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Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes

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Abstract

Research in autophagy continues to accelerate,[1] and as a result many new scientists are entering the field. Accordingly, it is important to establish a standard set of criteria for monitoring macroautophagy in different organisms. Recent reviews have described the range of assays that have been used for this purpose.[2,3] There are many useful and convenient methods that can be used to monitor macroautophagy in yeast, but relatively few in other model systems, and there is much confusion regarding acceptable methods to measure macroautophagy in higher eukaryotes. A key point that needs to be emphasized is that there is a difference between measurements that monitor the numbers of autophagosomes versus those that measure flux through the autophagy pathway; thus, a block in macroautophagy that results in autophagosome accumulation needs to be differentiated from fully functional autophagy that includes delivery to, and degradation within, lysosomes (in most higher eukaryotes) or the vacuole (in plants and fungi). Here, we present a set of guidelines for the selection and interpretation of the methods that can be used by investigators who are attempting to examine macroautophagy and related processes, as well as by reviewers who need to provide realistic and reasonable critiques of papers that investigate these processes. This set of guidelines is not meant

to be a formulaic set of rules, because the appropriate assays depend in part on the question being asked and the system being used. In addition, we emphasize that no individual assay is guaranteed to be the most appropriate one in every situation, and we strongly recommend the use of multiple assays to verify an autophagic response.

Keywords

autolysosome; autophagosome; flux; lysosome; phagophore; stress; vacuole

At the first Keystone Symposium on Autophagy in Health and Disease, one of the researchers in the audience, after listening to several comments detailing inadequacies in documenting autophagy, asked the question "What are the essential criteria for demonstrating autophagy?" This is a reasonable question, particularly considering that each of us may have his/her own opinion regarding the answer. Unfortunately, this presents something of a "moving target" for researchers who may think they have met those criteria, only to find out that the reviewer of their paper has different ideas. Conversely, as a reviewer, it is tiresome to raise the same objections repeatedly, wondering why researchers have not fulfilled some of the basic requirements for establishing the occurrence of an autophagic process. In addition, drugs that potentially modulate autophagy are increasingly being used in clinical trials, and screens are being carried out for new drugs that can modulate autophagy for therapeutic purposes. Clearly it is important to determine whether these drugs are truly affecting autophagy based on a set of accepted criteria. Accordingly, we describe here a basic set of contemporary guidelines that can be used by researchers to plan and interpret their experiments, by clinicians to decide which avenue of treatment is appropriate, and by both authors and reviewers to justify or criticize an experimental approach.

Several fundamental points must be kept in mind as we establish guidelines for the selection of appropriate methods to monitor autophagy. Importantly, there are no absolute criteria for determining the autophagic status that apply to every situation. This is because some assays are inappropriate, problematic or may not work at all in particular cells, tissues or organisms.[2] In addition, these guidelines may evolve as new methodologies are developed and current assays of the process are superseded. Nonetheless, it is useful to establish guidelines for acceptable assays that can reliably monitor autophagy in many experimental systems. It is important to note that in this set of guidelines the term "autophagy" generally refers to macroautophagy; other autophagy-related processes are specifically designated when appropriate.

An important point is that autophagy is a dynamic, multi-step process that can be modulated at several steps, both positively and negatively. In this respect, the autophagic pathway is not different from other cellular pathways. An accumulation of autophagosomes (be they measured by electron microscopy (EM) image analysis, as fluorescent GFP-LC3 dots, or as LC3 lipidation on a western blot), could, for example, reflect either increased autophagosome formation due to increases in autophagic activity, or to reduced turnover of autophagosomes (Fig. 1). The latter can occur by inhibiting their maturation to amphisomes or autolysosomes, which happens if there are defects in fusion with endosomes or lysosomes, respectively, or following inefficient degradation of the cargo once fusion has occurred.[4] For the purposes of this review, the autophagic compartments are referred to as the sequestering (preautophagosomal) phagophore,[5] the autophagosome,[6] the amphisome (generated by fusion of autophagosomes with endosomes, also referred to as an acidic late autophagosome[7])[8] and the autolysosome (generated by fusion of autophagosomes or amphisomes with a lysosome, also referred to as an autophagolysosome).[6] We note that the use of the term "phagophore" in this review has no implied meaning in regard to the origin of the autophagosomal membrane.

The word “phagophore” was originally coined to indicate that the initial sequestering structure was morphologically distinct from other organelles.[5] Other studies, however, suggest specific origins for the autophagosome sequestering membrane, most notably the endoplasmic reticulum.[9] Indeed, recent work suggests that the endoplasmic reticulum, and more generally membrane flow through the secretory pathway, is required for autophagosome formation.[10, 11] A complete understanding of the membrane source(s) for autophagy awaits further study and, accordingly, “phagophore” in the context of this review refers only to a particular structure.

Studies related to autophagic cell death or, more properly (because it is seldom verified that autophagy is the mechanism underlying such programmed cell death), autophagy-associated cell death, represent another important situation where it becomes necessary to distinguish whether the phenotypic defects arise due to the inhibition versus induction of autophagy. In some cases, this type of death is due to *reduced* autophagic flux, due to inhibition of the fusion of autophagosomes with lysosomes or to loss of the degradative functions of lysosomes.[12] Therefore, the use of autophagy markers such as LC3-II needs to be complemented by knowledge of overall autophagic flux to permit a correct interpretation of the results. In this case, one needs to measure the rate of general autophagic protein breakdown, or to arrest the autophagic flux at a given point to record the time-dependent accumulation of an organelle, an organelle marker, a cargo marker or the entire cargo at the point of blockage. Along the same lines, one can follow the time-dependent decrease of appropriate markers. In theory, this can be achieved by blocking autophagic sequestration at specific steps of the pathway (e.g., blocking further induction or nucleation of a new phagophore) and by measuring the decrease of markers behind the block point. The key issue is to differentiate between the formation versus accumulation of autophagosomes by measuring “steady state” levels and the rates of autophagic degradation of cellular components. Both processes have been used to estimate “autophagy” but unless the experiments can relate changes in autophagosome numbers to a direct or indirect measurement for autophagic flux (e.g., clearance of a substrate as a direct measurement, or changes in LC3-II as an indirect measurement), they may be difficult to interpret. A general caution regarding the use of the term “steady state” is warranted at this point. It should not be assumed that an autophagic system is at steady state as this implies that the level of autophagosomes does not change with time and the flux through the system is constant. Rather, in this review we use the term steady state to refer to measurements that are static in nature.

Autophagic flux refers to the complete process of autophagy including the delivery of cargo to lysosomes (via fusion of the latter with autophagosomes or amphisomes) and its subsequent breakdown and recycling. Thus, increases in the level of phosphatidylethanolamine-modified LC3 (LC3-II), or even the appearance of autophagosomes are not measures of autophagic flux per se, but can reflect the induction of autophagy and/or inhibition of autophagosome or amphisome clearance. Furthermore, the degradative capacity of a cell, which likely varies with cell type, age, transformation and/or disease, may determine the outcome of autophagy induction.[13] Finally, it is important to note that while formation of LC3-II correlates with the induction of autophagy, we do not know, at present, the actual mechanistic relationship between LC3-II formation and the rest of the autophagic process. Accordingly, it is essential to distinguish between autophagosome or LC3-II accumulation, and autophagic flux.

As a final note, we also recommend that authors refrain from the use of the expression “percent autophagy” when describing experimental results, as in “The cells displayed a 25% increase in autophagy.” In contrast, it is appropriate to indicate that a certain percentage of cells display punctate GFP-LC3, or that there is a particular increase or decrease in the rate of degradation of long-lived proteins, as these are the actual measurements being quantified.

Collectively, we propose the following guidelines for measuring these various aspects of autophagy in higher eukaryotes:

A. Monitoring Phagophore and Autophagosome Formation by Steady State Methods

The key reason for separating these guidelines into sections on steady state versus flux measurements is that the former rely on methods that indicate the induction of autophagy, but do not allow a determination of whether the process goes to completion. This is an important point because incomplete autophagy, which would lead to the accumulation of autophagosomes contributes to physiological dysfunction. In contrast, complete autophagy will generally exert a cytoprotective effect.

1. Electron microscopy

Autophagy was first detected by electron microscopy. The focal degradation of cytoplasmic areas sequestered by the phagophore (a specialized type of smooth, ribosome-free double membrane), which matures into the prelysosomal autophagosome is the hallmark of autophagy. Therefore, the use of electron microscopy is a valid and important method both for the qualitative and quantitative analysis of changes in various autophagic structures that sequentially form, the phagophore, autophagosome, amphisome and autolysosome (Fig. 1). The maturation from the phagophore through the autolysosome is a dynamic and continuous process,[14] and thus the classification of compartments into discrete morphological subsets can be problematic. Fortunately, for many biological and pathological situations, examination of both early and late autophagic structures yields valuable data regarding the overall autophagy/lysosomal status in the cells.[13]

Cautionary notes—Although EM is one of the most widely used methodologies to monitor autophagy, it is also one of the most problematic and prone to misinterpretation. Due to the large potential for sampling artifact, careful selection of appropriate nonbiased methods of quantification and morphometric/stereological analyses are essential.[15] For example, it is better to count autophagosome profiles than to just score for the presence or absence of autophagosomes in the section of a cell, but the preferred method is to quantify autophagosome volume as the percent of cytoplasmic volume using volumetric morphometry/stereology.[16] During quantification it is important to make sure that every cell profile in the thin section has equal probability to be included in the counting.

The reliable identification of the autophagosome is a prerequisite for a valid analysis. An additional complication, however, is that maturation of mammalian autophagosomes involves a transition to single-membrane structures (i.e., amphisomes and autolysosomes).[17] Thus, double membranes do not necessarily represent evidence for ultrastructural identification of autophagy-related structures, and it is important to employ expert analysis to avoid misinterpretation of micrographs. Even among experts, there is some disagreement as to the characteristics of an authentic autophagosome.[18] For example, starvation-induced autophagosomes should contain cytoplasm (i.e., cytosol and possibly organelles), but autophagosome-related structures involved in specific types of autophagy, such as selective peroxisome or mitochondria degradation (pexophagy or mitophagy, respectively) or targeted degradation of pathogenic microbes (xenophagy), may be relatively devoid of cytoplasm. Furthermore, some pathogenic microbes express membrane-disrupting factors during infection (e.g., phospholipases) that disrupt the normal double-membrane architecture of autophagosomes.[19] It is not even clear if the sequestering compartments used for specific organelle degradation or xenophagy should be termed autophagosomes or if alternate terms such as pexophagosome[20] and xenophagosome should be used, even though the membrane

and mechanisms involved in their formation may be identical to those for starvation-induced autophagosomes. It is also difficult to determine whether material present within a phagosomal structure derives from self-eating, or from a heterophagic process; when appropriate, specific analyses can be performed to assess the source of the engulfed material. Regardless, it is necessary to prove that the sequestered content becomes completely degraded within the membrane-bordered space. This is accomplished by demonstrating that sequential disintegration of well-recognizable sequestered structures (e.g., mitochondria or rough endoplasmic reticulum cisternae) proceeds to completion. The fact that the entire disintegration process remains focal is evidence for being completely bordered by a membrane in three dimensions. Demonstration of the presence of lysosomal enzymes in post-fusion autophagic compartments by traditional immunocytochemistry is also feasible. Finally, due to the cisternal structure of the endoplasmic reticulum, double membrane-like structures surrounding mitochondria or other organelles are often observed after sectioning, which actually correspond to cisternae of the ER coming into and out of the section plane. The presence of ribosomes associated with these membranes helps distinguish them from the ribosome-free double-membrane of the autophagosome.

In case of potential uncertainties, it is desirable to use immuno-EM with gold-labeling,[21,22] using antibodies to cargo proteins (of cytosolic origin; in this case the cargo should not be an abundant cytosolic protein or the background will be too high, but organelle markers work well) and to LC3 to verify the autophagic nature of the compartment. The success of this methodology, however, depends on the quality of the antibodies and also on the EM preparation and fixation procedures required. With immuno-EM, authors should provide controls showing that labeling is specific, by demonstrating that the signal is clearly above background. In addition, we recommend that statistical information be provided due to the necessity of showing only a selective number of sections. Again, we note that for quantitative data it is preferable to use proper volumetric analysis rather than just counting numbers of sectioned objects. It must be kept in mind, however, that even volumetric morphometry/stereology only shows steady state levels, and by itself is not informative regarding autophagic flux. On the other hand, quantitative analyses indicate that autophagosome volume in many cases does correlate with the rates of protein degradation.[23–25]

One additional caveat with EM, and to some extent with confocal fluorescence microscopy, is that the analysis of single sections of a cell can be misleading and may make the identification of autophagic structures difficult. One potential compromise is to perform whole cell quantification of autophagosomes using fluorescence methods, with qualitative verification by EM,[26] to show that the changes in fluorescent puncta reflect increases in autophagic structures. Confocal microscopy and fluorescence microscopy with deconvolution software (or with much more work, EM) can be used to generate multiple/serial sections of the same cell to reduce this concern, but this is generally unnecessary because analyzing single sections of multiple cells is more practical and provides more information. An additional methodology that is worth noting is correlative light and electron microscopy, CLEM, which is helpful in confirming that fluorescent structures are autophagosomes.[27] Finally, although an indirect measurement, a comparison of the ratio of autophagosomes to autolysosomes by EM can support alterations in autophagy identified by other procedures.[28] In this case it is important to always compare samples to the control of the same cell type, as the ratio of autophagosome/autolysosome varies in a cell context-dependent fashion, depending on their clearance activity. It may also be necessary to distinguish autolysosomes from telolysosomes/late secondary lysosomes (the former are actively engaged in degradation, whereas the latter have reached an end point in the breakdown of lumenal contents; see part B, section 10) because lysosome numbers generally increase when autophagy is induced.

2. Atg8/LC3 western blotting and ubiquitin-like protein conjugation systems

The Atg8/LC3 protein is a ubiquitin-like protein that can be conjugated to phosphatidylethanolamine (PE). In yeast, the conjugated form is referred to as Atg8—PE. The mammalian homologues of Atg8 constitute a family of proteins, with microtubule-associated protein 1 light chain 3 (LC3) being the most relevant for this discussion (this protein is referred to as "Atg8" in other systems, but for simplicity we primarily refer to it here as LC3 to distinguish it from the yeast protein). LC3 is initially synthesized in an unprocessed form, proLC3, which is converted into a proteolytically processed form lacking amino acids from the C terminus, LC3-I, and is finally modified into the PE-conjugated form, LC3-II (Fig. 2). Atg8—PE/LC3-II is the only protein marker that is reliably associated with completed autophagosomes, but is also localized to phagophores. In yeast, Atg8 amounts increase at least ten-fold when autophagy is induced.[29] In mammalian cells, however, the total levels of LC3 do not necessarily change, as there may be increases in the conversion of LC3-I to LC3-II, or a decrease in LC3-II relative to LC3-I if degradation of LC3-II via lysosomal turnover is particularly rapid. Furthermore, even if the total amount of LC3 does increase, the magnitude of the response is generally less than that documented in yeast. Western blotting can easily be used to monitor changes in LC3 amounts (Fig. 2). Note, however, that LC3-II western blotting has not been used successfully in *Drosophila melanogaster* (Baehrecke E, Neufeld T, unpublished results).

