Autophagy Overview

Autophagy is an evolutionarily conserved physiological process by which cellular components (cargo) are recycled or degraded in lysosomes. It plays a critical role in maintaining cellular homeostasis by protein degradation and turnover of damaged cell organelles. Under conditions of minimal cell stress, autophagy rates are low, however autophagy is induced under stressful conditions such as in a tumor microenvironment or during nutrient starvation. Autophagy has a complex dual role in cell survival and cell death and has been rigorously studied in relation to the immune system and cancer. Although crucial to the maintenance of a healthy cell, under certain circumstances autophagy-dependent cell death (ADCD) can occur, which is a form of regulated cell death (Denton and Kumar 2018), (Bialik et al. 2018).

Contents

1. Autophagy Overview

Autophagy plays a major role in the modulation of immune responses but also in malignancy protection and progression. Unlike some regulated cell death pathways, like apoptosis, autophagy can be difficult to detect and measure, as suitable reagents are limited. It is important to choose the most appropriate marker and application. More information on detecting autophagy can be found in section 3.

Autophagy Related Genes

Yeast studies during the 1990s uncovered several important genes, which in 2003 were named autophagy-related genes (ATGs) (Klionsky et al. 2003). Over 40 ATGs have since been discovered in yeast, many of which are conserved in animals and plants.

Research into autophagy has accelerated in recent years. A discovery made in 1999 connected autophagy to cancer, identifying downregulation of an autophagy gene, BECN1 (which encodes the protein Beclin 1), in breast tumor cells (Liang et al. 1999). The link between autophagy and cancer continues to be the main focus of autophagy research today but the role of autophagy in cancer is not straightforward. Autophagy induction may slow the development of tumors, and in some circumstances inhibition of autophagy limits the growth of established tumors and improves the response to cancer therapeutics (Amaravati et al. 2019). In 2016, the Nobel Prize in Physiology or Medicine was awarded to Yoshonori Ohsumi for his discoveries of the mechanisms for autophagy, bringing autophagy and its role in cellular processes to the scientific forefront.

Forms of Autophagy

Autophagy can occur in several forms depending on the induction signal, type of cellular components that have been destroyed (cargo), and how the cargo is delivered into the lysosome for degradation, these include:

- **Macroautophagy** – bulk degradation of nonselective cytoplasmic cargo; this is the most studied and best known autophagy pathway. It is important for recycling of damaged cell organelles and unused proteins and is upregulated under stress conditions
- **Microautophagy** – direct lysosomal engulfment of cytoplasmic cargo. This is the second type of nonselective autophagy and it provides the cell with essential amino acids and nutrients in stress conditions. In addition, it also delivers glycogen into lysosomes
- **Chaperone-mediated autophagy** – selective autophagy of target proteins. Proteins are trafficked across the lysosome membrane in complex with chaperone proteins and degraded upon recognition by a lysosomal membrane receptor, lysosomal-associated membrane protein 2A (LAMP-2A)
- **Mitophagy** – selective autophagy of the mitochondria. This occurs to remove excessive or defective mitochondria which serves to prevent an accumulation of dysfunctional mitochondria in a cell

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Other types of selective autophagy include golgiphagy, ribophagy, aggrephagy, ER-phagy, and lipophagy (Gatica et al. 2018).

This mini-review covers the mechanisms of macroautophagy (hereafter referred to as autophagy) and the key markers involved in this cellular process.

Stages of Autophagy

Autophagy can be divided into five main stages: (Figure 1)

1. Stress signals initiate formation of a double membrane structure called a phagophore.

2. Phagophore elongation and engulfment of cytosolic cargo.

3. The phagophore seals to form a spherical autophagosome.

4. Autophagosome fusion with a lysosome.

5. Degradation of the contents of the autophagosome by proteolytic enzymes contained within the lysosome.
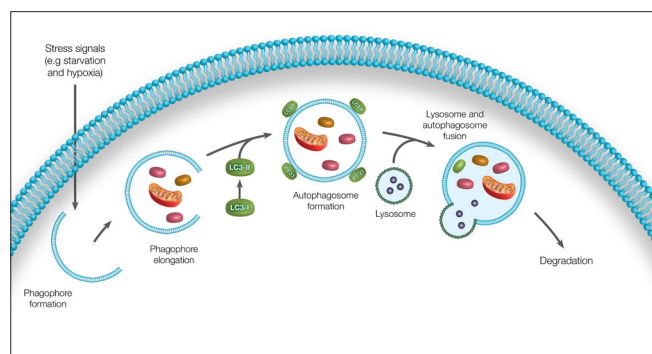

Fig. 1. Autophagy overview. The five main stages in the autophagy process.
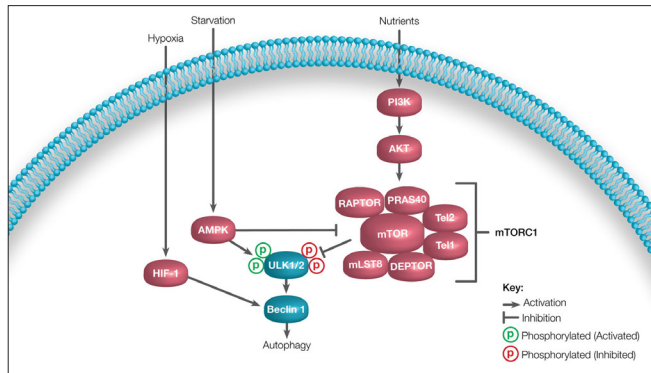
Autophagy Induction

