Review

This Review is in a thematic series on Autophagy in Health and Disease, which includes the following articles:

Molecular Mechanisms of Autophagy in the Cardiovascular System
Autophagy in Vascular Disease
The Role of Autophagy in Vascular Biology
Therapeutic Targeting of Autophagy: Potential and Concerns in Treating Cardiovascular Disease
Untangling Autophagy Measurements: All Fluxed Up
Mitochondria and Autophagy

Daniel Klionsky, Guest Editor

Untangling Autophagy Measurements
All Fluxed Up

Roberta A. Gottlieb, Allen M. Andres, Jon Sin, David P.J. Taylor

A photo is static, an instant in time,
Telling nothing that happened next or before;
Yet papers are published and conclusions are drawn
Claiming autophagy’s up when really it’s gone.

Like tea leaves or runes it’s not easy to read
When puzzling out blots of LC3B
When chloroquine’s there or when it’s left out
It’s the increase that matters to tell you what’s what.

Beclin is tricky when it’s gone half away,
Its effects on autophagy go every which way:
Nucleation is up or fusion’s not seen,
So consider with care what it all means.

Remember when calculating if flux is intact
It’s the ratio that gives you that crucial fact
LC3 levels of Baf over static
Will yield results that are not so erratic.

To understand the process you must think it through;
Autophagy requires you to use every clue.
Good data help you line up ducks,
Just please be sure to measure flux.

Abstract: Autophagy is an important physiological process in the heart, and alterations in autophagic activity can exacerbate or mitigate injury during various pathological processes. Methods to assess autophagy have changed rapidly because the field of research has expanded. As with any new field, methods and standards for data analysis and interpretation evolve as investigators acquire experience and insight. The purpose of this review is to summarize current methods to measure autophagy, selective mitochondrial autophagy (mitophagy), and autophagic flux. We will examine several published studies where confusion arose in data interpretation, to illustrate the challenges. Finally, we will discuss methods to assess autophagy in vivo and in patients. (Circ Res. 2015;116:504-514. DOI: 10.1161/CIRCRESAHA.116.303787.)

Key Words: autophagy ■ methods ■ microscopy, fluorescence ■ mitochondrial degradation ■ physiology
Overview of Autophagy

Autophagy emerged as a cardiac research topic starting in 2005 with the recognition of its role in hibernating myocardium and in the protective response to BNIP3; in the ensuing 8 years, the number of publications on cardiac autophagy have increased 10-fold. Thus, there is a substantial need to have reliable methods to measure the process of autophagy. Autophagy is an essential housekeeping function responsible for eliminating unwanted protein aggregates or organelles. Autophagy encompasses 3 primary pathways for lysosomal degradation: macroautophagy (the methodological focus of this review), microautophagy, and chaperone-mediated autophagy. Microautophagy is a nonspecific form of degradation in which lysosomal membranes directly engulf cytoplasm along the periphery of the organelle. Glycogen and other endosomal components are examples of material that is degraded by microautophagy. Chaperone-mediated autophagy targets cytosolic proteins and translocates them straight across the lysosomal membrane to be degraded. Proteins targeted by chaperone-mediated autophagy must contain a specific targeting motif that is recognized by HSPA8/HSC70, which subsequently directs the cargo for degradation. Ultimately, all 3 processes depend on lysosomal function, failure of which carries pathological consequences. Genetic defects in lysosomal function, such as Danon disease and Pompe disease, are recognized to result in cardiomyopathy because of glycogen accumulation. Although lysosomal function is essential to macroautophagy—the focus of this review—we do not cover specific methods to assess lysosomal function.

Macroautophagy involves the engulfment of a portion of the cytoplasm by a double-membrane phagophore, which elongates around both selectively and nonselectively targeted cargo. After phagophore closure, the newly formed vesicle, termed the autophagosome, fuses with the lysosome forming the autolysosome. In the acidic milieu of the lysosome, pH-dependent hydrolases degrade the proteins, lipids, nucleic acids, and carbohydrates of the cargo. The phagophore membrane is thought to arise from multiple membrane sources, including the endoplasmic reticulum. In a model, phagophore initiation begins with the distention of the endoplasmic reticulum into a phosphatidylinositol-3-phosphate (PtdIns3P)—rich structure called the omegasome. This process is initiated with the recruitment of the unc-51-like kinase (ULK) complex and the class III PtdIns-3-kinase complex, which are targeted to the omegasome via ATG13 and ATG14L1, respectively. From the omegasome emerges the phagophore membrane at which point WIPI2 and ATG16L1 are recruited. ATG12 is conjugated with ATG5 through the action of ubiquitin ligase-like enzymes ATG7 and ATG10; the covalently linked ATG5–ATG12 duo complexes with ATG16L1, enabling the processing of microtubule-associated protein 1A/1B-light chain 3 (LC3). The precursor form of LC3 is truncated by the cysteine protease ATG4 to expose the C-terminal glycine of LC3 to form LC3-I. This in turn is conjugated to the amino group of phosphatidylinositol-ethanolamine (forming membrane-associated LC3-II) by the action of E1- and E2-like enzymes ATG7 and ATG3. Once the LC3-II–decorated phagophore closes around its target, the ATG12–ATG5–ATG16L complex is released from the membrane. When the autophagosome fuses with the lysosome, LC3-II on the outer face of the autolysosome is released by the action of ATG4; however, LC3 on the inner face is retained and eventually degraded by lysosomal enzymes.

Examples of Confusing Autophagy Studies and Confounding Variables

To highlight the need for appropriate standards for measuring autophagy and interpreting results, we will first present some recent autophagy studies in which the results were contradictory, incomplete, or subject to alternative interpretation, leading to controversial and possibly incorrect conclusions.