Cautionary notes—There are two important caveats when using LC3-II to follow autophagy. First, changes in LC3-II amounts are tissue- and cell context-dependent.[30,31] Indeed, in some cases, autophagosome accumulation detected by electron microscopy does not correlate well with the amount of LC3-II (Tallóczy Z, de Vries RLA, and Sulzer D, and Eskelinen E-L, unpublished results). Conversely, a normal level of LC3-II is not sufficient evidence for autophagy. For example, homozygous deletion of *beclin 1* does not prevent the formation of LC3-II in embryonic stem cells even though autophagy is defective, whereas deletion of *atg5* does result in the complete absence of LC3-II (see Fig. 2B and suppl. data in ref. [32]). Thus, it is important to remember that not all of the autophagy-related proteins are required for Atg8/LC3 processing, including lipidation. Vagaries in the detection and amounts of LC3-I versus LC3-II present technical problems. For example, LC3-I is very abundant in brain tissue, and the intensity of the LC3-I band may obscure detection of LC3-II, unless the polyacrylamide crosslinking density is optimized. Conversely, certain cell lines have much less visible LC3-I compared to LC3-II. In addition, tissues may have asynchronous and heterogeneous cell populations, and this may present challenges when analyzing LC3 by western blotting.

Second, caution must be exercised in general when evaluating LC3 by western blotting, and appropriate standardization controls are necessary. For example, LC3-I may be less sensitive to detection by certain anti-LC3 antibodies, and LC3-I is more labile than LC3-II. LC3-I is also more sensitive to freezing-thawing and to degradation in SDS sample buffer, so fresh samples should be boiled and assessed as soon as possible and should not be subjected to repeated freeze-thaw cycles. Caveats regarding detection of LC3 by western blotting have been covered in a recent review,[33] but one important suggestion noted here is that one should measure levels of LC3-II relative to actin and not to that of LC3-I. In addition, Triton X-100 may not efficiently solubilize LC3-II.[34] Instead, heating in the presence of 1% SDS is needed to ensure complete solubilization, which is essential for correct interpretation of results from western blotting. Also, the utility of measuring LC3-I depends on the cells being analyzed. For example, in contrast to cells from peripheral tissues, LC3-I is abundant and stable in central nervous system tissue, and here both the ratio of LC3-II to LC3-I and the amount of LC3-II can be used to monitor autophagosome formation.[35] Finally, LC3 is expressed as three isoforms in mammalian cells, LC3A, LC3B and LC3C,[36] which exhibit different tissue

distributions, and it may be necessary to use different antisera or antibodies that distinguish among these isoforms. A point of caution along these lines is that the increase in LC3B-II levels, but not in LC3A-II, correlated with elevated levels of autophagic vesicles monitored either by electron microscopy or rat GFP-LC3 transfection in response to autophagy-inducing stress (Corcelle E, Mograbi B, personal communication). This supports the important notion that the LC3 isoforms display different functions, and we therefore advise anti-LC3B for western blotting and immunofluorescence experiments rather than anti-LC3A.

One additional point concerns the monitoring of Atg12—Atg5 conjugation, which has been used in some studies to measure autophagy. In some mammalian cells it appears that essentially all of the Atg5 and Atg12 proteins exist in the conjugated form and the expression levels do not change, at least during short-term starvation.[37,38] Therefore, monitoring Atg12—Atg5 conjugation per se may not be a useful method for following the induction of autophagy. It is worth noting, however, that in some cell lines free Atg5 can be detected,[39] suggesting that the amount of free Atg5 may be cell line-dependent. One final parameter that may be considered is that the total amount of the Atg12—Atg5 conjugate may increase following prolonged starvation as has been observed in hepatocytes and fibroblasts (Cuervo AM, personal communication).

Finally, we would like to point out one general issue with regard to any assay is that it could introduce some type of stress, for example, mechanical stress due to lysis, temperature stress due to heating or cooling a sample, or oxidative stress on a microscope slide, which could lead to potential artifacts. This point is not intended to limit the use of any specific methodology, but rather to point out there are no perfect assays. Therefore, it is important to verify that the positive (e.g., rapamycin treatment) and negative (e.g., inhibitor treatment) controls behave as expected in any assays being utilized.

3. Fluorescence microscopy

LC3B (hereafter referred to as LC3), or the protein tagged at its N terminus with a fluorescent protein such as GFP, GFP-LC3, has been used to monitor autophagy through indirect immunofluorescence (Fig. 3A) or direct fluorescence microscopy (Fig. 3B), measured as an increase in punctate LC3 or GFP-LC3.[40] The detection of GFP-LC3/Atg8 is also useful for in vivo studies using transgenic organisms such as *Caenorhabditis elegans*,[41] *Dictyostelium discoideum*,[42] *Drosophila melanogaster*,[43,44] *Arabidopsis thaliana*[45] and mice.[30] It is also possible to use anti-LC3 antibodies for immunocytochemistry or immunohistochemistry,[46–48] procedures that have the advantages of detecting the endogenous protein, obviating the need for transfection and transgenesis, as well as avoiding potential artifacts resulting from overexpression. Monitoring the endogenous protein, however, obviously depends on the ability to detect it in the system of interest. If the endogenous amount is below the level of detection, the use of an exogenous construct is warranted. In this case, it is important to consider the use of stable transformants versus transient transfections. Stable transformants may have reduced background resulting from the lower protein expression, and there is also the advantage of eliminating artifacts resulting from recent exposure to transfection reagents. Furthermore, with stable transformants more cells can be easily analyzed because nearly 100% of the population will express tagged LC3. On the other hand, one disadvantage of stable transfectants is that the integration sites cannot always be predicted, and expression levels may not be optimal. Furthermore, an important advantage of transient transfection is that this approach is better for examining the immediate effects of the transfected protein on autophagy. In addition, a double transfection can be used (e.g., with GFP-LC3 and the protein of interest) to visually tag the cells that express the protein being examined, an approach that may be more problematic with stable transfectants. In conclusion, there is no simple rule for the use of stable versus transient transfections. When stable transfections are utilized, it is worthwhile screening for clones that

give the best signal to noise ratio, and when transient transfections are used, it is worthwhile optimizing the GFP-LC3 DNA concentration to give the best signal to noise ratio. Optimization, together with including the appropriate controls, will help overcome the effects of the inherent variability in these analyses.

An additional use of GFP-LC3 is to monitor co-localization with a target during autophagy-related processes such as organelle degradation or the sequestration of pathogenic microbes.[49–51] For observing autophagy in *C. elegans*, it is best to use an integrated version of GFP-LC3 (GFP:LGG-1; Fig. 4) rather than an extra-chromosomal construct because the latter shows variable expression among different animals (Kang C, personal communication). In addition, with the integrated version it is still possible to perform a western blot analysis for lipidation.[52] Finally, we point out the increasing availability of instruments that are capable of nanoscale resolution for GFP-based microscopy, which will further enhance the value and possibilities afforded by this technology.[53]

Yeast Atg18 is required for both macroautophagy (i.e., non-specific sequestration of cytoplasm) and autophagy-related processes (e.g., the cytoplasm to vacuole targeting pathway,[54,55] specific organelle degradation,[56] and autophagic elimination of invasive microbes[57–61]).[62] A recent study shows that the human homologue of Atg18 (WIPI-1) accumulates at LC3-positive membrane structures when autophagy is induced, and the increase in Atg18 puncta correlates with elevated levels of LC3-II.[63] Endogenous levels of Atg18 can also be detected by indirect fluorescence microscopy and immunoelectron microscopy, and the distribution of transfected GFP-Atg18 appears similar. Accordingly, Atg18 puncta can be assessed as an alternative to LC3. With regard to other Atg proteins, Atg9 also displays partial co-localization with GFP-LC3.[64] Monitoring the localization of Atg9 has not been used extensively in higher eukaryotes, but this protein displays the same type of dependence for cycling on Atg1/Ulk1 as seen in yeast,[64,65] suggesting that it is possible to follow this protein as an indication of Atg1 function. Finally, Atg8/LC3 is the only protein known to remain associated with the autophagosome in higher eukaryotes, but additional proteins, in particular Atg5, Atg12 and Atg16, associate with the phagophore and have been detected by fluorescence or immunofluorescence.[37,38]

Cautionary notes—Although analysis of fluorescent GFP-LC3 is a useful approach, it is more tedious to quantify autophagy by measuring puncta of GFP-LC3 (or LC3 by immunofluorescence), than by monitoring LC3-II by western blot. Ideally, it is preferable to include both assays and to compare the two sets of results. In addition, if GFP-LC3 is being quantified, it is preferable to determine the number of puncta corresponding to GFP-LC3 on a per cell basis rather than simply the total number of cells displaying puncta. This latter point is critical because even cells in nutrient-rich conditions display some basal level of GFP-LC3 puncta, unless they are lacking autophagy-related genes (and even in the latter case it is possible to get puncta of GFP-LC3 depending on the specific conditions) (Fig. 3B). There are, however, practical issues with counting puncta manually and reliably, especially if there are large numbers per cell (although this may be more accurate than relying on a software program, in which case it is important to ensure that only appropriate dots are being counted). Also, when autophagosome-lysosome fusion is blocked, larger autophagosomes are detected, possibly due to autophagosome-autophagosome fusion. In many cell types it may be possible to establish a cut-off value for the number of puncta per cell in conditions of “low” and “high” autophagy.[66] This can be tested empirically by exposing cells to autophagy-inducing and -blocking agents. Thus, cell populations showing significantly greater proportions of cells with autophagosome numbers higher than the cut-off in perturbation conditions compared to the control cells could provide quantitative evidence of altered autophagy. It is then possible to score the population as the percentage of cells displaying numerous autophagosomes. This approach will only be feasible if the background number of puncta is relatively low, and, in

this case, it is particularly important to count a large number of cells (probably on the order of fifty or more, preferably in at least three different trials, depending on the particular system and experiment).

To allow comparisons by other researchers attempting to repeat these experiments, it is critical that the authors also specify the baseline number of puncta that are used to define "normal" or "low" autophagy. Furthermore, the cells should also be counted using unbiased procedures (e.g., using a random start point followed by inclusion of all cells at regular intervals), and statistical information should be provided for both baseline and altered conditions, as these assays can be highly variable. One possible method to obtain unbiased counting of GFP-LC3 puncta in a large number of cells is to perform multispectral imaging flow cytometry. This method allows characterization of single cells within a population by assessing a combination of morphology and immunofluorescence patterns, thereby providing statistically meaningful data.[67] An additional caution is that size determinations can be problematic by fluorescence microscopy unless careful standardization is carried out.[68] Furthermore, it is not clear that different sizes of GFP-LC3 puncta correlate with levels of autophagy.

One possible control to determine background levels of puncta is to examine fluorescence from untagged GFP. An important caveat in the use of GFP-LC3 is that this chimera can associate with aggregates, especially when expressed at high levels in the presence of aggregate-prone proteins, which can lead to a misinterpretation of the results.[69] Of note, GFP-LC3 can associate with ubiquitinated protein aggregates;[70] however, this does not occur if the GFP-LC3 is expressed at low levels (Rubinsztein DC, unpublished observations). These aggregates have been described in many systems, and are also referred to as Aggresome-Like Induced Structures or ALIS,[70,71] dendritic cell ALIS,[72] p62 bodies/sequestosomes[73] and inclusions. Inhibition of autophagy in vitro and in vivo leads to the accumulation of these aggregates, suggesting a role for autophagy in mediating their clearance.[70,71,74,75] The adaptor protein p62 is required for the formation of ubiquitinated protein aggregates in vitro.[73] In this case, the interaction of p62 with both ubiquitinated proteins and LC3 is thought to mediate delivery of these aggregates to the autophagy system.[76] Many cellular stresses can induce the formation of aggregates, including transfection reagents.[70] Moreover, calcium phosphate transfection of COS7 cells or lipofectamine transfection of MEFs (Pinkas-Kramarski R, personal communication) or neuronal cells (Chu CT, personal communication) transiently increases basal levels of GFP-LC3 puncta and/or the amount of LC3-II. One solution is to examine GFP-LC3 puncta in cells stably expressing GFP-LC3; however, as transfection-induced increases in GFP-LC3 puncta and LC3-II are often transient, another approach is to use cells transfected with GFP, and cells subjected to a mock time-matched transfection as background (negative) controls. A lipidation-defective LC3 mutant where glycine 120 is mutated to alanine is targeted to these aggregates independently of autophagy (likely via its interaction with p62, see above) and as a result this mutant can serve as another valuable control.[70]

Ubiquitinated protein aggregate formation and clearance appear to represent a cellular recycling process. Aggregate formation can occur when autophagy is either inhibited or when its capacity for degradation is exceeded by the formation of proteins delivered to the aggregates. In principle, formation of GFP-LC3-positive aggregates represents a component of the autophagy process. However, the formation of ubiquitinated GFP-LC3-positive protein aggregates does not directly reflect either the induction of autophagy (or autophagosome formation), or flux through the system. Indeed, formation of ubiquitinated protein aggregates can occur in autophagy-deficient cells.[70] Therefore it should be remembered that GFP-LC3 puncta likely represent a mix of ubiquitinated protein aggregates in the cytosol, ubiquitinated protein aggregates within autophagosomes and more "conventional" phagophores and autophagosomes bearing other cytoplasmic cargo. Moreover, a recent report shows that treatment with saponin and other detergents can provoke artifactual GFP-LC3 puncta

formation.[77] Saponin treatment has been used to reduce background fluorescence under conditions where no aggregation of GFP-LC3 is detected in both hepatocytes,[78] and in GFP-LC3 stably-transfected HEK-293 cells (Tooze S, unpublished data); however, controls need to be included in such experiments in light of these findings. In general, it is preferable to include additional assays that measure autophagy rather than relying solely on monitoring GFP-LC3. In addition, we recommend that researchers validate their assays at the start by demonstrating the absence or reversal of GFP-LC3 puncta formation in cells treated with pharmacological or RNA interference-based autophagy inhibitors. For example, 3-methyladenine (3-MA) is commonly used to inhibit starvation- or rapamycin-induced autophagy.