Autophagy normally occurs at low basal levels to maintain homeostasis, but is rapidly upregulated when cells require energy and nutrients, during high bioenergetic demand, structural remodeling, and to remove damaged cytoplasmic components. Autophagy needs to be carefully controlled as too much or too little can be detrimental to the cell. Autophagy is controlled by complex signaling pathways, key mediators for these are the Unc-51, like autophagy activating kinase (ULK1/2) complex and phosphoinositide 3-kinase class III (PI3K).

ULK1, a homolog of yeast Atg1, is a master nutrient sensor regulated by mammalian target of rapamycin complex (mTORC1) and 5'-AMP-activated protein kinase (AMPK). The PI3K and AKT (protein kinase B) signaling pathway regulates mammalian target of rapamycin (mTOR), a key component of mTORC1.
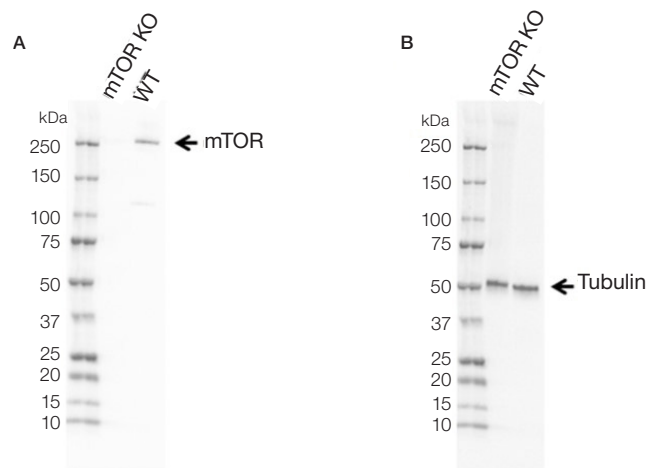
Fig. 2. Western blot analysis of mTOR CRISPR knockout HEK293 (mTOR KO) and wild type HEK293 (WT) whole cell lysates. A, Rabbit Anti-mTOR Antibody (Cat. #VPA00174) followed by detection with HRP conjugated Goat Anti-Rabbit IgG (H/L) (1/10,000 #STAR208P) and B, hFAB Rhodamine Anti-Tubulin Primary Antibody (#120004166) and visualized on the ChemiDoc MP Imaging System with a A, 37 sec and B, 30 sec exposure.

The PI3K pathway is activated under nutrient-rich conditions, resulting in mTORC1 activation and inhibition of autophagy by phosphorylating ULK1/2 at inhibiting sites. During starvation, AMPK levels increase. This results in inhibition of mTORC1 and phosphorylation of ULK1/2 at activating sites (Kim and Guan 2015). Downstream activation of Beclin 1 leads to phagophore formation and ultimately autophagy induction. This pathway is summarized in Figure 3.

Fig. 3. Simplified major pathways of autophagy induction. In nutrient-rich conditions, signaling occurs via PI3K and mTORC1 phosphorylates ULK1/2 at inhibiting sites (red P). During starvation, signaling via the AMPK pathway phosphorylates ULK1/2 at activating sites (green P) leading to Beclin 1 activation and autophagy activation. HIF-1 signaling induced by hypoxia can active Beclin 1 directly.

Autophagy can also be induced under cellular stress independent of mTORC1 (Figure 2). One example is HIF-1 activation of Beclin 1 under hypoxic conditions. In addition, eukaryotic initiation factor 2α (eIF2α) and mitogen-activated protein kinase 14 (MAPK14) are involved in the induction of autophagy under starvation conditions, DNA double-strand breaks and endoplasmic reticulum (ER) stress.

Visit bio-rad-antibodies.com/mtor-signaling-pathway for more information on the role of mTOR in mTORC1 and mTORC2 signaling.

**Phagophore and Autophagsome Formation**
Autophagy proteins, including phosphorylated phosphatidylinositol (PI), assemble at membrane sites. These areas of membrane form a double membrane phagophore. The localized synthesis of phosphatidylinositol-3 4,5-triphosphate (PI3P) plays a major role in autophagosome assembly, by recruiting a number of effector proteins that lead to membrane remodeling. One member of this family of effector proteins are the homologs of yeast ATG-18 called WD-repeat protein interacting with phosphoinositides (WIPIs). There are four mammalian proteins -WIPI 1-4. WIPI2b recruits ATG16L1, which in turn recruits the ATG5-12 complex to form the larger ATG5/12/16L1 complex. This complex is required for ATG8 lipidation and has a role in elongation of the autophagsome. Other ATG proteins are also important for autophagosome formation including ATG3, ATG7, ATG8, ATG9,ATG13, ATG16, and ATG18.

