Appetite for destruction

Studying macroautophagy with Premo fluorescent protein–based sensors.

Autophagy is a process of cellular self-digestion that involves the segregation and delivery of cytoplasmic cargo for degradation by hydrolytic enzymes through the lysosomal machinery. Critical to healthy cell functioning, this digestion process serves not only to recycle and repurpose intracellular material but also to dispose of misfolded proteins, damaged organelles, and other superfluous or aberrant entities.

The term autophagy covers three cellular processes that all share the lysosome as the ultimate endpoint. Microautophagy requires invagination of the lysosomal membrane to engulf cytoplasmic cargo, which is then delivered into the lysosomal lumen for degradation [1]. Chaperone-mediated autophagy utilizes transport mechanisms to transmit cytosolic proteins (containing a specific motif recognized by chaperones) across the lysosomal membrane, where they are degraded by hydrolases in the lumen [1]. Macroautophagy targets a wide range of cargo types (including dysfunctional or unnecessary proteins, organelles, and other cell components) for lysosomal digestion through formation of an autophagosome, an expanding double-membrane organelle that surrounds and engulfs the targeted cellular components and subsequently fuses with the lysosome itself [1]. It is this third process, macroautophagy, that has received the most attention and resulted in the award of the 2016 Nobel Prize in Physiology or Medicine to Yoshinori Ohsumi. Here we describe current fluorescence-based tools for studying macroautophagy, including the Invitrogen™ Premo™ Autophagy Sensors.

Studying autophagy with fluorescence-based methods

Fluorescence-based tools for identifying and monitoring cellular structures or processes include synthetic fluorescent probes, fluorescent protein chimeras (Figures 1A and 1B), and primary antibodies either labeled with a fluorescent dye or detected with a fluorescent secondary antibody (Figure 1C) [2]. All three of these methods have been harnessed to detect autophagy using a variety of fluorescence instrumentation,