Another general limitation of the GFP-LC3 assay is that it requires a system amenable to either transfection or transgenesis (e.g., infection). Accordingly, the use of GFP-LC3 in primary non-transgenic cells is more challenging. Here again, controls need to be included to verify that the transfection protocol itself does not artifactually induce GFP-LC3 puncta or cause LC3 aggregation. Furthermore, transfection should be performed with low levels of constructs, and the transfected cells followed to determine (1) when sufficient expression for detection is achieved, and (2) that during the time frame of the assay, basal GFP-LC3 puncta remain appropriately low. In addition, the demonstration of a reduction in the number of induced GFP-LC3 puncta under conditions of autophagy inhibition is helpful. For some primary cells, delivering GFP-LC3 to precursor cells by infection with recombinant lentivirus, adenovirus[79] or retrovirus, and subsequent differentiation into the cell type of interest, is a powerful alternative to transfection of the already differentiated cell type.[80]

An additional consideration is that transfection protocols, or viral infection, activate stress pathways in some cells and possibly induce autophagy, again emphasizing the importance of appropriate controls, such as control viruses expressing GFP.[78] When carrying out transfections it may be necessary to alter the protocol depending on the background. In addition, changing the medium and waiting 24 to 48 hours after the transfection can help to reduce the background level of GFP-LC3 puncta that is due to the transfection reagent (Colombo MI, personal communication). When using an mCherry-GFP-p62 double tag (see below under *Tandem RFP-GFP fluorescence microscopy*) in transient transfections it is best to wait 48 hours after transfection to reduce the level of aggregate formation and potential inhibition of autophagy (Johansen T, personal communication).

Finally, although LC3-II is primarily membrane associated, it is not necessarily associated with autophagosomes as is often assumed; the protein is also found on phagophores, the precursors to autophagosomes. In addition, the site of LC3 conjugation to PE is not known and levels of Atg8—PE/LC3-II can increase even in autophagy mutants that cannot form autophagosomes.[81] One method that can be used to examine LC3-II membrane association is differential extraction in Triton X-114, which can be used with mammalian cells.[79] Another approach is to examine co-localization of LC3 with Atg5 (or other Atg proteins); the Atg12—Atg5 conjugate does not remain associated with autophagosomes so co-localized structures would correspond to phagophores. Importantly, we stress again that numbers of GFP-LC3 puncta, similar to steady state LC3-II levels, reflect only a snapshot of the numbers of autophagy-related structures (e.g., autophagosomes) in a cell, and not autophagic flux.

With regard to detection of Atg18 or GFP-Atg18, it has not been demonstrated whether Atg18 puncta can be detected in systems other than human cells, and the level of puncta formation is cell context-dependent.[63] Additionally, Atg18 has not been detected on the completed (mature) autophagosome, so it may only decorate the phagophore. Accordingly, the formation of Atg18 puncta may only be useful to monitor autophagy induction and not flux.

4. TOR and Atg1 kinase activity

TOR complex I (TORC1) negatively regulates autophagy in a transcription-independent manner downstream of protein kinase B. In most systems, inhibition of TOR leads to induction of autophagy. TORC1 activity can be monitored by following the phosphorylation of its target protein(s) or downstream effectors, such as p70S6 kinase or the S6 protein.[82,83] For p70S6 kinase, it is important to examine phosphorylation at threonine 389, which is a direct target of TOR and is rapamycin-sensitive; the C-terminal phosphorylation sites do not always correlate with TOR activation (Murphy LO, personal communication). Accordingly, it is better to quantify p70S6 kinase activity in vitro, but this requires greater effort. A decrease in TORC1 activity can lead to autophagy induction, however, it is not a direct measurement. In contrast, in vitro Atg1 kinase activity towards an exogenous substrate appears to increase when autophagy is induced.[84] In yeast, and presumably in other organisms, it is possible to measure Atg1 kinase activity to verify the induction of autophagy.

Cautionary notes—There are TOR-independent mechanisms that induce autophagy.[85–88] Thus, it is necessary to verify that the pathway being analyzed displays TOR-dependent inhibition. At present, the use of Atg1 kinase activity as a tool to monitor autophagy is limited because an authentic substrate has not been characterized; the current assays rely on in vitro phosphorylation of the artificial substrate myelin basic protein. When a physiological substrate (s) of Atg1 is identified it will be possible to follow its phosphorylation in vivo as is done with analyses for TOR.

5. Transcriptional regulation

The induction of autophagy in certain scenarios is accompanied by an increase in the mRNA levels of certain autophagy genes, such as *Atg8/LC3*[89] and *Atg12*.[90] Thus, assessing the levels of LC3 mRNA by northern blot or qRT-PCR may provide correlative data relating to the induction of autophagy. It is not clear if these changes are sufficient to induce autophagy, however, and therefore these are not direct measurements. Of note, large changes in *Atg* gene transcription just prior to *Drosophila melanogaster* salivary gland cell death (that is accompanied by an increase in autophagy) are detected in *Atg2*, *Atg4*, *Atg5* and *Atg7*, whereas there is no significant change in *Atg8a* or *Atg8b*.[91,92] However, transcriptional upregulation of *Drosophila melanogaster Atg8a* and *Atg8b* is observed in fat bodies following induction of autophagy at the end of larval development,[93] and an increase in *Drosophila melanogaster Atg8b* is observed in cultured *Drosophila melanogaster l(2)mbn* cells following starvation (Gorski S, personal communication).

Cautionary notes—Most of the *Atg* genes do not show significant changes in mRNA levels when autophagy is induced. Even increases in *LC3* mRNA can be quite modest and are cell type- and organism-dependent.[94] In addition, it is generally better to follow protein levels because that is the ultimate readout that is significant with regard to the initiation and completion of autophagy, although Atg protein amounts do not always change significantly and the extent of increase is again cell type- and tissue-dependent. Finally, changes in autophagy protein levels are not sufficient evidence of autophagy induction, and must be accompanied by additional assays as described herein.

B. Monitoring Autophagy by Flux Measurements

Autophagy includes not just the increased synthesis or lipidation of Atg8/LC3, or an increase in the formation of autophagosomes, but most importantly flux, or flow, through the entire system, including lysosomes or the vacuole. Therefore, autophagic substrates need to be monitored to verify that they have reached this organelle, and, when appropriate, degraded.

1. Autophagic protein degradation

Protein degradation assays represent a well-established methodology for measuring autophagic flux, and they allow good quantification. The general strategy is first to label cellular proteins by incorporation of a radioactive amino acid (e.g., [^{14}C]-leucine or [^{14}C]-valine), preferably for a long time to achieve sufficient labeling of the long-lived proteins that best represent autophagic substrates, and then to follow this with a long cold-chase so that the assay starts well after labeled short-lived proteins are degraded. Next, the time-dependent release of acid-soluble radioactivity from the labeled protein in intact cells or perfused organs is measured.[2,95] A considerable fraction of the measured degradation will, however, be non-autophagic, and thus one should also measure, in parallel, cell samples treated with autophagy-suppressive concentrations of 3-MA or amino acids; these values are then subtracted from the total. The complementary approach of using compounds that block other degradative pathways, such as proteasome inhibitors, may cause unexpected results due to crosstalk among the degradative systems. For example, blocking proteasome function may activate autophagy.[96–98] Thus, when using inhibitors it is critical to know whether the inhibitors being used alter autophagy, in the particular cell type and context being examined. In addition, because 3-MA could have some autophagy-independent effects in particular settings it is advisable to verify that the 3-MA-sensitive degradation is also sensitive to general lysosomal inhibitors (such as ammonium chloride or leupeptin).

Another assay that could be considered relies on the limited proteolysis of a betaine homocysteine methyltransferase (BHMT) fusion protein. Previous studies show that the 44 kDa full-length BHMT protein is cleaved in hepatocytic lysosomes in the presence of leupeptin to generate a 32 kDa fragment.[99,100] Accumulation of the 32 kDa species is time-dependent and is blocked by treatment with autophagy inhibitors. A modified version of this marker, GST-BHMT, can be expressed in other cell lines where it behaves similar to the wild-type protein (Mercer C, Kaliappan A, Dennis PB, personal communication). Other substrates may be considered for similar types of assays. For example, the neomycin phosphotransferase II-GFP (NeoR-GFP) fusion protein is a target of autophagy.[101] Transfection of lymphoblastoid cells with a plasmid encoding NeoR-GFP followed by incubation in the presence of 3-MA leads to an accumulation of the NeoR-GFP protein as measured by flow cytometry.[102]

Cautionary notes—Measuring the degradation of long-lived proteins requires prior radiolabeling of the cells (and subsequent separation of acid-soluble from acid-insoluble radioactivity), and although the labeling can be done with relative ease in cultured cells, such pulse-chase experiments are not possible in animals, although they can be done in perfused organs. In cells, it is also possible to measure the release of an unlabeled amino acid by chromatographic methods, thereby obviating the need for prelabeling.[103] In either case, one potential problem is that the released amino acid may be further metabolized. For example, branched chain amino acids are good indicators of proteolysis in hepatocytes, but not in muscle cells where they are further oxidized (Meijer AJ, personal communication). Furthermore, the amino acid can be reincorporated into protein; for this reason, such experiments can be carried out in the presence of cycloheximide, but this raises additional concerns (see *Turnover of autophagic compartments* below). In the case of labeled amino acids, a non-labeled chase is added where the tracer amino acid is present in excess (being cautious to avoid using an amino acid that inhibits autophagy), or by use of single pass perfused organs or superfused cells.[104,105] The perfused organ system also allows for testing the reversibility of effects on proteolysis and the use of autophagy-specific inhibitors in the same experimental preparation, which are crucial controls for proper assessment.

If the autophagic protein degradation is low (as it will be in cells in replete medium), it may be difficult to measure it reliably above the relatively high background of non-autophagic

degradation. It should also be noted that the usual practice of incubating the cells under "degradation conditions," that is, in a saline buffer, indicates the potential autophagic *capacity* (maximal attainable activity) of the cells rather than the autophagic *activity* that prevails in vivo or under rich culture conditions. Finally, inhibition of a particular degradative pathway is typically accompanied by an increase in a separate pathway as the cell attempts to compensate for the loss of degradative capacity.[98,106] This compensation might interfere with control measurements under conditions that attempt to inhibit macroautophagy; however, as the latter is the major degradative pathway, the contributions of other types of degradation over the course of this type of experiment are most often negligible.

2. Turnover of LC3-II

Autophagic flux can be measured by inferring LC3-II turnover by western blot (Fig. 2)[31] in the presence and absence of lysosomal degradation. Preventing lysosomal degradation can be achieved through the use of protease inhibitors (e.g., leupeptin and E64d) or drugs such as bafilomycin A_1 that alter the lysosomal pH,[107] or by treatment with agents that block fusion of autophagosomes with lysosomes.

One of the most recent additions to methodologies for monitoring autophagy relies on the observation that a subpopulation of LC3-II exists in a cytosolic form (LC3-IIs) in some cell types.[108] The amount of cytosolic LC3-IIs and the ratio between LC3-I and LC3-IIs appears to correlate with changes in autophagy and provides a more accurate measure of autophagic flux than ratios based on the total level of LC3-II.[108] The validity of this method has been demonstrated by comparing autophagic proteolytic flux in rat hepatocytes and hepatoma cells. One advantage of this approach is that it does not require the presence of autophagic or lysosomal inhibitors to block the degradation of LC3-II.

Cautionary notes—The main caveat regarding the measurement of LC3-IIs/LC3-I is that it is not yet known whether this method is generally applicable to other cell types, and a soluble form of LC3-II is not observed in many standard cell types including HeLa, HEK293 and PC12. In addition, the same concerns apply regarding detection of LC3-I by western blotting. It should be noted that the LC3-IIs/LC3-I ratio must be analyzed using the cytosolic fractions rather than the total homogenates. In addition, the same caveats mentioned above regarding the use of LC3 for qualitatively monitoring autophagy also apply to the use of this marker for following flux.

The use of a radioactive pulse-chase analysis provides an alternative to lysosomal protease inhibitors,[29] although such inhibitors should still be used to verify that degradation is lysosome-dependent. In addition, drugs must be used at concentrations and for time spans that are effective in inhibiting fusion or degradation, but that do not provoke cell death. Thus, these techniques may not be practical in all cell types or in tissues from whole organisms where the use of protease inhibitors is problematic, and where pulse labeling requires artificial short-term culture conditions that may induce autophagy.

It may not be absolutely necessary to follow LC3-II turnover if other substrates are being monitored simultaneously. For example, an increase in LC3-II levels in combination with the lysosomal (or ideally autophagy-specific) removal of an autophagic substrate (such as a polyQ-expanded protein for researchers studying neurodegeneration, or an organelle[109]) that is not a good proteasomal substrate provides an independent assessment of autophagic flux.

3. GFP-Atg8/LC3 lysosomal delivery and proteolysis

GFP-LC3B (GFP-LC3) has also been used to follow flux. First, when GFP-Atg8 or GFP-LC3 is delivered to a lysosome the Atg8/LC3 part of the chimera is sensitive to degradation, whereas the GFP protein (although not necessarily GFP fluorescence) is relatively resistant to

hydrolysis. Therefore, the appearance of free GFP on western blots can be used to monitor lysis of the inner autophagosome membrane and breakdown of the cargo (Fig. 5).[110–112] The movement of GFP-LC3 to lysosomes also can be monitored by fluorescence microscopy, although the GFP fluorescent signal is more sensitive to acidic pH than other fluorophores. In either case, it can be problematic to use GFP fluorescence to follow flux, as new GFP-LC3 is being synthesized. A potential solution to this problem for following fluorescence is to use a photoactivatable version of the fluorescent protein,[113] which allows this assay to be performed essentially as a pulse/chase analysis. Another alternative is to follow flux using GFP-LC3 fluorescence by adding lysosomal protease inhibitors to cells expressing GFP-LC3 and monitoring changes in the number of puncta. In this case, the presence of lysosomal inhibitors should increase the number of GFP-LC3-positive structures, and the absence of an effect on the total number of GFP-LC3 puncta or on the percentage of cells displaying numerous puncta is indicative of a defect(s) in autophagic flux.[114] The combination of protease inhibitors (to prevent the degradation of GFP) or compounds that modify lysosomal pH such as ammonium chloride or chloroquine, or drugs such as bafilomycin A_1 along with compounds that block fusion of autophagosomes with lysosomes (e.g., vinblastine) may be most effective in preventing lysosome-dependent decreases in GFP-LC3 puncta. However, because the stability of GFP is affected by lysosomal pH, we advise the use of protease inhibitors whether or not lysosomotropic compounds or fusion inhibitors are included. Finally, a new method was recently developed utilizing the fluorescence activated cell sorter to allow quantitative analysis of GFP-LC3 turnover (Shvets E, Fass E, Elazar Z, personal communication).

Cautionary notes—The main limitation of the GFP-LC3 processing assay is that it seems to depend on cell types and culture conditions (Hosokawa N, Mizushima N, unpublished data). Apparently, GFP is more sensitive to mammalian lysosomal hydrolases than the degradative milieu of the yeast vacuole. Alternatively, the lower pH of lysosomes relative to that of the vacuole may contribute to differences in detecting free GFP. Therefore, if this method is used it should be accompanied by immunoblotting including controls to address the stability of non-lysosomal GFP such as GFP-LC3-I. Along these lines, a caution concerning the use of the eGFP fluorescent protein for microscopy is that this fluorophore has a relatively neutral pH optimum for fluorescence,[115] so that its signal may diminish quickly at a reduced pH. Thus, it may be preferable to use an alternate fluorophore such as red fluorescent protein (RFP) or mCherry, which retain fluorescence even at acidic pH.[116] Another alternative to RFP or mCherry is to use the Venus variant of YFP, which is brighter than mRFP and less sensitive to pH than GFP.[117] The pH optimum of eGFP is important to consider when using GFP-LC3 constructs, as the original GFP-LC3 marker[40] uses the eGFP variant, which may result in a reduced signal upon the formation of amphisomes or autolysosomes. An additional caveat when using the photoactivatable construct PA-GFP[115] is that the process of activation by photons may induce DNA damage, which could, in turn, elicit induction of autophagy. Finally, GFP is relatively resistant to denaturation, and boiling for 5 min may be needed to prevent folded protein from being trapped in the stacking gel during SDS-PAGE.