Yeast only has one ATG8 gene, but in mammalian cells there are at least seven ATG8 paralogs which can be grouped into two categories:

- Gamma aminobutyric acid receptor associated proteins (GABARAP's), GABARAP-L1/GEC1/ATG8L, GABARAP-L2/GATE-16, and GABARA-L3
- MAP1LC3 (LC3) family: LC3A, LC3B, LC3B2, and LC3C

GABARAP/GATE16 is involved in autophagosome sealing whereas LC3 is required for autophagosome expansion. Cytosolic forms of LC3 (LC3-I) are lipidated by conjugation to phosphatidylethanolamine (PE) to form a LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes. LC3-II is widely used as a marker of autophagosomes as it is the only protein that remains attached to the autophagosome following its completion, and is therefore a crucial marker when detecting autophagy (Mannack 2015).

p62 is a protein involved in selective autophagy and is also used as a marker of autophagy (Figure 4). It interacts via its C-terminal to ubiquitin or polyubiquitin chains on cargo to LC3 via a specific sequence motif resulting in the delivery of cargo to be degraded to the autophagosome. p62 accumulates when autophagy is inhibited and decreases under conditions of autophagy induction, as p62 is degraded along with the cargo in autolysosomes.
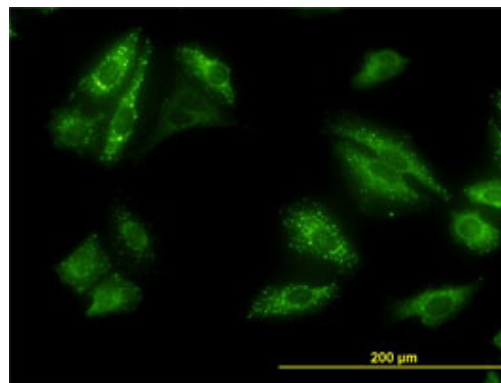
Fig. 4. Detection of p62 by immunofluorescence. Staining of HeLa cells with Mouse Anti-Human p62 (SQSTM1) Antibody (#MCA4265Z).

Fusion with Lysosomes and Degradation
Autophagosomes fuse with early and late endosomes as they travel along the microtubule network towards the lysosome, to form an amphisome. The structure acidifies as it matures due to the fusion and the actions of proton pumps on its outer membrane. It is thought that this acidification allows more efficient degradation of its engulfed contents. It then fuses with a lysosome to form an autolysosome, this fusion is dependent on recognition of phosphoinostide PI(3,5)P2 on the autophagosome membrane. Lysosomal membrane proteins, key to the fusion process, include LAMP1 and LAMP2, and the fusion event is controlled by soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE). An important SNARE protein on the autophagosome is syntaxin17 which forms a complex with two additional SNARE proteins - vesicle associated membrane protein 8 (VAMP8) on the lysosome and synaptosomal-associated protein 29 (SNAP29) in the cytoplasm. Ras-associated binding (Rab) proteins on membranes are also critical for fusion as they act as bridges to bring the compartments together (Nakamura and Yoshimori 2017). This final step in the autophagy process occurs when the contents of the autolysosome are degraded by lysosomal proteases such as cathepsins.

Summary
Autophagy is a cellular process that removes and recycles contents of the cell. This process occurs at a basal level, but is increased during cellular stress. There are several forms of autophagy, here we have described macroautophagy. It is a multistep process leading to the formation of autophagosomes which fuse with lysosomes to degrade the cellular content for recycling.

2. Autophagy in the Immune System and in Cancer

Introduction
Autophagy has a complex dual role in cell survival and cell death and has been rigorously studied in relation to the immune system and in cancer, particularly in the role that the immune system plays in the removal of malignant cells.

Autophagy can promote or inhibit tumor development depending on the cell type and tumor stage. How autophagy regulates the immune system to attack malignant cells is therefore of interest (Jiang et al. 2019). Many anticancer therapies attempt to modulate autophagy, for example using autophagy related gene (ATG) inducers/inhibitors or preventing autophagosome-lysosome fusion, with the aim to enhance the immune response and antitumor effects of immunotherapies (Cuomo et al. 2019).

There have been many recent studies on autophagy, demonstrating the importance of this process in modulating immune cells of both the innate and adaptive immune system. A brief summary is outlined below.

Innate Immunity
In innate immunity, autophagy is upregulated downstream of pattern recognition receptors by activation of innate immune receptors such as Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD) — like receptors (NLRs), where it facilitates a number of effector responses. These include natural killer T (NKT) cell activation, cytokine production in neutrophils, phagocytosis, and differentiation of neutrophils, NK cells, and macrophages (Jiang et al. 2019), (Germic et al. 2019a), (Germic et al. 2019b). Autophagy has also been shown to modulate NK cell migration into tumor tissue, where targeting autophagy increases NK infiltration into melanoma tissues and improves patient survival (Mgrdtichian et al. 2017).