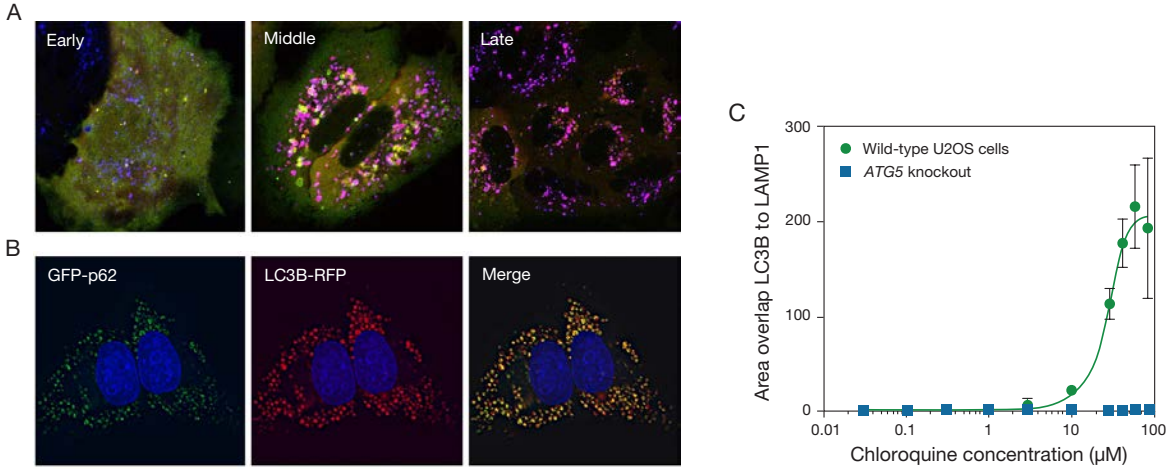

Figure 1. Detection of the autophagosomal marker LC3B using fluorescent protein chimeras or immunocytochemistry. (A) To monitor autophagosome–lysosome fusion and subsequent acidification, A549 cells were transduced with Invitrogen™ Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B, stained with Invitrogen™ LysoTracker™ Deep Red dye (Cat. No. L12492), and subjected to nutrient deprivation with Gibco™ EBSS (Earle's Balanced Salt Solution, Cat. No. 14155-063). Images were captured immediately following nutrient deprivation (at 2 min, early), as well as at 20 min (middle) and 1 hr (late) after nutrient deprivation. In cells at rest (early), there are numerous discrete lysosomes (blue), with few autophagosomes (yellow puncta) or autolysosomes (pink puncta); upon nutrient deprivation, there is an increase in the number of autophagosomes (middle), followed by a decrease in the number of lysosomes and autophagosomes and an increase in the number of autolysosomes (late). **(B)** U2OS cells were transduced with Invitrogen™ Premo™ Autophagy Sensor GFP-p62 and Premo™ Autophagy Sensor LC3B-RFP and cultured for 24 hr. Chloroquine was added at a final concentration of 60 µM, and cells were cultured for a further 16 hr before counterstaining with Hoechst™ 33342 (blue). In the merged image, p62-positive protein aggregates (green) can be seen associated with LC3B-positive autophagosomes (red) and appear yellow. **(C)** Wild-type or *ATG5* knockout (KO) U2OS cells were treated with a range of chloroquine concentrations overnight to block autophagic flux. Cells were fixed, permeabilized, and then immunostained with anti-LC3B and anti-LAMP1 antibodies to label autophagosomes and lysosomes, respectively. Cells were imaged on the Thermo Scientific™ CellInsight™ CX5 High-Content Screening (HCS) Platform and analyzed with the colocalization bioapplication using Thermo Scientific™ HCS Studio™ Cell Analysis Software. Blocking autophagic flux with chloroquine leads to autolysosome accumulation in wild-type but not *ATG5* KO cells. Identification of the LC3B-positive puncta as autolysosomes is confirmed by the colocalizaiton of anti-LC3B and anti-LAMP antibodies.

and, as with all experimental approaches, they are not without caveats.

Despite the enormous breadth of proteins that can be detected using specific antibodies, immunoassays all require a similar time-consuming protocol that involves fixation, permeabilization, and blocking of the cells, followed by incubation with the primary antibody of choice and potentially a secondary antibody, with wash steps in between. The process of chemical fixation itself has been shown to affect cellular structures, leading to potential artifacts [3] and, more importantly, precludes analysis of living cells. Synthetic fluorescent dyes are popular alternatives to antibodies; however, these probes are susceptible to photobleaching and are often found not to be specific for autophagy.

An alternative to fluorescent dye– and antibody-based monitoring of autophagy is the use of cells transformed with a fluorescent protein chimera (i.e., a biosensor that contains both a fluorescent protein and a functional autophagy marker). Once generated, stable cell lines are ready to assay; however, the process of generating stably transformed cell lines is expensive and time consuming, and assaying different cell types requires the creation of new cell lines. Fortunately, the delivery of fluorescent protein–based sensors can be performed quickly and easily using the BacMam gene delivery and expression system, which is incorporated in the Invitrogen™ Premo™ Autophagy Sensor Kits. The add-and-read Premo reagents can be used to transiently transduce cells with a simple overnight incubation, after which the cells are ready to be used in autophagy assays.

Five Premo Autophagy Sensors…

The Premo Autophagy Sensors combine the specificity and live-cell compatibility of fluorescent protein–based reporters with easy delivery and expression using the BacMam gene delivery platform. Premo sensors are available as GFP (i.e., Emerald GFP) or RFP (i.e., TagRFP) constructs with either the autophagosomal marker LC3B or the autophagy receptor p62 (SQSTM1) (Figures 1 and 2). These Premo sensors can be combined with fluorescent markers of specific cargo to image its sequestration by the autophagosome (Figure 1A). Alternatively, the Premo LC3B and Premo p62 sensors can be used together to visualize the incorporation of autophagy receptors into the autophagosome (Figure 1B). Furthermore, the Premo Autophagy Tandem Sensor RFP-GFP-LC3B—which combines the pH-sensitive fluorescence of GFP with the pH-insensitive fluorescence of RFP and the autophagosomal marker LC3B—can be used to image the maturation of the autophagosome into an autolysosome and the associated increase in acidity after lysosome fusion (Figure 1A).