4. p62 western blot

In addition to LC3, it is also possible to use p62/SQSTM1 as a marker, at least in certain settings.[33] The p62 protein serves as a link between LC3 and ubiquitinated substrates.[118] p62 becomes incorporated into the completed autophagosome and is degraded in autolysosomes (Fig. 6A). A recent study shows that inhibition of autophagy correlates with increased levels of p62, suggesting that steady state levels of this protein reflect the autophagic status.[119,120] Interestingly, another report shows that p62 is involved in inclusion body formation and that loss of p62 attenuates the liver injury that results from a deficiency in autophagy.[121] In contrast, loss of p62 had little effect on neuronal degeneration, suggesting a cell-type specific nature to inclusion body-related pathologies.

Cautionary notes—One problem with p62 is that it is presently not known if this protein is a general marker for autophagy, although it binds strongly to LC3 as well as to ubiquitinated substrates. In addition, it is most easily used to assess the down-regulation rather than the induction of autophagy (i.e., p62 levels only increase when autophagy is blocked; Fig. 6B). For example, there is no obvious difference in p62 amounts after 30 minutes of autophagy induction, whereas a change in LC3-II can be detected by this time.[33] Furthermore, it is necessary to examine endogenous p62 because overexpression of this protein leads to the formation of protein inclusions. In fact, even endogenous p62 becomes Triton X-100-insoluble in the presence of protein aggregates and when autophagic degradation is inhibited; thus, results with this protein are often context-dependent. In addition, p62 participates in proteasomal degradation, and its level may also increase when the proteasome is inhibited.[122] Finally, p62 may be transcriptionally upregulated under certain conditions,[123] further complicating the interpretation of results. In conclusion, although analysis of p62 can assist in assessing the impairment of autophagy, we do not recommend using p62 alone to monitor flux.

5. Autophagic sequestration assays

Autophagic activity can also be monitored by the sequestration of autophagic cargo, using either an (electro)injected, inert cytosolic marker such as [^{3}H]raffinose,[124] or an endogenous cytosolic protein such as lactate dehydrogenase,[125] in the latter case along with treatment with a proteinase inhibitor (e.g., leupeptin) to prevent intralysosomal degradation of the protein marker. The assay simply measures the transfer of cargo from the soluble (cytosol) to the insoluble (sedimentable) cell fraction (which includes autophagic compartments), with no need for a sophisticated subcellular fractionation (a filtration assay would presumably work just as well as centrifugation, although it would be necessary to verify that the filtration membrane does not destroy the integrity of the post-nuclear supernatant compartments). The cargo marker can be quantified by an enzymatic assay, or by western blotting. In principle, any intracellular component can be used as a cargo marker, but cytosolic enzymes having low sedimentable backgrounds are preferable. Membrane-associated markers are less suitable, and proteins such as LC3, which are part of the sequestering system itself, will have a much more complex relationship to the autophagic flux than a pure cargo marker such as lactate dehydrogenase.

Sequestration assays can be designed to measure flux through individual steps of the autophagy pathway. For example, microtubule inhibitors such as vinblastine will block autophagosome-lysosome fusion, and intralysosomally degraded sequestration probes such as [^{14}C]lactate will mark only prelysosomal compartments in cells treated with this inhibitor,[126] and these have been used to obtain background control data for monitoring of the overall autophagic pathway (autophagic lactolysis).[127] One caveat, however, is that some of these inhibitors promote sequestration through an unknown mechanism (see *Autophagy inhibitors and inducers*).

A variation of this approach applicable to mammalian cells includes live cell imaging. Autophagy induction is monitored as the movement of cargo, such as mitochondria, to GFP-LC3-colocalizing compartments, and then fusion/flux is measured by delivery of cargo to lysosomal compartments.[79,128] In addition, sequestration of fluorescently tagged cytosolic proteins into membranous compartments can be measured, as fluorescent puncta become resistant to the detergent digitonin.[129]

Cautionary notes—The electro-injection of radiolabeled probes is technically demanding, but the use of an endogenous cytosolic protein probe is very simple and requires no pretreatment of the cells other than with a protease inhibitor. Another concern with electro-injection is that it can affect cellular physiology, so it is necessary to verify that the cells behave properly under control situations such as amino acid deprivation. An alternate approach for incorporating exogenous proteins into mammalian cell cytosol is to use “scrape-loading,” a method that

works for cells that are adherent to tissue culture plates.[130] Finally, these assays work well with hepatocytes but may be problematic with other cell types, and it can be difficult to load the cell while retaining the integrity of the compartments in the post-nuclear supernatant (Tooze S, unpublished results). General points of caution to be addressed with regard to live cell imaging relate to photo-bleaching of the fluorophore, cell injury due to repetitive imaging, autofluorescence in tissues containing lipofuscin, and the pH sensitivity of the fluorophore.

6. Turnover of autophagic compartments

Inhibitors of autophagic sequestration (e.g., amino acids, 3-MA or wortmannin) can be used to monitor the disappearance of autophagic elements (phagophores, autophagosomes, autolysosomes) to estimate their half-life by electron microscopy morphometry/stereology. The turnover of the autophagosome or the autolysosome will be differentially affected if fusion or intralysosomal degradation is inhibited.[131–134] The duration of such experiments is usually only a few hours; therefore, long-term side effects or declining effectiveness of the inhibitors can be avoided. It should be noted that fluorescence microscopy has also been used to monitor the half-life of autophagosomes, monitoring GFP-LC3 in the presence and absence of bafilomycin A1 or following GFP-LC3 after starvation and recovery in amino acid-rich medium.[107]

Cautionary notes—The inhibitory effect must be strong and the efficiency of the inhibitor needs to be tested under the experimental conditions to be employed. Cycloheximide is frequently used as an autophagy inhibitor, but this is problematic because of the many potential indirect effects. For example, cycloheximide decreases the efficiency of protein degradation in several cell types (Cuervo AM, personal communication). In addition, at high concentrations (in the millimolar range) cycloheximide inhibits complex I of the mitochondrial respiratory chain,[135] but this is not a problem, at least in hepatocytes, at low concentrations (10–20 μM) that are sufficient to prevent protein synthesis (Meijer AJ, personal communication).

7. Autophagosome-lysosome colocalization and dequenching assay

Another method to demonstrate the convergence of the autophagic pathway with a functional degradative compartment is to incubate cells with the bovine serum albumin derivative de-quenched (DQ)-BSA that has been labeled with the red-fluorescent BODIPY TR-X dye; this conjugate will accumulate in lysosomes. The labeling of DQ-BSA is so extensive that the fluorophore is self-quenched. Proteolysis of this compound results in de-quenching and the release of brightly fluorescent fragments. Thus, the use of DQ-BSA is useful for detecting intracellular proteolytic activity as a measure of a functional lysosome (Colombo MI, personal communication). Furthermore, DQ-BSA labeling can be combined with GFP-LC3 to monitor colocalization and thus visualize the convergence of autophagosomes with a functional degradative compartment. This method can also be used to visualize fusion events in real time experiments by confocal microscopy (live cell imaging). Along similar lines, other approaches for monitoring convergence are to follow the colocalization of RFP-LC3 and LysoSensor Green (Bains M, Heidenreich KA, personal communication) or tagged versions of LC3 and LAMP-1 (Macleod K, personal communication) as a measure of the fusion of autophagosomes with lysosomes.

Cautionary notes—Some experiments require the use of inhibitors (e.g., 3-MA or wortmannin) or overexpression of proteins (e.g., Rab7 dominant negative mutants) that may also affect the endocytic pathway or the delivery of DQ-BSA to lysosomes. In this case, the lysosomal compartment can be labeled with DQ-BSA overnight before treating the cells with the drugs or prior to the transfection.

8. Sequestration and processing assays in plants

The fluorophore of the red fluorescent protein shows a relatively high stability under acidic pH conditions. Thus, chimeric RFP fusion proteins that are sequestered within autophagosomes and delivered to the plant vacuole can be easily detected by fluorescence microscopy. Furthermore, fusion proteins with some versions of RFP tend to form intracellular aggregates, allowing the development of a visible autophagic assay for plant cells.[136] For example, fusion of cytochrome b5 and the original (tetrameric) RFP generate an aggregated cargo protein that displays cytosolic puncta of red fluorescence and, following vacuolar delivery, diffuse staining throughout the vacuolar lumen. This system allows autophagy to be monitored through fluorescence microscopy with minimum damage to intact plant cells. In addition, the size difference between the intact and processed cargo protein allows the quantification of autophagic degradation through the detection of RFP after separation of total protein by gel electrophoresis, similar to the GFP-Atg8/LC3 processing assay described above. As with other systems, autophagosome formation in plants can also be monitored through the use of fluorescent protein fusions to Atg8, and electron microscopy (Fig. 7).

In some systems, including fungi and plants, the size of the vacuole is sufficiently large such that fusion of the autophagosome results in the release of the inner vesicle into the organelle lumen; the resulting single-membrane vesicle is termed an autophagic body (Fig. 8). The accumulation of autophagic bodies can be detected by light microscopy in cells that lack vacuolar hydrolase activity (e.g., the *pep4*Δ yeast mutant) or in the presence of inhibitors that interfere with hydrolase activity (e.g., PMSF or concanamycin). Using Nomarski optics (differential interference contrast) it is easy to distinguish and quantify yeast vacuoles that lack autophagic bodies from those that have accumulated them, and the same is true for plants.

Cautionary notes: Although the detection of vacuolar RFP can be applied to both plant cell lines and to intact plants, it is not practical to measure RFP fluorescence in intact plant leaves, due to the very high red fluorescence of chloroplasts. Furthermore, different autophagic induction conditions cause differences in protein synthesis rates; thus, special care should be taken to monitor the efficiency of autophagy by quantifying the intact and processed cargo proteins. With regard to autophagic body accumulation, it is difficult to quantify their number and/or volume, although their presence or absence can be examined by light or electron microscopy. In addition, the accumulation of autophagic bodies requires the inhibition of vacuolar hydrolase activity. Therefore, to demonstrate turnover the assay must be performed either in the absence and presence of appropriate inhibitors or in a strain with a deletion in a gene encoding a vacuolar hydrolase(s). Otherwise, accumulation of autophagic bodies could instead indicate a defect in the lysis/degradation step of autophagy. Finally, this method is not well suited for systems other than plants or fungi because lysosomes are too small for detection by standard (i.e., non-fluorescence) light microscopy, and fusion with autophagosomes does not generate autophagic bodies (Fig. 8).

9. Tandem RFP-GFP fluorescence microscopy

A new assay that can be used to monitor flux relies on the use of a tandem monomeric RFP-GFP-tagged LC3 (tfLC3; Fig. 9).[116] The GFP signal is sensitive to the acidic and/or proteolytic conditions of the lysosome lumen, whereas mRFP is more stable. Therefore, colocalization of both GFP and RFP fluorescence indicates a compartment that has not fused with a lysosome, such as the phagophore or an autophagosome. In contrast, an mRFP signal without GFP corresponds to an amphisome or autolysosome. Other fluorophores such as mCherry are also suitable instead of mRFP.[73] One of the major advantages of this method is that it enables simultaneous estimation of both the induction of autophagy and flux through autophagic compartments in essentially native conditions, without requiring any drug treatment.

Cautionary notes—This is a new assay that has not been tested in a wide range of cell types. Accordingly, the sensitivity and the specificity of the method must be verified independently until this method has been tested more extensively.

10. Tissue fractionation

The study of autophagy in the organs of larger animals, in large numbers of organisms with very similar characteristics, or in tissue culture cells provides an opportunity to use tissue fractionation techniques as has been possible with glucagon-induced autophagy in rat liver.[137–141] For the purpose of this section, it is important to clarify some of the terms used to identify components of the autophagic system.[6] "Primary lysosomes" refer to small vesicles containing acid hydrolases that have not participated in a previous digestive process, whereas "secondary lysosomes" are somewhat larger particles containing hydrolases and, in the case of late secondary lysosomes/telolysosomes, residues of previous digestions. "Autophagosomes" contain cytoplasmic components but no hydrolases, and finally, "autolysosomes" or "autophagolysosomes" result from the fusion of autophagosomes with primary or secondary lysosomes. It has been shown that with proper homogenization techniques,[142] populations of particles making up the autophagic process in cells (autophagosomes, autolysosomes and telolysosomes) are present in tissue homogenates and are randomly distributed.[139,143] Because of their sizes (smaller than nuclei but larger than membrane fragments (microsomes)), differential centrifugation can be used to obtain a subcellular fraction enriched in mitochondria and organelles of the autophagic-lysosomal system (usually the classical Mitochondrial Fraction + Light Mitochondrial Fraction [M+L Fraction]; the L Fraction contains the highest activity for lysosomal enzymes, but the main component is still mitochondria), which can then be subjected to discontinuous density gradient centrifugation to separate autophagosomes, autolysosomes and lysosomes.[143–145] Any part of such a fraction can be considered to be a representative sample of tissue constituents and used in quantitative biochemical, centrifugational and morphological studies of autophagic particle populations. The data obtained can be further evaluated using sophisticated statistical analysis.