Adaptive Immunity
In adaptive immunity, autophagy has a role in degrading intracellular bacteria, parasites, and viruses, providing a source of antigens for loading onto MHC class II molecules. Autophagy may also be important in dendritic cells for cross-priming to CD8^{+} T cells. Viral nucleic acids are transferred by autophagy from the cytoplasm to intracellular compartments containing Toll-like receptor 7 which signals the production of type 1 interferon (Jiang et al. 2019). Autophagy is essential for T cell homeostasis, survival, activation, differentiation, and metabolic regulation in T cells. Both CD4^{+} and CD8^{+} T cells upregulate autophagy in response to T cell receptor (TCR) engagement which ultimately results in proliferation, differentiation, and cytokine production (Macian 2019). Autophagic activity declines in aged T cells which causes defects in TCR signaling and contributes to immune senescence (Zhang et al. 2016). Autophagy is also crucial to the survival, function, and homeostasis of B cells and B cell progenitors. This is particularly important in secreting plasma cells which need to maintain their endoplasmic reticulum, remodel their proteome, and secrete large amounts of correctly folded antibodies (Cui et al. 2019).

The Role of Autophagy in Malignancy
A link between autophagy and cancer was first identified in 1999 when an autophagy gene, Beclin 1, was discovered to be deleted in breast tumor cells (Liang et al. 1999). At present the role of autophagy in cancer is far from clear, but is considered to be a double-edged sword. In the early stages of cancer, autophagy is generally beneficial to the host and protects against tumorigenesis, however once a tumor is established, it contributes to cancer progression (Jiang et al. 2019).

Autophagy in Early Malignancy

In the early stages of cancer, autophagy acts as a tumor suppressive mechanism. It helps maintain genome stability and cell injury by removing damaged proteins and mitochondria, reducing damage by reactive oxygen species (ROS), and inhibiting inflammation, which are features of early cancer development. This maintains cell quality, preventing tumor initiation and proliferation. Since the initial identification of a link between Beclin 1 and breast cancer, other autophagy genes have been associated with tumorigenesis, including ATG5 and ATG12 mutations (Li et al. 2020).

Autophagy in Established Malignancy

In established cancer, autophagy can act to progress tumors to late stage and promote cancer metastasis. In the majority of reports, autophagy enhances the survival of cells in established tumors by removing toxic oxygen radicals and damaged proteins, and allows cells to grow in stress conditions such as hypoxia and nutrient deprivation. Different tumor cells have a range of reliance on autophagy; in vitro studies have shown that responses to autophagy inhibition in tumor cells range from no effect, slower growth, or causing the tumor cells to die (Towers et al. 2020).

Autophagy in Treatment for Malignancy

During cancer treatments — radiation therapy, chemotherapy and immunotherapies, malignant cells upregulate autophagy further as a mechanism to prevent their death (Li et al. 2019). It is therefore thought that using autophagy inhibitors for the treatment of cancers that rely on autophagy could enhance and increase the effectiveness of conventional therapies, especially when given in combination. Many such clinical trials have taken place or are in progress (for more information, visit ClinicalTrials.gov). The most common autophagy inhibitors used in clinical trials are chloroquine (CQ) and hydroxychloroquine (HCQ), as both of these have been approved for use in other indications. They work by inhibiting the autophagosome-lysosome fusion in the autophagy pathway. They have showed positive efficacy and have demonstrated a good safety profile in combination with cytotoxic chemotherapies (Jiang et al. 2019), (Pérez-Hernández et al. 2019). However, none are currently approved for use in a clinical setting as an anticancer therapy. This is in part due to clinical trials highlighting the need for a reliable biomarker for autophagy detection to help determine efficacy.

The dual role of autophagy in cancer means that it will be vital to identify patients with cancers susceptible to autophagy inhibition/induction, with treatments most likely to be administered in combination with conventional cancer treatments (Pérez-Hernández et al. 2019).

Summary

Both the innate and adaptive immune systems are critical for health, fighting pathogens, and removing malignant cells. Autophagy is upregulated downstream of PRRs in innate immunity cells and is crucial for cell homeostasis, survival, and development of adaptive immune cells. Autophagy maintains normal physiological function and allows cells to respond correctly to a threat. It has been shown to be helpful in preventing cancer progression but also promotes survival of established cancers. Modulation of both the immune response and autophagy could be crucial in finding new treatments, and improving current treatments when combined with chemotherapy and radiotherapy.

To find out more about how Bio-Rad can help with your autophagy, immunology, and cancer research visit bio-rad-antibodies.com. Bio-Rad has a wide range of reagents including the highly validated PrecisionAb Antibodies, and resources including posters, webinars, mini-reviews, and protocols to help you get the most out of your experiments.

3. Effective Detection of Autophagy

Introduction

Autophagy is a vital cellular process involved in keeping the immune system working properly and has been linked to a number of diseases, including malignancies. Multiple components of the autophagy pathway can be detected, allowing researchers to efficiently study the process.