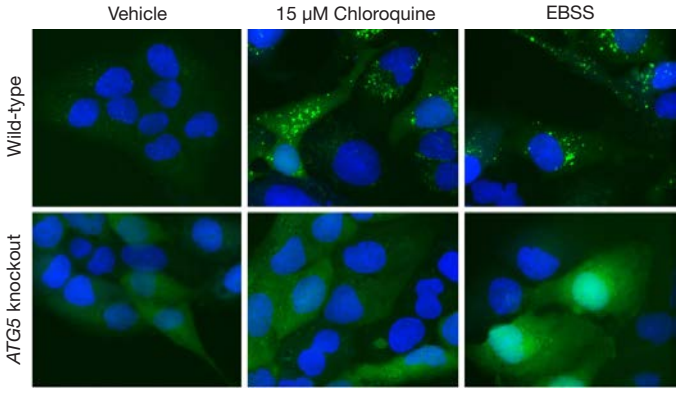

Figure 2. Specificity of labeling with Premo Autophagy Sensors. Wild-type or *ATG5* knockout (KO) U2OS cells were transduced with Invitrogen™ Premo™ Autophagy Sensor LC3B-GFP (green) and cultured for 48 hr. Cells were then incubated with vehicle or 15 µM chloroquine or subjected to nutrient deprivation with Gibco™ EBSS (Earle's Balanced Salt Solution, Cat. No. 14155-063) for 24 hr, counterstained with Hoechst™ 33342, and imaged on an Invitrogen™ EVOS™ FL Imaging System. Blocking autophagic flux with chloroquine or inducing autophagy with EBSS causes the formation of puncta (LC3B-positive autophagosomes), as compared with vehicle-treated wild-type cells. This increase in LC3B-positive puncta in response to chloroquine or EBSS is absent in *ATG5* KO cells.

… all of which are highly specific for autophagy

Of the three types of fluorescence-based autophagy tools, antibodies and fluorescent protein chimeras are the most reliable because they either bind to or mimic specific proteins involved in the autophagy pathway. When using synthetic fluorescent probes, care must be taken to ensure their specificity and determine any off-target effects using autophagy models.

Genetic models represent the most effective method for blocking the autophagy pathway (through deletion of gene products required for macroautophagy), and therefore for testing reagent specificity. In contrast, use of cell treatments that block one or more autophagy processes—e.g., inhibitors of phosphoinositide 3-kinase (PI3K), which can block the formation of autophagosomes—are typically not truly specific for autophagy because they show broad efficacy against members of multiple classes of PI3K, affecting many signaling pathways [4]. During the development of the Premo Autophagy Sensors, we used *ATG5* knockout (KO) cell lines to test for specificity. Cell lines carrying a deletion of the *ATG5* gene (encoding a protein in the Atg12–Atg5-Atg16 complex, which acts in part as an E3 ligase in the macroautophagy pathway [5]) show a loss of multiple →

macroautophagy-related events (reviewed in [6]). In cells lacking *ATG5* and transduced with Premo Autophagy Sensor LC3B-GFP, no puncta (i.e., LC3-containing autophagosomes) are seen following treatment with chloroquine to block autophagic flux or nutrient deprivation with Earle's Balanced Salt Solution (EBSS) to stimulate autophagy via mTOR inhibition (Figure 2).

Probes not specific for autophagy

Given the interest in autophagy, many fluorescent probes have been reported to be able to detect autophagosome formation, including Invitrogen™ reagents such as LysoTracker™ dyes [7], fluorescent cadaverines (e.g., monodansylcadaverine [8] and BODIPY™ TR [9] and Alexa Fluor™ derivatives [9]), and FluoZin™ ion indicators [10], as well as the CYTO-ID™ Autophagy Detection Kits (Enzo Life Sciences) [11]. Using the *ATG5* KO model, we have been able to show that these probes are not specific autophagy reporters. While many of them may report cellular events associated with autophagy, none of them can be used as specific markers for the induction of macroautophagy. For example, we observed puncta (i.e., puncta we would have concluded were autophagosomes by appearance) in *ATG5* KO cells stained with either the CYTO-ID Autophagy Detection Kit (Figure 3) or with Alexa Fluor 488 cadaverine (data not shown) following stimulation of autophagy or inhibition of autophagic flux, demonstrating the lack of specificity of these probes for macroautophagy events.