The simplest studies of the autophagic process take advantage of sequestered marker enzymes, changes in location of these enzymes, differences in particle/compartment size and differential sensitivity of particles of different sizes to mechanical and osmotic stress (acid hydrolases are found primarily in membrane-bound compartments and their latent activities cannot be measured unless these membranes are lysed). For example, autolysosomes/early secondary lysosomes are much larger than telolysosomes/late secondary lysosomes[139] and the location of enzymes in the former can be detected by an increase in the release of these enzymes by osmotic shock or mechanical disruption.[137,139,141] Such a change in enzyme accessibility can be used to follow the time course of an exogenously induced, or naturally occurring, autophagic process.[137,139,141]

Quantitative localization of enzymatic activity (or any other marker) to specific cytoplasmic particle populations and changes in the location of such markers during autophagy can be carried out using rate sedimentation ultracentrifugation.[143] Application of a centrifugal force to a sample of homogeneously distributed particles results in their migration at different speeds away from the axis of rotation, carrying their markers with them. This results in a distribution of marker activity, dependent on particle size and relative density, in fractions taken at different distances from the axis of rotation.[143] These distributions can be used to determine which markers are in the same particles[137,143] and whether or not the markers have moved to particles with different physical properties.[139] Similar results can be obtained with isopycnic centrifugation where particles enter a density gradient (sometimes made with sucrose but iso-osmotic media such as iodixanol, metrizamide and Nycodenz may be preferred as discussed

below under *Cautionary notes*) and are centrifuged until they reach locations in the gradient where their densities are equal to those of the gradient.[143]

Particle populations in subcellular fractions evaluated with quantitative biochemical and centrifugational approaches can also be studied with quantitative morphological methods. Detailed morphological study of the particle populations involved in the autophagic process usually requires the use of electron microscopy. The thin sections required for such studies pose major sampling problems in both intact cells[146] and subcellular fractions.[143] With the latter, 2,000,000 sections can be obtained from each 0.1 ml of pellet volume, so any practical sample size is an infinitesimally small subsample of the total sample.[143] However, through homogenization and resuspension, complex and heterogeneous components of subcellular fractions become randomly distributed throughout the fraction volume. Therefore, as mentioned above, any aliquot of that volume can be considered a random sample of the whole volume. What is necessary is to conserve this property of subcellular fractions in the generation of a specimen that can be examined with the electron microscope. This can be done with the use of a pressure filtration procedure[147] to deposit the contents of an aliquot of fraction volume on a filter, which is subsequently covered with a layer of red blood cells, processed for electron microscopy, embedded and sectioned.[143] Because the direction of pressure is perpendicular to the plane of the filter, any section containing the full pellet thickness can be considered a random sample of the pellet volume. Because of the thinness of the sections, multiple sections of individual particles are possible so morphometric/stereological methods[146] must be used to determine the volume occupied by a given class of particles, as well as the size distribution and average size of the particle class. From this information the number of particles in a specific particle class can be calculated.[148] If these data are obtained for all classes of particles in the autophagic system, the kinetics of particle interaction can be evaluated.[138] Examination of individual profiles gives information on the contents of different types of particles and their degree of degradation, as well as their enclosing membranes.[137,139] By combining the quantitative biochemical and morphological methods described above, it is possible to show that most of the populations of certain marker enzymes and specific cellular organelles have similar sedimentation properties, confirming the location of these enzymes.[137,148] Furthermore, these approaches permit the identification of compartments such as autophagosomes and autolysosomes in the same subcellular fraction without cytochemistry.[139]

Cautionary notes—When isolating organelles from tissues and cells in culture it is essential to use disruption methods that do not alter the membrane of lysosomes and autophagosomes, compartments that are particularly sensitive to some of those procedures. For example teflon/glass motor homogenization is suitable for tissues with abundant connective tissue, such as liver, but for circulating cells or cells in culture, disruption by nitrogen cavitation is the best method to preserve lysosomal membrane stability.[149] During the isolation procedure it is essential to always use iso-osmotic solutions (e.g., 0.25 M sucrose) to avoid hypotonic or hypertonic disruption of the organelles. In that respect, because lysosomes are able to take up sucrose if it is present at high concentrations, the use of sucrose gradients for the isolation of intact lysosome-related organelles is strongly discouraged. Other density media such as Nycodenz, metrizamide and Percoll, cannot be transported inside lysosomes and subsequently are more suitable for their isolation.

As with the isolation of any other intracellular organelle, it is essential to assess the purity of each preparation, as there is often considerable variability from experiment to experiment due to the many steps involved in the process. Correction for purity can be done through calculation of recovery (percentage of the total activity present in the homogenate) and enrichment (multiplying by the specific activity in the homogenate) of enzymes or protein markers for those compartments (e.g., β-hexosaminidase is routinely used to assess lysosomal purity).

[149] Along these lines, it is essential to keep a balance sheet when using markers in biochemical studies of autophagy. This is necessary to insure that all marker activity is accounted for and that excessive damage to particles of interest has not occurred. Because of the time-consuming nature of quantitative morphological studies, such studies should not be carried out until simpler biochemical procedures have established the circumstances most likely to give meaningful morphometric/stereological results.

Finally, it is worthwhile noting that not all lysosomes are alike. For example, as noted above, there are differences among primary lysosomes, autolysosomes and telolysosomes. Furthermore, what we refer to as "lysosomes" are actually a very heterogeneous pool of organelles that simply fulfill five classical criteria, having a pH <5.6, cleaved cathepsins, the presence of LAMP proteins, a single membrane, and the absence of endosomal and recycling compartment markers (e.g., the mannose-6-phosphate receptor or Rab5). But even applying those criteria we can separate lysosomes with clear differences in their proteome and other properties, and these distinct populations of lysosomes are likely to participate in different functions in the cell.[150]

11. Analyses in vivo

Monitoring autophagic flux in vivo or in organs is one of the least developed areas at present, and ideal methods relative to the techniques possible with cell culture may not exist. One of the most useful methods is the analysis of GFP-LC3/Atg8 (see *Fluorescence microscopy* above). The morphological detection of autophagic structures (e.g., autophagosomes) by fluorescence or electron microscopy is one approach, although there are practical limits to the number of sections that can be prepared and analyzed. Extensive autophagy can result in obvious changes in cell morphology and the elimination of sufficient amounts of cytoplasm to cause cells to appear "clear."[151,152] Another useful approach is immunohistochemical staining, an important procedure considering the role of autophagy in protecting against certain neurodegenerative diseases, and its potential roles in myopathies, heart disease and the response to ischemia/reperfusion, where samples may be limited to biopsies; however, this methodology has not received extensive evaluation, and does not lend itself well to dynamic assays. One method that has been used to monitor autophagy in myocytes is the detection of granular ubiquitin inclusions in the cytosol;[46–48] however, it is important to note that the presence of such inclusions may actually indicate decreased autophagy or autophagic flux. Along these lines, it is worth noting that immunodetection of LC3 is also possible using tissue sections.[153] It is likely, however, that in vivo analyses will be relatively complex. For example, in skeletal muscle the induction of autophagy by starvation appears to occur more actively in very young animals (e.g., in 4 to 5 week old mice) than in more elderly organisms (e.g., in four month old mice) (Raben N, personal communication).

Some biochemical assays may be used to at least provide indirect correlative data relating to autophagy, in particular when examining the role of autophagy in cell death. For example, cellular viability is related to high cathepsin B activity and low cathepsin D.[154] Therefore, the appearance of the opposite levels of activities may be one indication of the initiation of autophagy (lysosome)-dependent cell death. The question of "high" versus "low" activities can be determined by comparison to the same tissue under control conditions, or to a different tissue in the same organism, depending on the specific question. Finally, certain molecular biology analyses are also possible, such as the detection of some cytokeratins that appear under autophagic conditions.[152,155]

With regard to living animals, a minimally invasive method that may be used even in humans is to measure the arteriovenous amino acid exchange rate in the peripheral tissues as a measure of post-absorptive protein catabolism. In humans, the insulin- and amino acid-sensitive postabsorptive (autophagic) net protein catabolism in the peripheral (mostly skeletal muscle)

tissue can be conveniently measured by determining the amino acid exchange rate across the lower extremities, as defined by the difference between the plasma amino acid concentrations in the femoral artery and femoral vein multiplied by the blood flow.[156–158] Amino acid exchange studies have shown that the peripheral tissues take up amino acids during the post-prandial (fed) state and release amino acids in the postabsorptive (fasted) state, i.e., in a state with relatively low plasma insulin and amino acid levels. This postabsorptive release of amino acids is strongly inhibited by infusion of insulin or by exogenous supply of amino acids suggesting that it is mainly mediated by a lysosomal/autophagic mechanism of protein catabolism.[156–163]

Finally, to obtain flux data it is necessary to include a time course parameter to follow changes in substrate accumulation. An example of this approach is seen with the study of *Drosophila melanogaster blue cheese* mutants, which accumulate ubiquitin-positive inclusions in a time-dependent manner.[164]

Cautionary notes—One caution in using approaches that monitor ubiquitinated aggregates is that the accumulation of ubiquitin may indicate a block in autophagy, inhibition of proteasomal degradation, or may correspond to structural changes in the substrate proteins that hinder their degradation. In addition, only cytosolic and not nuclear ubiquitin is subject to autophagic degeneration. When analyzing cathepsin D, it is advisable to use both western blots and activity assays; activity measurements alone can be misleading because procathepsin D is also active. In addition, it is important to realize that the level of mature cathepsin D is usually lower than expected in tissue that is undergoing autophagy; procathepsin D is matured in lysosomes, and extensive vacuolization resulting from autophagy interferes with trafficking of the enzyme through the endosome (Coto-Montes A, personal communication). Therefore, indirect measures of autophagy may be a higher ratio of procathepsin D to cathepsin D, or an alteration in the cathepsin B:cathepsin D activity ratio (potentially indicating the onset of autophagic cell death).

C. Methods Warranting Special Caution

1. Acidotropic dyes

One of the most frequently used methods for following autophagy is staining with acidotropic dyes such as monodansylcadaverine (MDC),[165] acridine orange,[166] LysoSensor Blue[167] and LysoTracker Red.[44]

Cautionary notes—Although MDC was first described as a specific marker of autophagic vacuoles[168] subsequent studies have suggested that this, and other acidotropic dyes, are not specific markers for early autophagosomes,[79] but rather label later stages in the degradation process. For example, autophagosomes are not acidic, and MDC staining can be seen in autophagy-defective mutants[38] and in the absence of autophagy activation.[169] MDC may also show confounding levels of background labeling unless narrow bandpass filters are used. On the other hand, in the presence of vinblastine, which blocks fusion with lysosomes, MDC labeling increases, suggesting that under these conditions MDC can label late stage autophagosomes.[170] Along these lines, cells that overexpress a dominant negative version of Rab7 (the T22N mutant) show colocalization of this protein with MDC; in this case fusion with lysosomes is also blocked[171] indicating that MDC does not just label lysosomes. Finally, MDC labeling is blocked by treatment with the autophagy inhibitors wortmannin or 3-MA.

Overall, staining with MDC or its derivative monodansylpentane (MDH) is not, by itself, a sufficient method for monitoring autophagy. Similarly, LysoTracker Red and acridine orange are not ideal markers because they primarily detect lysosomes. For example, LysoTracker Red has been used to provide correlative data on autophagy in *Drosophila melanogaster* fat body

cells (Fig. 10).[43,44] However, additional assays, such as GFP-Atg8/LC3 fluorescence or EM, should be used to substantiate results obtained with acidotropic dyes whenever possible.

Some of the confusion regarding the interpretation of results with these dyes stems in part from the nomenclature in this field. Indeed, the discussion of acidotropic dyes points out why it is advisable to differentiate between the terms "autophagosome" and "autophagic vacuole," although they are occasionally, and incorrectly, used interchangeably. The autophagosome is the sequestering compartment generated by the phagophore. The fusion of an autophagosome with an endosome or a lysosome generates an amphisome or an autolysosome, respectively. The early autophagosome is not an acidic compartment, whereas amphisomes and autolysosomes are acidic. Archaic names for these compartments are "initial autophagic vacuole (AVi)," "intermediate autophagic vacuole (AVi/d)" and "degradative autophagic vacuole (AVd)," respectively. Thus, acidotropic dyes can stain late autophagic vacuoles, but not the initial autophagic vacuole, the early autophagosome. With the above caveats in mind, the combined use of early and late markers of autophagy is highly encouraged, and when quantifying mammalian lysosomes, it is important to keep in mind that increases in both lysosome size and number are frequently observed. Finally, in order to avoid confusion with the plant and fungal vacuole, the equivalent organelle to the lysosome, we recommend the use of the term "autophagosome" instead of "autophagic vacuole," and the use of "autophagic compartment" when the specific nature of the structure is not known.

2. Autophagy inhibitors and inducers

In many situations it is important to demonstrate an effect resulting from inhibition or stimulation of autophagy (see ref. [172] for a partial listing of regulatory compounds), and a few words of caution are worthwhile in this regard. Most chemical inhibitors of autophagy are not entirely specific, and it is preferable to analyze specific loss-of-function *Atg* mutants. However, it must be kept in mind that some apparently specific *Atg* gene products may have autophagy-independent roles (e.g., Atg5 in cell death). Therefore, the experimental conditions of inhibitor application and their side effects must be carefully considered. In addition, it must be emphasized once again that autophagy, as a multi-step process, can be inhibited at different stages. Sequestration inhibitors include for example, 3-MA, LY294002 and wortmannin, which inhibit class I as well as class III phosphatidylinositol 3-kinases.[132,173] The class I enzymes generate products that inhibit autophagic sequestration, whereas the class III products generally stimulate autophagic sequestration. The overall effect of these inhibitors is typically to block autophagy because the class III enzymes that are required to activate autophagy act downstream of the negative regulatory class I enzymes, although cell death may ensue in cell types that are dependent upon high levels of protein kinase B for survival. Although ineffective in isolated hepatocytes,[174] cycloheximide is a well established inhibitor of sequestration in vivo,[131,133,134,175–181] and in certain cell types in vitro,[182] and it has been utilized to investigate the dynamic nature of the regression of various autophagic elements.[132–134, 176–179] The mechanism of action of cycloheximide in short term experiments is not clear, but it has no direct relation to the inhibition of protein synthesis.[174]

Most other inhibitory drugs act at post-sequestration steps. These types of agents have been used in many experiments to both inhibit endogenous protein degradation and to increase the number of autophagic compartments. They cause the accumulation of sequestered material in either autophagosomes or autolysosomes, or both, because they allow autophagic sequestration to proceed. The main categories of these types of inhibitors include the vinca alkaloids (e.g., vinblastine) and other microtubule poisons that inhibit fusion, inhibitors of lysosomal enzymes (e.g., leupeptin, pepstatin A and E64d), and compounds that elevate lysosomal pH (e.g., inhibitors of vacuolar-type ATPases such as bafilomycin A_1, and weak base amines including ammonia, methyl- or propylamine, chloroquine, and Neutral Red, some of which slow down

fusion). It is worth noting that lysosomal proteases fall into the three general groups (cysteine, aspartic acid and serine). Therefore, the fact that leupeptin, a serine and cysteine protease inhibitor, has little or no effect does not necessarily indicate that lysosomal degradation is not taking place; a combination of leupeptin, pepstatin and E64d may be a more effective treatment.