These include:

- Signaling molecules involved in autophagy induction, such as mTOR and AMPK
- LC3-I and LC3-II. LC3-I is a protein found in the cytosol which becomes lipidated to form LC3-II and is inserted into the membrane of autophagosomes
- Other autophagy-related proteins, like ATG5-12 complex and WIPI1
- Lysosome mass. Lysosomal markers, such as LAMP1 and LAMP2

These markers tell us about specific components of the autophagy pathway. An alternative method is to measure autophagy activity, also known as autophagy flux. This is a measurement of the whole autophagic process from induction, autophagosome formation, fusion with lysosomes, and breakdown of the autophagosome contents. The different techniques and assays that can be used to investigate autophagy, including the pros and cons of each method, are discussed in more details.

Techniques Used for Autophagy Detection

The best method to detect autophagy will depend on the experimental question and conditions. No individual assay is appropriate for every situation and it is best practice to confirm findings using more than one method. The use of proper controls is also vital, including positive and negative experimental controls. Below is a summary of the most common techniques used to detect autophagy.

Electron Microscopy

Electron microscopy (EM) is the only technique able to identify the morphology and cellular position of autophagy structures, as such this is the most comprehensive method that can be used to measure the whole process. Prior to the discovery of specific autophagy markers, it was the only way to detect autophagy (Jung et al. 2019). EM is the most sensitive method for autophagy detection, but it is time consuming to set up and requires technical expertise for both generation of images and data interpretation. The characteristic double membrane morphology of autophagsosomes can be difficult to distinguish and depends on the quality of the samples and/or fixative used. In good quality samples, autophagosomes can be identified as well as the single membrane autolysosomes. Autophagy can be quantified simply by scoring for the presence or absence of autophagic structures in a section of a cell, in the total cytoplasm of a cell, or by estimating the cell volume occupied by autophagosomes. However, it can be difficult to accurately quantify autophagy levels due to cellular artefacts derived from the methodology and operator bias. Furthermore this technique is low-throughput (Martinet et al. 2014), (Klionsky et al. 2016).

Western Blotting

The most widely used antibodies to detect autophagy by western blotting are those that detect LC3. LC3 lipidation (LC3-I to LC3-II) results in an increase in molecular weight that can be detected by western blot (Figure 5). Although LC3-II has a greater molecular weight than LC3-I, it migrates faster than LC3-I in SDS-PAGE, which is thought to be due to its increased hydrophobicity (Tanida et al. 2008). The amount of LC3-II detected by western blot (Figure 5) increases with autophagosome number and is a good indicator of autophagosome formation. However, results may be misleading as this assay does not take into account LC3-II turnover. Autophagy induction causes LC3-II levels to increase. This would not be detected by this assay though if there was also an increase in the rate of autophagy flux in the cell, resulting in rapid recycling of LC3-II. In this situation, LC3-II levels may stay the same or increase (Gottlieb et al. 2015).
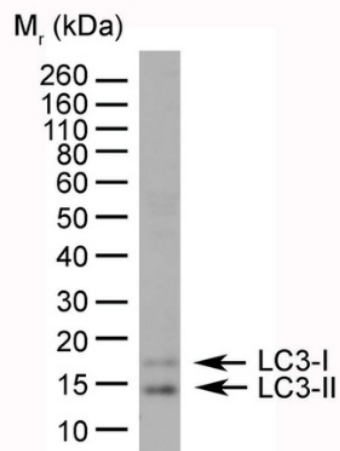
Fig. 5. Detection of LC31/11 by western blotting. HeLa lysate probed with Rabbit Anti-Human LC3A/B (N-terminal) **(#AHP2167)** followed by horseradish peroxidase conjugated Sheep Anti-Rabbit IgG Antibody. As can be seen in the blot, LC3-I and LC3-II can be clearly distinguished from each other.

When prepared in SDS sample buffer (e.g. Laemmli buffer), LC3-II is more sensitive to degradation due to freeze-thawing than LC3-I. Therefore, fresh samples should be prepared and assessed as soon as possible, and repeated freeze-thaw cycles avoided (Klionsky et al. 2016). When quantifying LC3-I / LC3-II by western blotting, as with other proteins, a housekeeper protein is used as a loading control. However, the housekeeper protein actin can be reduced during autophagy induction and should not be used as a loading control for LC3 western blots (Klionsky et al. 2016). Other housekeeping proteins such as GAPDH or tubulin should be considered. An alternative method of quantification is to use Bio-Rad stain-free gels, which allow normalization of your protein of interest against total cellular protein in each lane. Another point to be aware of is that LC3 binds more strongly to PVDF membranes than to nitrocellulose membranes and is lost after stripping and reprobing from both membranes, therefore LC3 should be the first protein probed for on a membrane if you plan to strip and reprobe (Gottlieb et al. 2005).

In addition to detecting LC3, other key autophagy-related proteins can be detected by western blotting (Table 1). While they are not definitive markers of autophagy, these proteins can give useful information on the status of autophagy pathways within the cell. These include other proteins expressed on autophagosomes such as WIPI2 and the phosphorylation status of signaling molecules mTOR and ULK1.

