQSTM1/p62 reporter and not endogenous SQSTM1/p62, thus demonstrating the potential issue with exogenous reporters. Following this, Jia and Bonifacino used gene editing to knock-in the mCherry-GFP tag into the endogenous *MAPILC3B* locus as a way to track endogenous LC3 flux in a whole-genome CRISPRn screen [23]. Importantly, this strategy uncovered a novel role for UBA6-BIRC6 in the regulation of LC3 expression, which was not identified from previous screens using exogenous tfLC3B, even when this reporter was integrated within a safe-harbor genome locus [83]. These studies collectively underscore the importance of using endogenous readouts in the primary screen if at all possible.

One important lesson from some of the recent CRISPRn screens is that cell context and treatment sensitizations can expose novel regulatory mechanisms. For example, Xu and colleagues elegantly

uncovered a novel role for the V-ATPase in *Salmonella typhimurium*-induced noncanonical LC3 lipidation by performing a genome-scale CRISPRn screen in *FIP200*-deficient cells [84]. By taking this approach they ruled out any contribution from autophagy in the generation of LC3-II, thus enhancing the probability of finding genes relevant to the host–pathogen response being studied. Given the molecular complexity of autophagy, there is a major opportunity to exploit CRISPR-based gene modulation to advance our understanding of this pathway. To this end, the genome-wide screens discussed earlier have been performed in cancer cell lines and under standard cell culture conditions, which could limit one's ability to make discoveries relevant for normal physiology. Thus, functional genomic approaches should be applied to non-cancer cell models and performed under more physiological- and disease-relevant conditions. Here, the use of induced pluripotent stem cells (iPSCs) and subsequent differentiation into distinct lineages could be helpful to discover cell type-specific components of the autophagy pathway. Importantly, CRISPRn-mediated double-stranded breaks (which are required for gene knockout) are not compatible with gene modulation in iPSCs; however, CRISPR interference (CRISPRi) is a robust alternative [89,90] and has been used to discover survival pathways in human iPSC-neurons [91]. CRISPRi is also useful when cross-validating small molecule effects, since partial downregulation with CRISPRi would more likely approximate effects of a drug relative to CRISPRn, which will result in 100% ablation of the target. Finally, CRISPR activation is another complementary approach to CRISPR loss of function and could be considered for autophagy pathway discovery [92].

Autophagy Biomarkers: The Next Frontier

One of the major challenges in studying autophagy is the availability of appropriate assays to quantitate flux in animals. Moreover, given the high interest in developing autophagy therapeutics to treat human disorders, this aspect of the field needs further attention. However, the current assays used for animals require tissue isolation and can suffer from sensitivity and dynamic range issues. For example, Pietrocola and colleagues showed changes in LC3 lipidation using western blotting in circulating leukocytes from individuals who were starved for 1–2 days [93]. However, the endpoint for LC3 analysis came after culturing these cells *ex vivo*, which could impact normal physiology. More recently, high-dimensional flow analysis and cytometry were used to measure autophagy flux in intrahepatic lymphocytes from human liver biopsies but even with this approach some amount of cell handling *ex vivo* was required [94]. We propose that the evaluation of autophagy therapeutics both in preclinical and clinical studies should be accompanied by procedures that quantitate flux in a noninvasive manner through the use of target engagement biomarkers. Examples of approaches, discussed later, might involve detection of circulating factors that are modulated by cellular autophagy or direct imaging of autophagic substrates.

Primary endpoint measures in clinical trials for chronic disease can take several months or sometimes years and it would be hugely advantageous to have confirmation of pharmacodynamic target engagement in the hours and days following administration of an experimental autophagy therapeutic. An approach here might be to employ nuclear imaging technologies such as **positron emission tomography (PET)** to quantitate the abundance of a specific autophagy substrate and, ideally, the driver of the disease in question (Figure 4). PET relies on having a radioactive small molecule or ligand, delivered intravenously, that binds to the target of interest. The gamma rays derived from this ligand can be detected in a PET scanner and provide a direct readout for target abundance. For example, PET ligands have been used to track levels of pathological misfolded tau in a collection of neurodegenerative disorders referred to as tauopathies [95]. Should one wish to test the effect of an autophagy-inducing drug on tau lowering, then PET could be used to visualize this in the brain of patients, before and after treatment. In the future, small molecule discovery could enable the development of novel PET ligands that bind to specific autophagy substrates, allowing their use for quantitating flux in humans. Alternatively, where modulation of 'bulk' autophagy is desired, for