As with the phosphatidylinositol 3-kinase inhibitors, many of these autophagy compounds are not specific. For example, okadaic acid is a powerful inhibitor of type 1 (PP1) and type 2A (PP2A) protein phosphatases.[183] Bafilomycin A_1 and other compounds that raise the lysosomal pH may have indirect effects on any acidified compartments. Thus, although these various agents can inhibit different steps of the autophagic pathway, their potential side effects must be considered in interpretation of the secondary consequences of autophagy inhibition, especially in long-term studies. For example, lysosomotropic compounds can increase the rate of autophagosome formation by inhibiting TORC1 (Høyer-Hansen M, unpublished observation). Along these lines, chloroquine treatment may cause an apparent increase in the formation of autophagosomes possibly by blocking fusion with the lysosome (Dorsey FC, Cleveland JL, personal communication). Furthermore, in addition to causing the accumulation of autophagic compartments, many of these drugs seem to be stimulators of sequestration in many cell types, especially in vivo.[78,131,177,182,184–189] Although it is clear why these drugs cause the accumulation of autophagic compartments, it is not known why they stimulate sequestration. One possibility, at least for hepatocytes, is that the inhibition of protein degradation reduces the intracellular amino acid pool, which in turn upregulates sequestration. A time-course study of the changes in both the intra- and extracellular fractions may provide accurate information regarding amino acid metabolism. For these various reasons, it is important to include appropriate controls; along these lines, rapamycin or amino acid deprivation can be utilized as positive controls for inducing autophagy. In many cell types, however, the induction of autophagy by rapamycin is relatively slow, allowing more time for indirect effects. Finally, it has recently been shown that a specialized class of compounds with α, β-unsaturated ketone structure tends to induce autophagic cell death, accompanied by changes in mitochondrial morphology.[190] Due to the potential pleiotropic effects of various drug treatments, it is incumbent upon the researcher to demonstrate that autophagy is indeed inhibited, by using the methodologies described herein.

The use of gene deletions (e.g., in primary or immortalized *Atg*$^{-/-}$ MEFs,[38] or in vivo using transgenic knockout models[191,192] including Crelox based “conditional” knockouts[74,75]) or functional knockdowns (e.g., with RNAi) is the preferred approach when possible because these methods allow a more direct assessment of the resulting phenotype. In certain contexts, it is advisable when using a knockout or knockdown approach to examine multiple autophagy-related genes to exclude the possibility that the phenotype observed is due to effects on a non-autophagic function(s) of the corresponding protein, especially when examining the possibility of autophagic cell death (in contrast, if examining whether perturbation induces clearance of a substrate via autophagy, a single Atg gene knockout is probably sufficient). This is particularly the case in evaluating Beclin 1, which interacts with anti-apoptotic Bcl-2 family proteins,[193] or when low levels of a target are sufficient for maintaining autophagy as is the case with Atg5.[111] Along these lines, and as stated above for the use of inhibitors, when employing a knockout or especially a knockdown approach, it is again incumbent upon the researcher to demonstrate that autophagy is actually inhibited, by using the methodologies described herein. Finally, we note that the long-term secondary consequences of gene knockouts or knockdowns are likely much more complex than the immediate effects of the actual autophagy inhibition. To overcome this concern, tetracycline-regulated reversible *Atg5* knockout cells might be useful.[111] Another strategy to specifically interfere with autophagy is to use dominant negative inhibitors. Delivery of these agents by transient transfection, adenovirus, or TAT-mediated protein transduction offers the possibility of their use in cell culture or in vivo.[194] However, since autophagy is an essential metabolic process

for many cell types and tissues, loss of viability due to autophagy inhibition always has to be a concern when analyzing cell death-unrelated questions.

There are fewer compounds that act as inducers of autophagy, but the initial characterization of this process was due in large part to the inducing effects of glucagon, which appears to act through indirect inhibition of mTOR via the activation of LKB1-AMPK.[140,141,195] Currently, the most commonly used and specific inducer of autophagy is rapamycin, which directly inhibits mTOR.[84,196–198] TOR-independent regulation can be achieved through lithium, sodium valproate and carbamezapine, compounds that lower the myo-inositol-1,4,5-triphosphate levels.[199] Relatively little is known about direct regulation via the Atg proteins, but there is some indication that tamoxifen acts to induce autophagy by increasing the expression of Beclin 1.[200] Finally, new screens have identified small molecules that induce autophagy independently of rapamycin, and allow the removal of misfolded or aggregateprone proteins,[201,202] suggesting that they may prove useful in therapeutic applications.

3. Experimental systems

Throughout these guidelines we have noted that it is not possible to state explicit rules that can be applied to all experimental systems. For example, some techniques may not work in particular cell types or organisms. In some cases this must be empirically determined, which is one reason why it is important to include appropriate controls. Differences may also be seen between in vivo or perfused organ studies and cell culture analyses. For example, insulin has no effect on proteolysis in suspended rat hepatocytes, in contrast to the result with perfused rat liver. The insulin effect reappears, however, when isolated hepatocytes are incubated in stationary dishes[203,204] or are allowed to settle down on the matrix (Häussinger D, personal communication). The reason for this might be that autophagy regulation by insulin and some amino acids requires volume sensing via integrin-matrix interactions and also intact microtubules.[205–207] Therefore, it is important to keep in mind that results from one particular system may not be generally applicable to others.

Conclusions and future perspectives

In conclusion, we suggest a set of recommended methods for monitoring macroautophagy in higher eukaryotes (Table 1). Importantly, investigators need to determine whether they are evaluating autophagosome levels or autophagic flux. If the question being asked is whether a particular condition changes autophagic flux (i.e., the rate of delivery of autophagy substrates to lysosomes or the plant vacuole, followed by degradation), then assessment of steady state levels of autophagosomes (e.g., by counting GFP-LC3 puncta, monitoring the amount of LC3-II without examining turnover, or by electron microscopy) is not sufficient as an isolated approach. In this case it is also necessary to directly measure the flux of autophagosomes and/or autophagy cargo (e.g., in wild-type cells compared to autophagy-deficient cells, the latter generated by treatment with an autophagy inhibitor or resulting from *Atg* gene knockdowns). Collectively, we strongly recommend the use of multiple assays whenever possible, rather than relying on the results from a single method.

As a final reminder, we stated at the beginning of this review that this set of guidelines is not meant to be a formulaic set of rules, because the appropriate assays depend in part on the question being asked and the system being used. Rather, these guidelines are presented primarily to emphasize key issues that need to be addressed such as the difference between measuring autophagy components, and flux or substrate clearance; they are not meant to constrain imaginative approaches to monitor autophagy. Hopefully, new methods for monitoring autophagy will continue to be developed, and new findings may alter our view of the current assays. For example, one area that shows promise is the use of nanoparticles as tools for monitoring autophagy,[208] as they could be used in EM (e.g., providing contrast by

using different sizes and shapes of nanoparticles) or to follow autophagic flux in living cells (e.g., relying on the stable fluorescence of quantum dots) allowing the tracking of autophagosomes and amphisomes. Similar to the process of autophagy, this is a dynamic field, and we need to remain flexible in the standards we apply.

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Glossary

Amphisome

Intermediate compartment formed by the fusion of an autophagosome with an endosome; this compartment has not yet fused with a lysosome (also referred to as an acidic late autophagosome)

Autolysosome/autophagolysosome

A degradative compartment formed by the fusion of an autophagosome or amphisome with a primary lysosome or telolysosome. Upon completion of degradation, or when degradation has reached an end point, this compartment (again) becomes a telolysosome (also referred to as a residual body)

Autophagosome

A cytosolic membrane bound compartment typically denoted by a limiting double membrane. The early autophagosome in particular contains cytoplasmic components and organelles that are morphologically unchanged because the compartment has not fused with a lysosome and lacks proteolytic enzymes

Autophagy

A collection of processes typically involving degradative delivery of a portion of the cytoplasm to lysosomes or the plant or fungal vacuole that does not involve direct transport through the endocytic or vacuolar protein sorting, Vps, pathways

Chaperone-mediated autophagy (CMA)

Import and degradation of soluble cytosolic proteins by chaperone-dependent, direct translocation across the lysosomal membrane

Cytoplasm to vacuole targeting (Cvt)

A biosynthetic pathway in fungi that transports resident hydrolases to the vacuole through a selective autophagy-related process

Lysosome

A degradative organelle in higher eukaryotes that compartmentalizes a range of hydrolytic enzymes and maintains a highly acidic pH. A primary lysosome is a relatively small compartment that has not yet participated in a degradation process, whereas secondary lysosomes are sites of present or past digestive activity. The secondary lysosomes include autolysosomes and telolysosomes. Autolysosomes/early secondary lysosomes are larger compartments actively engaged in digestion, whereas telolysosomes/late secondary lysosomes do not have significant digestive activity and contain residues of previous digestions. Both may contain material of either autophagic or heterophagic origin

Macroautophagy

The largely nonspecific autophagic sequestration of cytoplasm into a double- or multiple-membrane-delimited compartment (an autophagosome) of nonlysosomal/vacuolar origin and its subsequent degradation by the lysosomal system. Note that certain proteins and organelles may be selectively degraded via a macroautophagy-related process, and conversely, some cytosolic components such as cytoskeletal elements are selectively excluded

Microautophagy

Uptake and degradation of cytoplasm by protrusion, invagination or septation of the lysosome or vacuole membrane

Mitophagy

The selective autophagic sequestration and degradation of mitochondria

Pexophagy

A selective type of autophagy involving the sequestration and degradation of peroxisomes; can occur by a micro- or macroautophagic process

Phagophore

Membrane cisterna that has been implicated in an initial event during formation of the autophagosome. Also referred to as the “isolation membrane.”

Phagophore assembly site (PAS)

A perivacuolar compartment or location that is involved in the formation of Cvt vesicles and autophagosomes in yeast. The PAS may supply membranes during the formation process or may be an organizing center where most of the autophagic machinery resides, at least transiently

Phosphatidylinositol 3-kinase

A family of enzymes that add a phosphate group to the 3′ hydroxyls on the inositol ring of phosphoinositides. The class III phosphatidylinositol 3-kinases are stimulatory for autophagy, whereas class I enzymes are inhibitory

Programmed cell death (PCD)

Regulated self-destruction of a cell. Type I is associated with apoptosis and is marked by cytoskeletal breakdown and condensation of cytoplasm and chromatin followed by fragmentation. Type II is associated with autophagy and is characterized by the presence of enhanced levels of autophagosomes. Type III is marked by the absence of condensation, and does not involve the lysosomal system but rather is proteasome-dependent

Vacuole

The fungal and plant equivalent of the lysosome; this organelle also carries out storage and osmoregulatory functions

Xenophagy

The selective degradation of microbes (e.g., bacteria, fungi, parasites and/or viruses) through an autophagy-related mechanism

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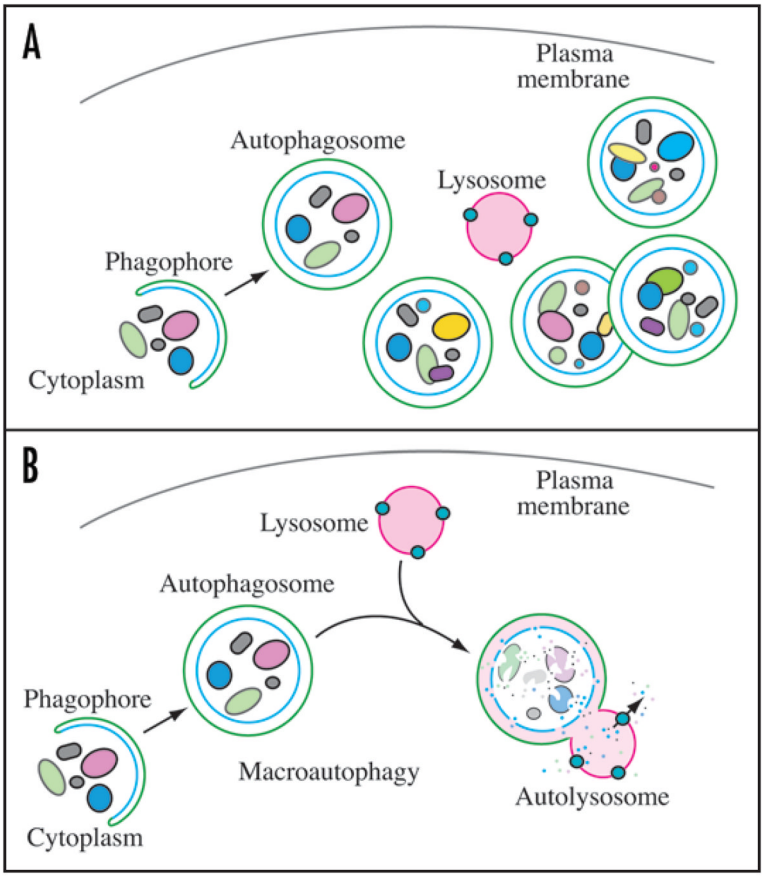

Figure 1.
Schematic model demonstrating the induction of autophagosome formation when turnover is blocked versus normal autophagic flux. (A) Induction results in the initiation of autophagy including the formation of the phagophore, the initial sequestering compartment, which expands into an autophagosome. A defect in autophagosome turnover due, for example, to a block in fusion with lysosomes or disruption of lysosomal functions will result in an increased number of autophagosomes. In this scenario, autophagy has been induced, but there is no or limited autophagic flux. This is a different outcome than the situation shown in (B) where autophagosome formation is followed by fusion with lysosomes and degradation of the contents, allowing complete flux, or flow, through the entire pathway.

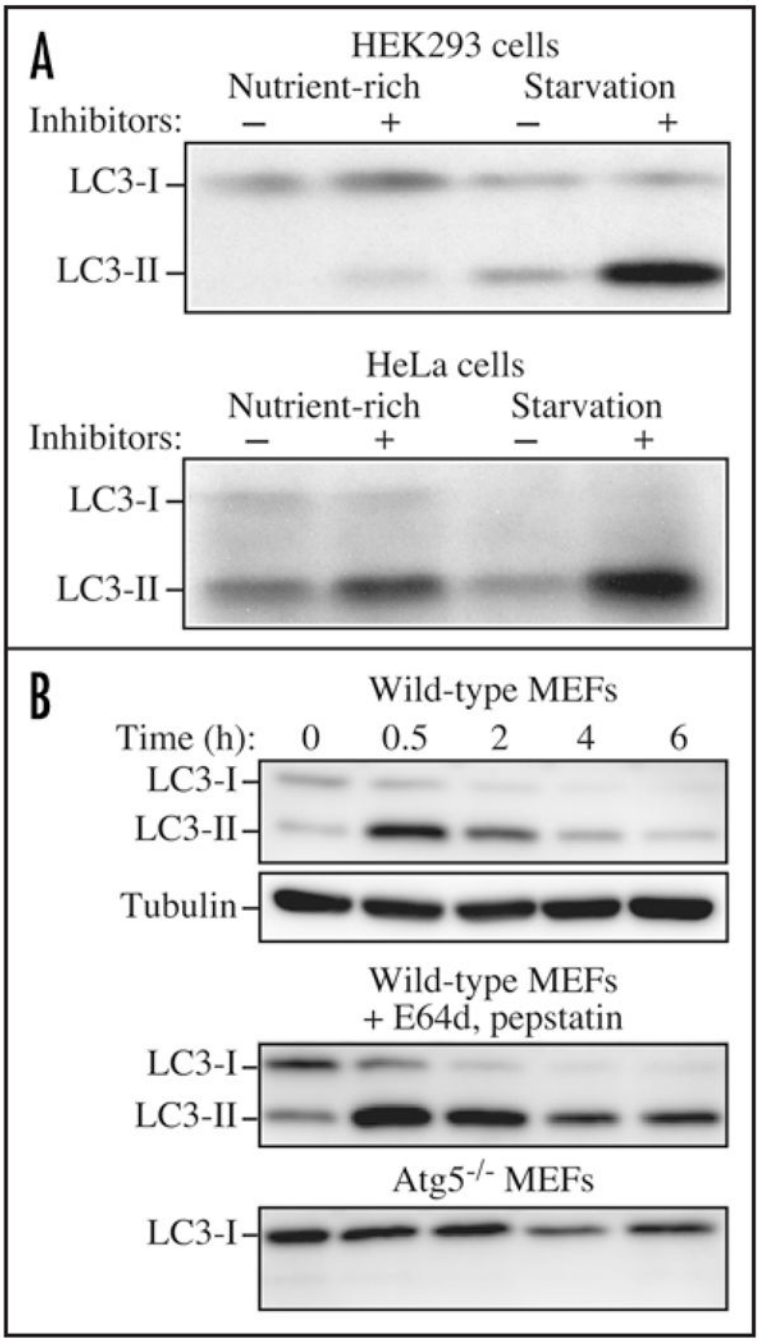

Figure 2.