Table 1. Anti-human and anti-mouse antibodies available to study the autophagy pathway by western blotting.

| Target	Reactivity	Catalog Number
AMBRA1	Human, mouse	AHP1882, AHP1889
AMPKα	Human	VMA00246, AHP2429, VPA00425
AGT1 / ULK1	Human	AHP2214
ATG3	Human	VMA00280, AHP2219
ATG4B	Human	AHP2440, AHP2098
ATG5	Human	AHP2160
ATG5-ATG12 complex	Mouse	AAM79
ATG7	Human, mouse	AHP1651
ATG9A	Human, mouse	AHP2248
ATG9B	Human, mouse	AHP2241
ATG12	Human, mouse	AHP1891, AHP1892
ATG13	Human, mouse	AHP2242, AHP2438
ATG16	Human, mouse	AHP1894, VPA00086
ATG16L1	Human, mouse	AHP2439
ATG101	Human, mouse	AHP2243
Beclin 1	Human	VMA00721, AHP1009
DRAM	Human, mouse	AHP1694
LAMP1	Human	AHP2448, VPA00561, MCA6113
LAMP2	Human	MCA6114, VPA00316
LAMP2	Human, mouse	AHP2324, AHP2325, APH1634
LAMP2	Mouse	MCA2293
MAP1LCA/B	Human	AHP2167
mTOR	Human	VPA00171, AHP2489, AHP2657 (pSer2448)
P62	Human	MCA4265
WIPI2	Human	MCA5780GA

Microscopy

Immunofluorescence (IF)

Microscopy can reveal the cellular localization of proteins associated with autophagy. LC3 is a target often studied due to its distinct redistribution during autophagy induction. Cells can be stained using anti-LC3 antibodies or transfected with fluorescently tagged LC3 and analyzed by fluorescence microscopy. Cells with basal levels of autophagy show a diffuse cytosolic LC3 expression pattern. Following autophagy induction, LC3 becomes associated with the membrane of autophagosomes, and LC3 can be seen as a dotted/punctate expression pattern (Figure 6).

A
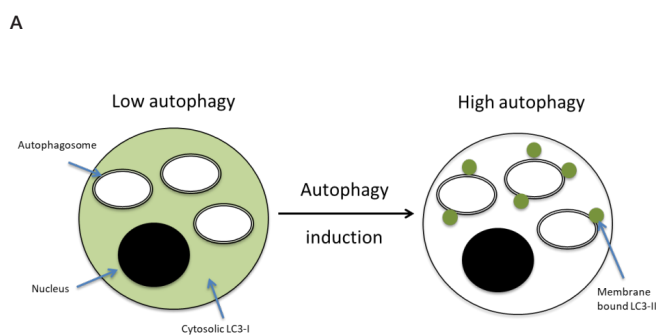
B
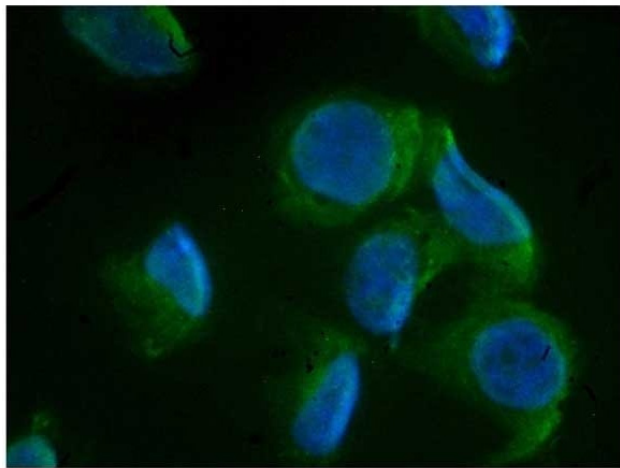
Fig. 6. Distribution of basal LC3 and high autophagy conditions. A, during levels of low autophagy, LC3-I is present in the cytoplasm. Following autophagy induction, LC3-I is lipidated to form LC3-II which is bound to autophagosome membranes. **B,** IF staining of HeLa cells stained with Anti-Human LC3A/B.

Other key autophagy proteins can also be studied by immunofluorescence, such as p62 (also known as SQSTM1). As p62 is involved in a number of cellular pathways, results should be confirmed by other methods. Lysosomes can also be detected by staining for LAMP1 or LAMP2 or by staining for cathepsins using our easy-to-use kits (Figure 7).
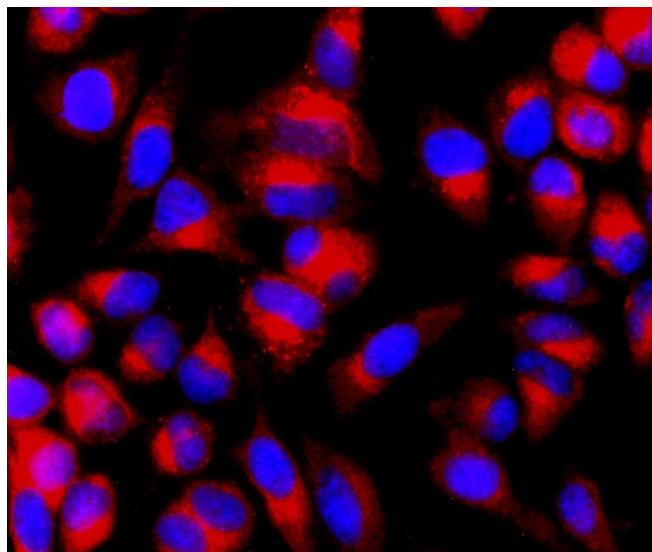
Fig. 7. Capthepsin B staining in HeLa cells. HeLa cells were stained with Magic Red Cathepsin B Kit for 1 hr and counterstained with Hoechst.