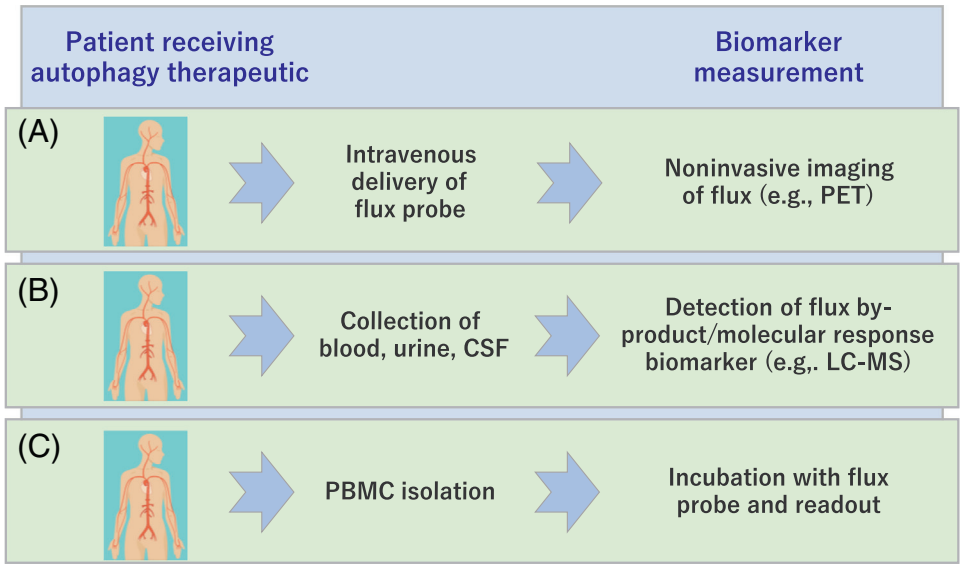

Figure 4. Potential Settings for the Use of Autophagy Biomarkers in the Clinic. Three paradigms are illustrated in which autophagy flux is evaluated. First, administration to patients of a probe that enables molecular imaging of flux in specific tissues and organs (A). Second, measurement of specific biomarkers in patient-derived biofluids using LC-MS (B). Third, *ex vivo* treatment of peripheral blood mononuclear cells (PBMCs) with a flux probe followed by rapid measurement (C). In each case significant effort will be required to discover and validate biomarker endpoints using preclinical models (see text for details). Abbreviations: CSF, cerebrospinal fluid; LC-MS, liquid chromatography-mass spectrometry; PET, positron emission tomography.

example, in aging or immune disorders, having a generic flux biomarker might be desirable. Perhaps exogenous, cell permeable probes could be developed to capitalize on the transit of autophagy substrates from the neutral pH of the autophagosome to the acidic environment of the autolysosome. Similar to PET ligands, these probes could be delivered to patients intravenously or incubated with patient-derived peripheral blood cells at collection, allowing rapid evaluation of autophagy flux (Figure 4).

Another promising area might be the development of biomarkers that capitalize on the role for autophagy in the regulation of unconventional secretion [96,97]. This is an attractive strategy because secreted biomarkers could be measured in biological fluids such as blood, cerebrospinal fluid, and urine. In support of this, unbiased metabolomics has been used to discover circulating metabolites that are altered in mice with liver-specific ablation of *Atg7* [98]. While metabolites found in this way are likely a consequence of long-term adaption to autophagy deficiency, they nevertheless could serve as surrogates for pathway inhibition or induction. The discovery of lysosomal-derived metabolites is another exciting approach [99] that could be performed with *in vitro* and *in vivo* preclinical models to discover biomarker candidates before validation in humans.

Discovery of biomarkers to facilitate the testing of an autophagy therapeutic should include a strategy to identify molecular species that are derived from the diseased compartment where autophagy modulation is the goal. This will allow drug development teams to administer a systemic drug and to determine the extent of autophagy modulation in the disease-relevant site as opposed to nondiseased tissues. The approaches discussed earlier should also encompass use of biomarker assays to measure autophagy pre- and post-therapy, which will ensure a more objective assessment of the role autophagy may have in the overall clinical response. In

summary, the discovery of autophagy biomarkers will also not only provide critical support for drug development but these tools will significantly impact the ability of basic researchers to study autophagy across many biological settings. Meaningful progress in this area will require dedicated collaborations between autophagy scientists and experts from the biomarker development, imaging, and drug development fields.

Concluding Remarks

Identifying the best assay with which to assess autophagy is challenging. In reality there is no one assay that can unambiguously answer the following question: Is autophagy activated or inhibited? Here we have highlighted some of the critical assays, and lessons learned, that can help scientists answer this question and guide future efforts. By combining the use of fluorescent- and pH-sensitive reporters with mechanism-based readouts one can rapidly determine if and how a given experimental condition is perturbing the autophagy pathway (Figure 2). In this way, the investigator does not rely on a single readout and minimizes the potential for misinterpreting experimental observations. Through functional screening, some of the assays highlighted have been instrumental in expanding our view of how autophagy is regulated. CRISPR-based approaches have been especially useful as they have faithfully revealed known but also many novel pathway components and new mechanisms.

Should we aspire to have a definitive autophagy assay? It depends. Given that this pathway is part of a larger endocytic and metabolic signaling network, there will always be a need to investigate mechanistic questions using different assays. With that said, the field could benefit from having an endogenous flux readout that works across cell lineages without the need to engineer cells up-front. Imaging and analytical probes delivered exogenously to cells, animals, and patients could be envisioned here. Moreover, detection of circulating autophagy by-products and/or molecular responses to pathway modulation could be investigated as candidate biomarkers (see Outstanding Questions). Taking future innovative steps towards the measurement of autophagy will greatly enable the discovery of fundamental mechanisms and the development of autophagy-based therapies. In the clinic, such biomarkers will provide an early and robust view of therapeutic target modulation, which will drive decisions around adapting dose schedule and intensity, which will bring greater benefit to patients.

Outstanding Questions

How can we measure autophagic flux without reporter gene transfection? The field eagerly awaits new methods to measure autophagic flux more quantitatively in cultured cells, in model organisms using endogenous markers, and eventually in humans.

What novel autophagy substrates can be identified to better represent autophagic flux? Ideal substrates would be those generated constantly and degraded mainly by autophagy.

What are the limitations to overcome reliable measurement of autophagic flux in animals and how can we overcome them?

What innovative approaches should be investigated to develop autophagy biomarkers in patients and what biospecimens will be applicable for each disease indication?

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