LC3-I conversion and LC3-II turnover. (A) HEK293 and HeLa cells were cultured in nutrient-rich medium (DMEM containing 10% FCS) or incubated for 4 h in starvation conditions (Krebs-Ringer medium) in the absence (−) or presence (+) of E64d and pepstatin at 10 μg/ml each (Inhibitors). Cells were then lysed and the proteins resolved by SDS-PAGE. Endogenous LC3 was detected by immunoblotting. Positions of LC3-I and LC3-II are indicated. In the absence of lysosomal protease inhibitors, starvation results in a modest increase (HEK293 cells) or even a decrease (HeLa cells) in the amount of LC3-II. The use of inhibitors reveals that this apparent decrease is due to lysosome-dependent degradation. This figure was modified from data previously published in reference [31], and is reproduced by permission of Landes

Bioscience, copyright 2005. (B) Expression levels of LC3-I and LC3-II during starvation. *Atg5*$^{+/+}$ (wild-type) and *Atg5*$^{-/-}$ MEFs were cultured in DMEM without amino acids and serum for the indicated times, and then subjected to immunoblot analysis using anti-LC3 antibody and anti-tubulin antibody. E64d (10 μg/ml) and pepstatin A (10 μg/ml) were added to the medium where indicated. Positions of LC3-I and LC3-II are indicated. Similar to the result in (A), the inclusion of lysosomal protease inhibitors reveals that the apparent decrease in LC3-II is due to lysosomal degradation as easily seen by comparing samples with and without inhibitors at the same time points (the overall decrease seen in the presence of inhibitors may reflect decreasing effectiveness of the inhibitors over time). Monitoring autophagy by following steady state amounts of LC3-II without including inhibitors in the analysis can result in an incorrect interpretation that autophagy is not taking place (due to the apparent absence of LC3-II). Conversely, if there are high levels of LC3-II but there is no change in the presence of inhibitors this may indicate that induction has occurred but that the final steps of autophagy are blocked, resulting in stabilization of this protein. This figure was modified from data previously published in reference [33], and is reproduced by permission of Landes Bioscience, copyright 2007.

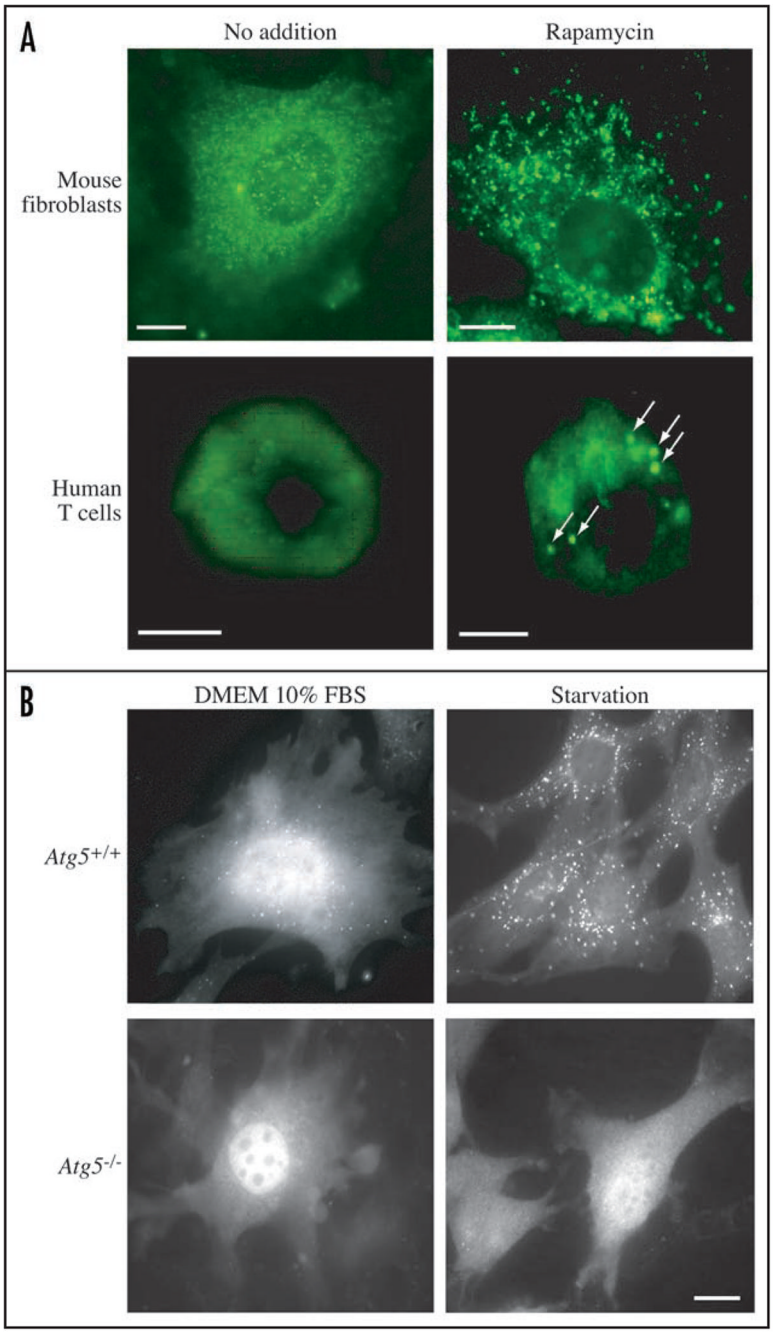

Figure 3.

Changes in the localization of LC3 and GFP-LC3 upon the induction of autophagy. (A) Immunofluorescence in mouse fibroblasts and human T cells. The indicated cells were left untreated or were treated with 100 μM rapamycin for 4 h and were subjected to immunofluorescence with a selective antibody against LC3. LC3-stained autophagic compartments in T cells are indicated with arrows. Quantification of 20 cells similar to the ones shown here indicated that rapamycin-treated cells had 165 ± 8 vesicles per fibroblast and 6 ± 2 vesicles per T cell. Bar, 5 μm. This figure was previously published in reference [2], and is reproduced by permission of Landes Bioscience, copyright 2007. (B) Direct fluorescence in stable MEF transformants. GFP-LC3-expressing *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ MEFs were cultured in

DMEM with 10% FBS or DMEM without amino acids and serum for 1.5 h. Cells were fixed with 3% PFA and analyzed by fluorescence microscopy. Bar, 20 μm. This figure was previously published in reference [69], and is reproduced by permission of Landes Bioscience, copyright 2007.

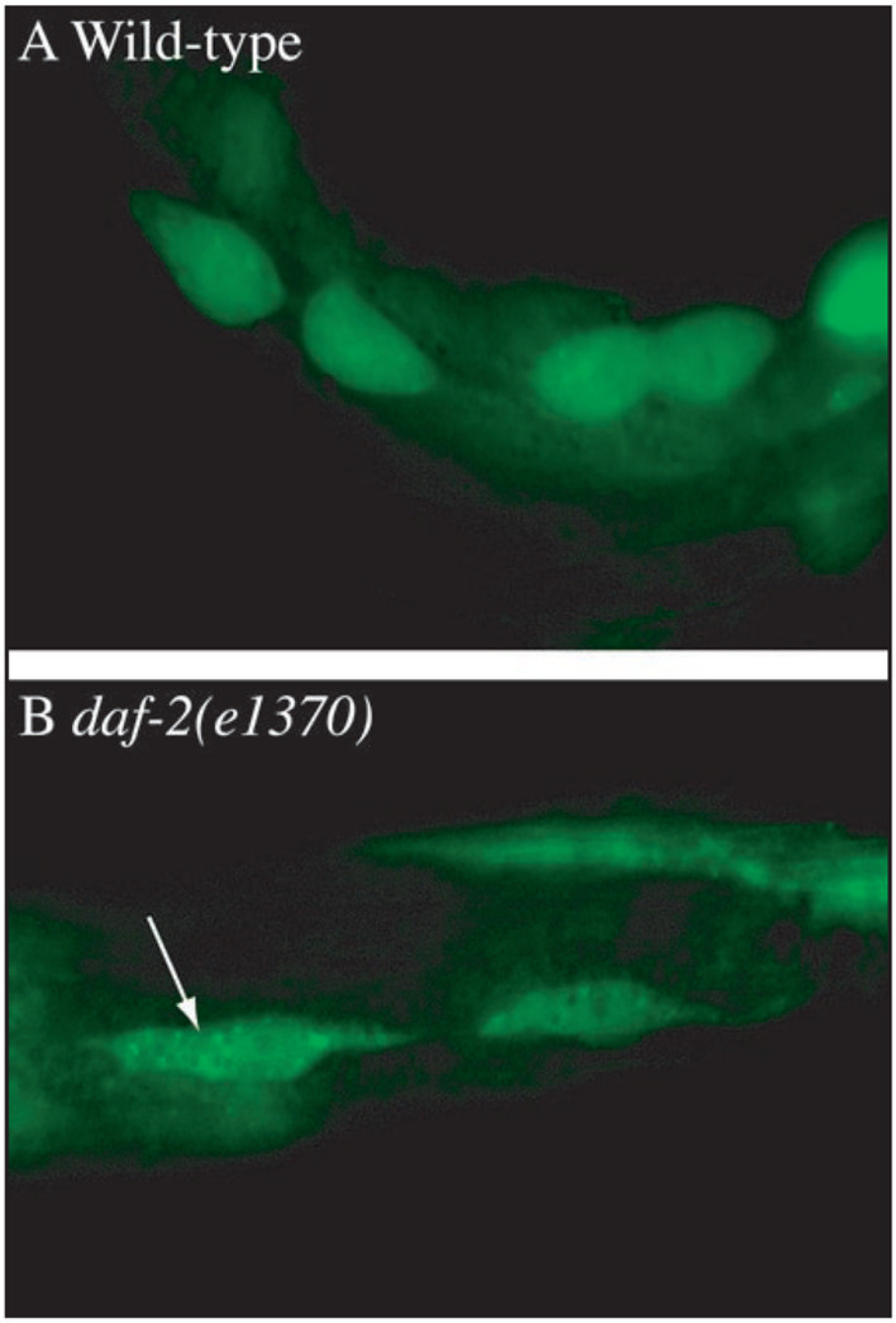

Figure 4.
GFP::LGG-1 is an autophagy marker in *C. elegans*. GFP::LGG-1 expression in the hypodermal seam cells of (A) wild-type N2 animals and (B) *daf-2(e1370)* animals that have an increase in autophagy. The arrow shows representative GFP-positive punctate areas that label pre-autophagosomal and autophagosomal structures. This figure was modified from data previously published in Meléndez A, Tallóczy Z, Seaman M, Eskelinen E-L, Hall DH, Levine B. Autophagy genes are essential for dauer development and life-span extension in *C. elegans*. Science 2003; 301:1387–91. Reprinted with permission from AAAS.

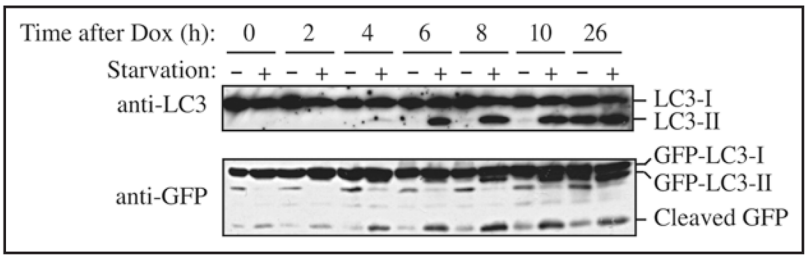

Figure 5.

GFP-LC3 processing can be used to monitor delivery of autophagosomal membranes. *Atg5*$^{-/-}$ MEFs engineered to express Atg5 under the control of the Tet-off promoter were grown in the presence of doxycyline (10 ng/ml) for one week to suppress autophagy. Cells were then cultured in the absence of drug for the indicated times, with or without a final 2 h starvation. Protein lysates were analyzed by western blot using anti-LC3 and anti-GFP antibodies. The positions of GFP-LC3-I, GFP-LC3-II and free GFP are indicated. This figure was modified from data previously published in reference [111], FEBS Letters, 580, Hosokawa N, Hara Y, Mizushima N, Generation of cell lines with tetracycline-regulated autophagy and a role for autophagy in controlling cell size, pp. 2623–9, copyright 2006, with permission from Elsevier.