Immunohistochemistry (IHC)

Of the techniques covered here to study autophagy, IHC is the common technique due to a lack of biomarkers and its susceptibility to misinterpretation. LC3 is the most commonly used marker to evaluate autophagy levels by IHC but requires careful analysis. High levels of cytosolic LC3 make it difficult to clearly see LC3 associated with autophagosomes. It is also common to see false positives due to nonspecific granular staining pattern of cytoplasmic components (Martinet et al. 2017). Staining of frozen tissues has poorer morphology and resolution, but also the added complication of lipid droplets staining positive for LC3 antibodies when using some fixatives. LC3 is involved in lipid droplet formation and in paraformaldehyde-fixed frozen sections can be seen as small dot like structures around lipid droplets, but not those fixed in acetone (Martinet 2013). Therefore, staining of frozen tissue sample with LC3 is not recommended (Martinet et al. 2013).

When studying autophagy markers in IHC, you should use several markers or additional assays with the right controls, as no one marker can give a complete picture of the state of autophagy in a cell.

p62 is used in IHC, but as previously mentioned in the immunofluorescence section, p62 is also involved in other cellular pathways. Other markers you could use include ULK-1, LAMP1, Beclin 1, and ATG5/7 and 12. Figure 8 shows staining for LAMP1. While the staining is clear, differences can also indicate general changes in lysosomal activity rather than increases in autophagy (Klionsky et al. 2016).

A list of IHC and IF validated antibodies suitable for studying autophagy can be found in Table 2.

Fig. 8. Detection of LAMP-1 by IHC. Staining of paraffin-embedded human liver cancer using Rabbit Anti-LAMP1 (CD107a) Antibody (#AHP2448) at a dilution of 1/100.
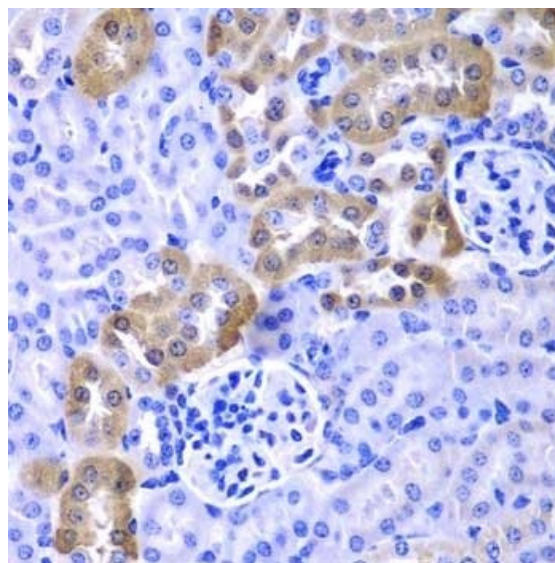
Table 2. Anti-human and anti-mouse antibodies available to study the autophagy pathway by IF and IHC.

| Target	Reactivity	Catalog Number	Application
AMBRA1	Human, mouse	AHP1889	IF
ATG7	Human, mouse	AHP1651	IHC
ATG9B	Human, mouse	AHP2241	IHC
ATG16	Human	AHP1894	IHC
ATG16L1	Human, mouse	AHP2439	IF
ATG101	Human, mouse	AHP2243	IHC
LAMP1	Human, mouse	AHP2448	IHC
LAMP1	Human	MCA2881Z	IF
LAMP1	Human	MCA6113	IHC
LAMP1	Mouse	MCA4707	IHC, IF
LAMP2	Human	MCA6114	IHC
LAMP2	Mouse	MCA2293	IHC
LAMP2	Human, mouse	AHP1634	IHC
MAP1LCA/B	Human	AHP2167	IF
p62	Human	MCA4265	IF
PI-3 Kinase p85	Human, mouse	MCA1167	IHC
Subunit	Human	MCA1170	IHC
WIPI2	Human	MCA5780GA	IF

Flow Cytometry

Flow cytometry is a powerful technique that allows you to look at autophagy in a highly quantitative manner. In addition to just measuring autophagy, cells can be labeled with several antibodies at the same time allowing multiple cell types to be identified in a mixed cell population. This would only be possible for the western blotting technique by presorting the cells into their respective subpopulations and only a limited number of different cell types could be detected by microscopy. Flow cytometry also allows the detection of several components of the autophagy pathway simultaneously. Another advantage is that large numbers of cells can be analyzed allowing rapid quantification. The drawback for using standard flow cytometry is that it does not provide information on the cellular localization of proteins, however, imaging flow cytometry can be used to overcome this issue (Phadwal et al. 2012).