Tools for autophagy research

In addition to the Premo Autophagy Sensors, we offer a variety of lysosome-selective probes, as well as antibodies that recognize autophagy markers. Learn more about our cell analysis products for autophagy at **thermofisher.com/autophagybp76.** ■

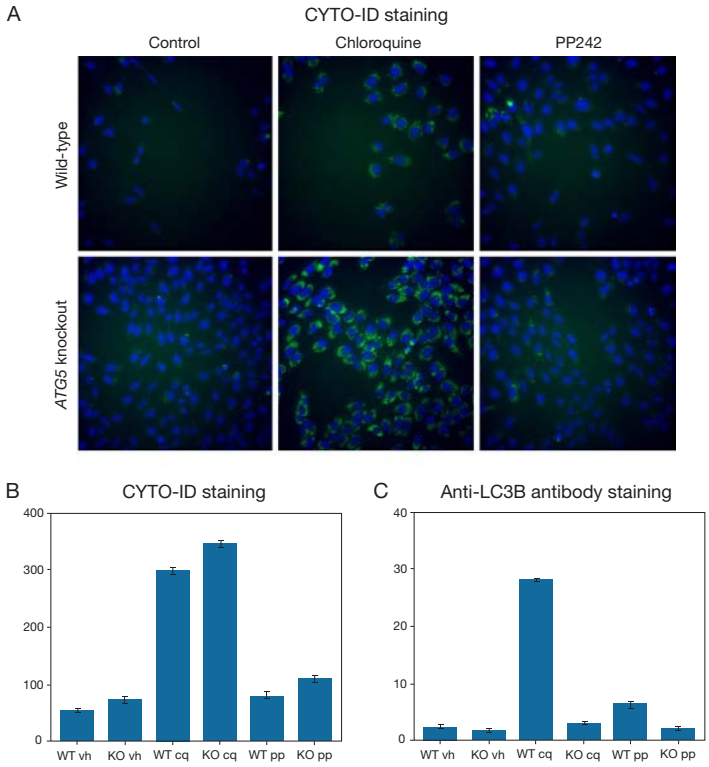

Figure 3. Use of an *ATG5* knockout cell line to determine probe specificity for macroautophagy. (A, B) Wild-type (WT) and *ATG5* knockout (KO) U2OS cells were plated, cultured for 48 hr, and stained with CYTO-ID™ Autophagy Detection Kit (Enzo Life Sciences) according to the manufacturer's instructions. As compared with treating with vehicle (vh) only, inhibiting autophagic flux with chloroquine (cq) or induction of autophagy with the mTOR inhibitor PP242 (pp) caused a significant increase in CYTO-ID staining in both wild-type and *ATG5* KO cells, indicating that the CYTO-ID probe is not specific for macroautophagy. **(C)** Following the experiments, the cells were fixed, permeabilized, and immunostained with anti-LC3B antibody (Cat. No. L10382) in the same microplate wells used for the CYTO-ID staining. LC3B immunostaining revealed that only the *ATG5* KO cells lack LC3B-positive puncta following chloroquine or PP242 treatment. Chloroquine and PP242 caused a significant increase in LC3B-positive puncta in wild-type cells.

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Product	Quantity	Cat. No.
Premo™ Autophagy Sensor GFP-p62 Kit	1 kit	P36240
Premo™ Autophagy Sensor RFP-p62 Kit	1 kit	P36241
Premo™ Autophagy Sensor LC3B-GFP Kit	1 kit	P36235
Premo™ Autophagy Sensor LC3B-RFP Kit	1 kit	P36236
Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B Kit	1 kit	P36239