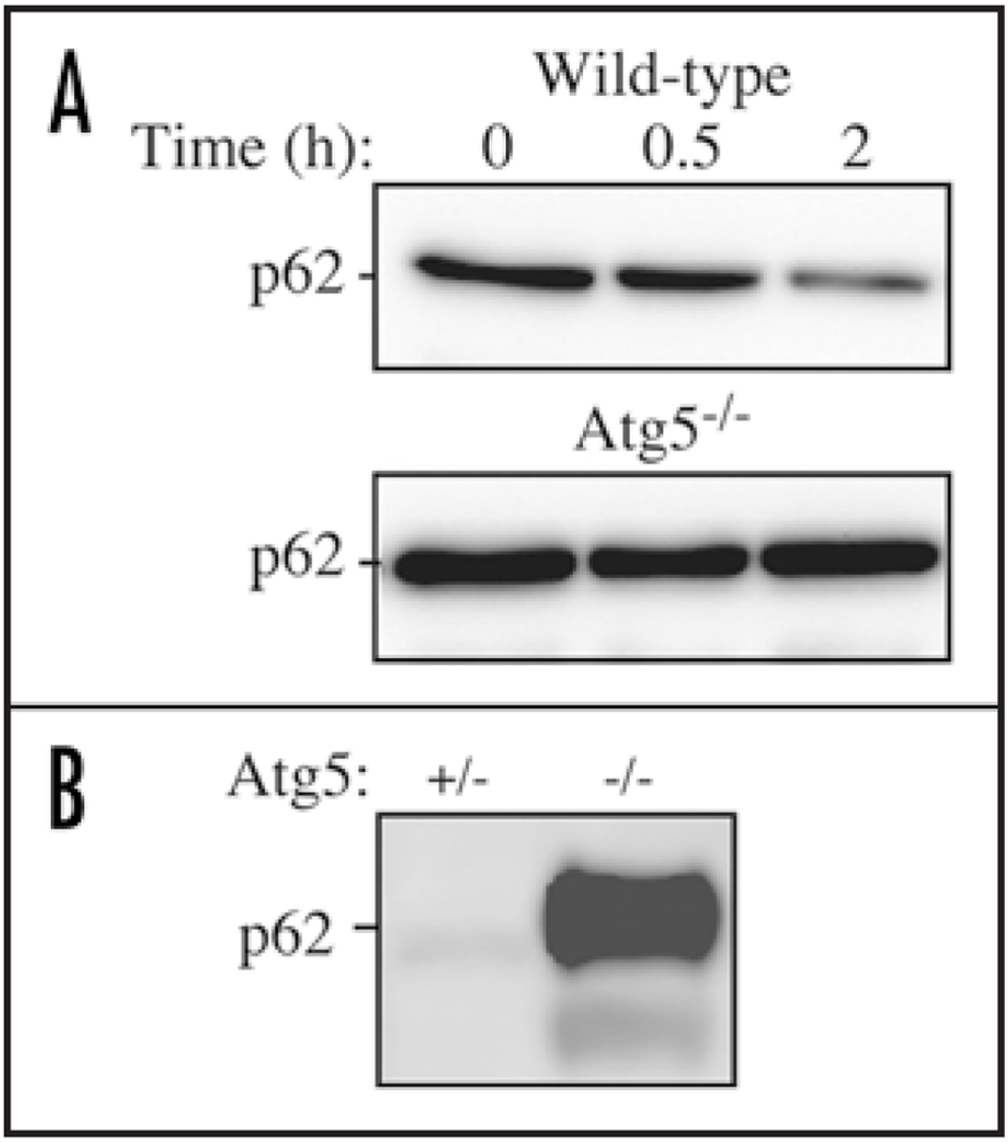

Figure 6.

Regulation of the p62 protein during autophagy. (A) The level of p62 during starvation. *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ MEFs were cultured in DMEM without amino acids and serum for the indicated times, and then subjected to immunoblot analysis using anti-p62 antibody (Progen Biotechnik). This figure was previously published in reference [33], and is reproduced by permission of Landes Bioscience, copyright 2007. (B) The level of p62 in the brain of neural-cell specific Atg5 knockout mice. This image was generously provided by Dr. Taichi Hara (Tokyo Medical and Dental University).

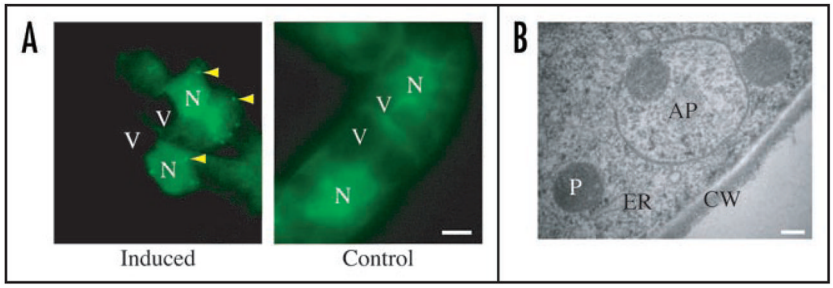

Figure 7.

Detection of macroautophagy in tobacco BY-2 cells. (A) Induction of autophagosomes in tobacco BY-2 cells expressing YFP-NtAtg8 (shown in green for ease of visualization) under conditions of nitrogen limitation (Induced). Arrowheads indicate autophagosomes that can be seen as a bright green dot. No such structure was found in cells grown in normal culture medium (Control). Bar, 10 μm. N, nucleus; V, vacuole. (B) Ultrastructure of an autophagosome in a tobacco BY-2 cell cultured for 24 h without a nitrogen source. Bar, 200 μm. AP, autophagosome; P, plastid; CW, cell wall. This image was provided by Dr. Kiminori Toyooka (RIKEN Plant Science Center).

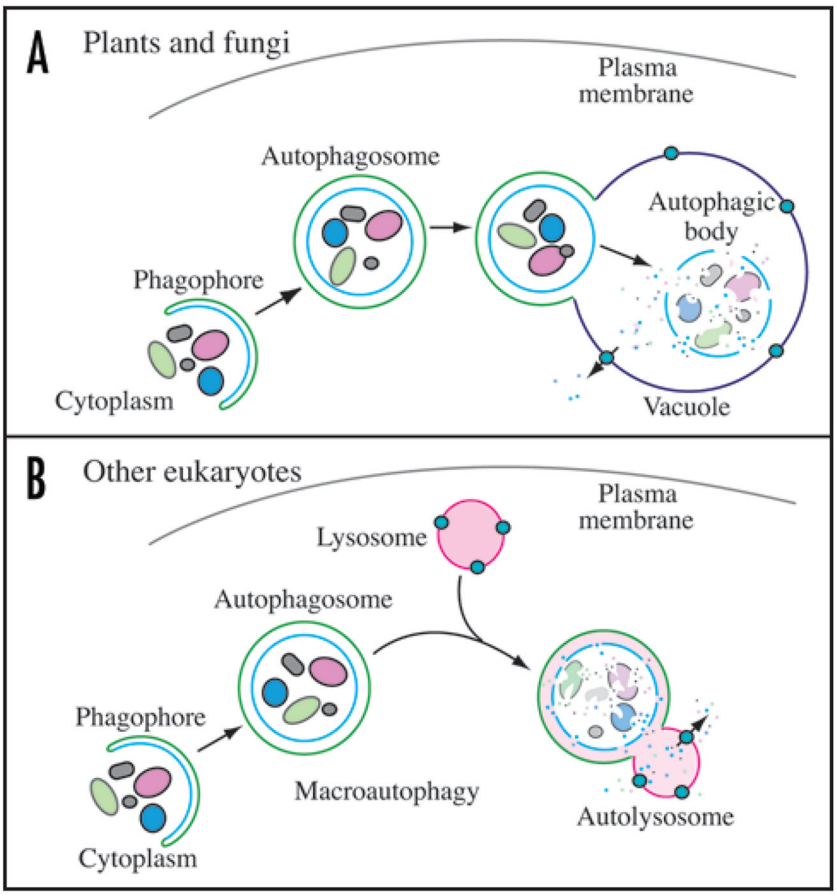

Figure 8.

Schematic drawing showing the formation of an autophagic body in plants and fungi. The large size of the plant and fungal vacuole relative to autophagosomes allows the release of the single-membrane autophagic body within the vacuole lumen. In cells that lack vacuolar hydrolase activity, or in the presence of inhibitors that block hydrolase activity, intact autophagic bodies accumulate within the vacuole lumen and can be detected by light microscopy. The lysosome of most higher eukaryotes is too small to allow the release of an autophagic body.

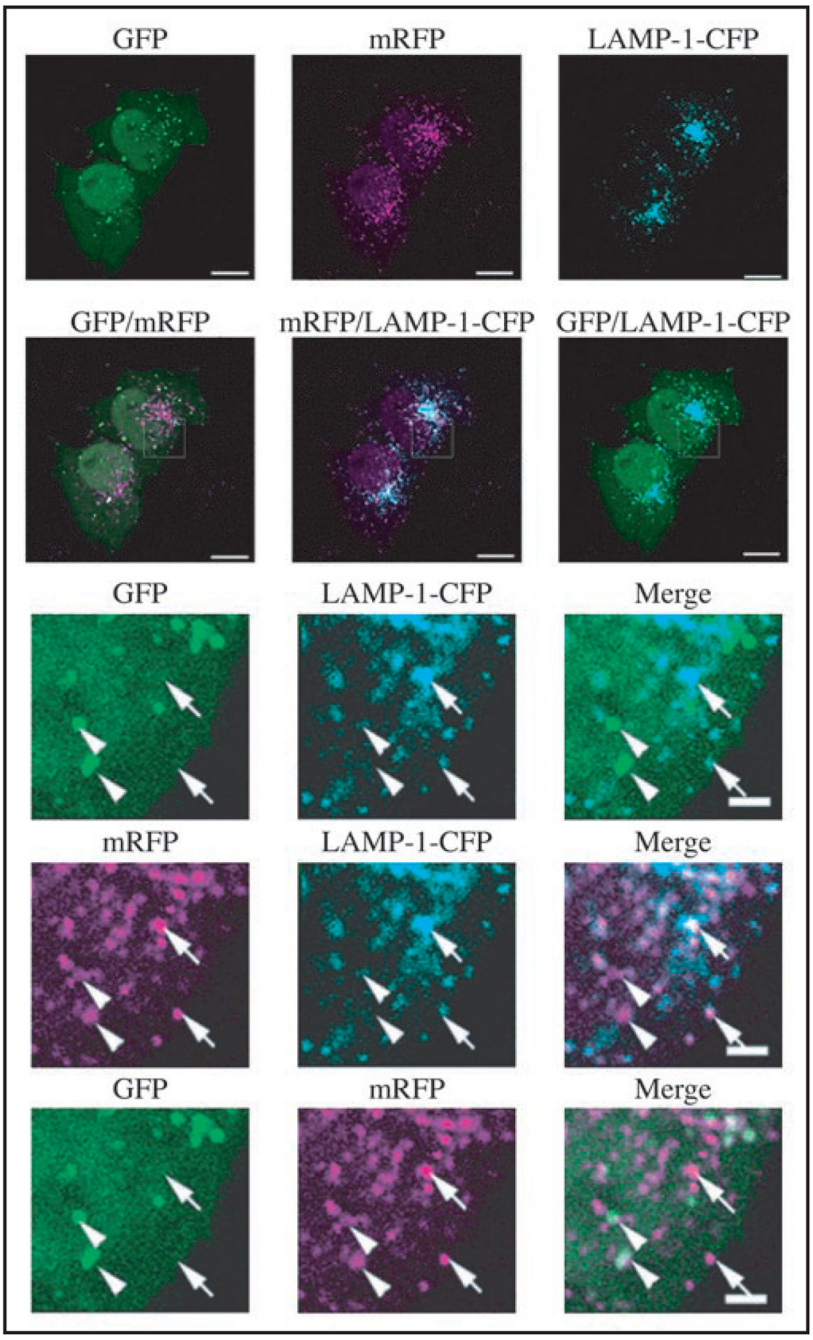

Figure 9.

The GFP and mRFP signals of tandem fluorescent LC3 (tfLC3, mRFP-GFP-LC3) show different localization patterns. HeLa cells were cotransfected with plasmids expressing either tfLC3 or LAMP-1-CFP. Twenty-four hours after the transfection, the cells were starved in Hanks' solution for 2 hours, fixed and analyzed by microscopy. The lower panels are a higher magnification of the upper panels. Bar, 10 μm in the upper panels and 2 μm in the lower panels. Arrows in the lower panels point to (or mark the location of) typical examples of colocalized signals of mRFP and LAMP-1. Arrowheads point to (or mark the location of) typical examples of colocalized particles of GFP and mRFP signals. This figure was previously published in reference [116], and is reproduced by permission of Landes Bioscience, copyright 2007.

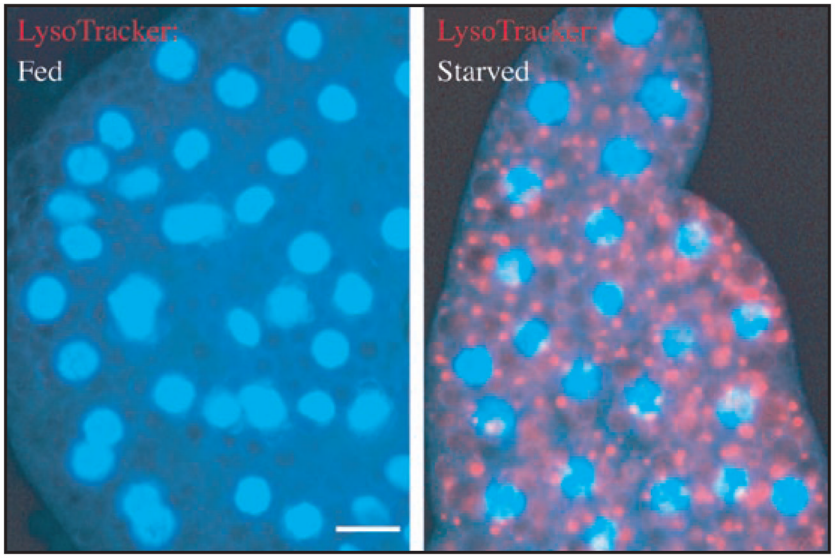

Figure 10.

LysoTracker Red stains lysosomes and can be used to monitor autophagy in *Drosophila melanogaster*. Live fat body tissues from *Drosophila melanogaster* were stained with LysoTracker Red (red) and Höechst 33342 (blue) to stain the nucleus. Tissues were isolated from fed (left) or 3 h starved (right) animals. Bar, 25 μm. This figure was modified from data presented in reference [44], Dev Cell, 7, Scott RC, Schuldiner O, Neufeld TP, Role and regulation of starvation-induced autophagy in the *Drosophila* fat body, pp. 167–78, copyright 2004, with permission from Elsevier.

Table 1

Recommended methods for monitoring autophagy in higher eukaryotes

Criteria	Methods
Monitoring Phagophore and Autophagosome Formation by Steady State Methods	
1. Electron microscopy (increase in autophagosome quantity)	Quantitative electron microscopy, immunoelectron microscopy
2. Atg8/LC3 western blotting and ubiquitin-like protein conjugation systems (increase in the amount of LC3-II, and Atg12—Atg5 conjugation)	Western blot
3. Fluorescence microscopy (increase in punctate LC3 (or Atg18)	Fluorescence, immunofluorescence and immunoelectron microscopy
4. TOR and Atg1 kinase activity	Western blot, immunoprecipitation or kinase assays
5. Transcriptional regulation	Northern blot, or qRT-PCR
Monitoring Autophagy by Flux Measurements	
1. Autophagic protein degradation	Turnover of long-lived proteins
2. Turnover of LC3-II	Western blot +/− lysosomal fusion or degradation inhibitors
3. GFP-Atg8/LC3 lysosomal delivery, and proteolysis (to generate free GFP)	Fluorescence microscopy, FACS Western blot +/− lysosomal fusion or degradation inhibitors
4. p62 western blot	Western blot with qRT-PCR or northern blot to assess transcription
5. Autophagic sequestration assays	Lysosomal accumulation by biochemical or multilabel fluorescence techniques
6. Turnover of autophagic compartments	Electron microscopy morphometry/stereology
7. Autophagosome-lysosome colocalization and dequenching assay	Fluorescence microscopy
8. Sequestration and processing assays in plants	Chimeric RFP fluorescence and processing, light and electron microscopy
9. Tandem mRFP-GFP fluorescence microscopy	Fluorescence microscopy of tandem mRFP-GFP-LC3
10. Tissue fractionation	Centrifugation, western blot and electron microscopy
11. Analyses in vivo	Fluorescence microscopy and immunohistochemistry