A common method to study autophagy by flow cytometry involves transfecting cells with a fluorescent LC3 reporter tag (e.g. GFP-LC3). Cytosolic LC3-I can be removed by incubation with saponin, so that only the membrane bound LC3-II remains, which can then be quantified by flow cytometry. Anti-LC3 antibodies can also be used using the same principle of removing cytosolic LC3-I. Saponin interacts and removes membrane cholesterol leaving holes in the cell membrane allowing LC3-I to be removed from the cell. Careful titration of saponin and time of treatment is required to ensure LC3-I is removed without excessive damage to the cell membrane. Including autophagy controls, such as an activator, will indicate if enough saponin is used; an activator will not show an increase in LC3 levels over basal conditions.

Measuring endogenous levels of LC3 has an advantage over transfection using an LC3 reporter tag, as it avoids the need for transfection and potential artefacts that can result from overexpression. However, in some cell types or assays, endogenous levels may be below the level of detection, in which case another method will need to be used. Other proteins of the autophagic pathways can be analyzed by flow cytometry including LAMP1/2 and Beclin 1. A more comprehensive list is shown in Table 3.

In addition to measuring specific protein levels, dye-based commercial kits are available to measure autophagy by flow cytometry, such as Bio-Rad's Autophagy Assay Kit Red (#APO010) (Figure 9). This contains a cell-permeant aliphatic molecule that fluoresces brightly when inserted in the lipid membranes of autophagosomes and autolysomes.
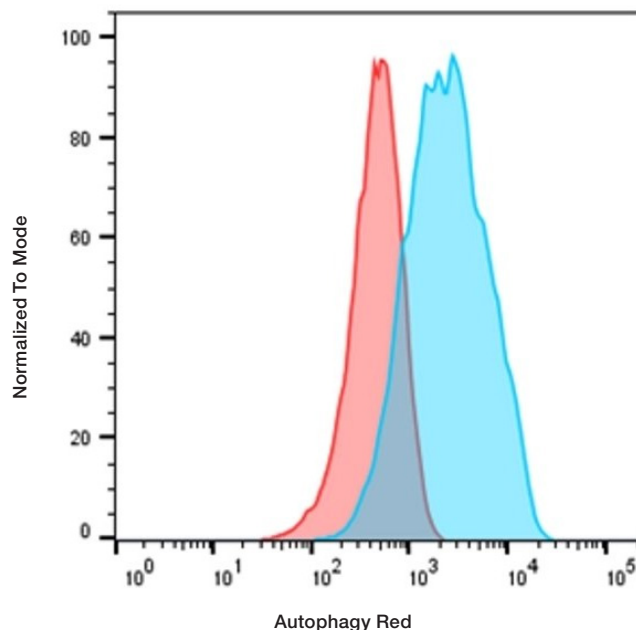
Fig. 9. Autophagy induction in Jurkats measured by flow cytometry using Autophagy Assay Kit Red. Jurkat cells were either untreated for basal conditions (red) or incubated in HBSS for 2 hr and 10 nM bafilomycin A for 2 hr (blue) to induce autophagy. After staining with Autophagy Probe Red for 30 min, cells were washed and analyzed on the ZE5 Cell Analyzer using the 561 nm laser and 640/20 filter.

Table 3. Anti-human and anti-mouse antibodies and kits available to study the autophagy pathway by flow cytometry. Antibodies are available in a purified format (Pur) or conjugated to PE, APC, or FITC.

Target/Kit	Reactivity	Catalog Number	Formats
Autophagy Assay Kit Red	Human	APO010	Red (Ex 590 nm, Em 620 nm)
Beclin 1	Human	AHP1009	Pur.
LAMP1	Human	AHP28817	Pur.
LAMP1	Human	AHP6113	Pur., PE, APC
LAMP1	Mouse	AHP4707	Pur.
LAMP2	Human	AHP6114	Pur., APC, FITC, PE
LAMP2	Mouse	MCA2293	Pur.

Abbreviations: APC, allophycocyanin; FITC, fluorescein isothiocyanate; Pur., purified; PE, phycoerythrin.

Careful consideration needs to be given to the experimental design, including sample preparation, choice of fluorophore, the most appropriate positive control, and how best to determine autophagy flux when detecting autophagy by flow cytometry. This is particularly important in primary cells as changes seen by flow cytometry are often small (Cossarizza et al. 2019). This means positive signals can be hard to detect and false positives need to be minimized.

For more information on how to detect autophagy by flow including experimental setup, multiplexing and appropriate controls, visit our best practices for detecting autophagy at bio-rad-antibodies.com/autophagy-flowcytometry

Summary

Autophagy can be difficult and time consuming to detect. Take some time to determine which is the most appropriate application and marker for your experiment, and ensure the proper controls are performed. This will depend upon the conditions and results required. Whichever you choose, consider confirming your results in another application or detecting another component of the autophagy pathway, as this will give you more confidence in your results and is often required for peer reviewed publication.

Autophagy is just one of the pathways in regulated cell death. Bio-Rad offers reagents for western blotting, microscopy, and flow cytometry, and resources to help you study these pathways including mini-reviews and a downloadable poster.

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