Difficulties in Interpreting Autophagic Changes in the Setting of Diet-Induced Obesity

At a recent meeting, contradictory results were reported by 2 groups using mCherry-LC3 transgenic mice, in which the fusion protein is expressed under the control of the α-MHC promoter. The group of Abel observed an increase in the number of mCherry-labeled puncta in mice fed a high-fat diet, whereas the group of Mentzer observed a decrease (Figure 1). Diet composition differed as follows: the group of Mentzer used the D12492 60% fat diet, whereas the group of Abel used a Western diet of 40% fat and increased sucrose. Mentzer study enrolled mice at 5 to 6 weeks and maintained for 15 weeks, whereas the Abel study enrolled mice at 8 weeks and maintained them on the diet for 12 weeks. Another important difference was the genetic background of the mice. The Mentzer laboratory used mCherry-LC3 mice in the FVB/N background, whereas the group of Abel backcrossed the line into the C57BL/6 background. Interestingly, both groups arrived at the same conclusion that the obesogenic diet impaired cardiac autophagy. The group of Abel used chloroquine to show that autophagic flux was attenuated. They noted elevated p62/SQSTM1 that did not increase further when flux was inhibited with chloroquine. The group of Mentzer also observed elevated p62/SQSTM1 in the high-fat diet group, and similarly concluded that autophagic flux was impaired. This illustrates the difficulties in determining autophagic activity based on snapshot measurements of LC3 puncta or Western blot and emphasizes the need to assess flux directly (eg, by chloroquine) or indirectly (by p62/SQSTM1).

Beclin 1 Haploinsufficient Mice

Deletion of Atg5 results in death in the newborn period unless nutritional support is provided during the first 12 to 24 hours of postnatal life, but surviving pups are relatively normal although they develop heart failure and other abnormalities with time. Subsequent studies revealed an alternative autophagic pathway independent of ATG5 and ATG7 but dependent on

Nonstandard Abbreviations and Acronyms

<table>
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<th>Acronym</th>
<th>Description</th>
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<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
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<tr>
<td>LC3</td>
<td>light chain 3</td>
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<tr>
<td>LGE</td>
<td>late gadolinium enhancement</td>
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<tr>
<td>SAHA</td>
<td>suberoylanilide hydroxamic acid (vorinostat)</td>
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<td>ULK</td>
<td>unc-51-like kinase</td>
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BECN1, ULK1, and RAB9. BECN1, which participates in both pathways, is essential, because the deletion of the corresponding gene is embryonic lethal. Subsequently, BECN1 haploinsufficient mice were used as a model of attenuated autophagy in several studies; however, BECN1 also regulates autophagosome–lysosome fusion and when partnered with KIAA0226/Rubicon, it can suppress fusion. These complexities have led to several contradictory findings: BEcn1+/- mice showed less hypertrophy in response to aortic banding, which was used as evidence that autophagy contributed to pathological hypertrophy. BEcn1+/- mice are observed to have smaller infarcts after ischemia/reperfusion injury, leading the authors to conclude that BECN1-dependent autophagy contributes to reperfusion injury. In contrast, chloramphenicol administered to pigs before ischemia or at reperfusion profoundly reduces infarct size and is associated with a substantial upregulation of BECN1. Thus, contradictory roles for BECN1 and autophagy emerged from these studies, largely dependent on the interpretation of LC3 autophagic markers.

In the study by Zhu et al of BEcn1+/- mice subjected to aortic banding, they observed fewer autophagosomes in heart tissue and concluded that this indicated decreased autophagy. However, they did not measure autophagic flux; increased autophagic flux would also be consistent with their observation. Subsequent work by Ma et al used neonatal rat ventricular myocytes showed that BECN1 knockdown results in accelerated autophagic flux, presumably by reducing the BECN1-KIAA0226 impediment to autophagosome–lysosome fusion. A criticism of the work of Ma et al is that it is limited to cell culture. However, a study by Xu et al examined autophagic flux in normal and diabetic mice, comparing wild-type and BECN1 haploinsufficient mice. They measured LC3 levels in the absence and presence of the lysosomal inhibitor bafilomycin A1 (BAF) and reported flux as the difference in LC3-II content between BAF and vehicle, reaching the conclusion that flux was not different between Becn1+/− and wild-type mice. However, when flux is calculated as the fold increase over vehicle control as originally described by Tanida et al, they might have reached a different conclusion that would have supported the idea that BECN1 can slow autophagic flux. These controversies over interpretation of the data and the functional effects of BECN1 are reflected in previous work with OVE26 diabetic mice (BECN1 suppresses flux) and other studies with the Becn1+/- mice (BECN1 deficiency results in insufficient autophagy initiation). Whether diminished autophagy initiation or accelerated flux is the dominant effect may depend on the tissue and the context of pathological stress. Taken together, these studies exemplify the difficulties associated with using BECN1 to modulate autophagy and the challenges associated with interpreting results.

If the dominant effect of BECN1 in the heart is to slow autophagic flux, then it becomes necessary to revisit the conclusions reached by Zhu et al and Matsui et al: BECN1 haploinsufficiency might have accelerated autophagic flux to reduce hypertrophy and infarct size in their models. This illustrates the importance of measuring autophagic flux correctly and emphasizes the profound limitations of static measurement of LC3.

Because BECN1 also has the potential to regulate apoptosis through interaction with Bcl-2, its downregulation might benefit the heart through reduction of apoptosis. To examine this, Ma et al used chloroquine and found that suppression of autophagic flux was sufficient to exacerbate autophagy after hypoxia/reoxygenation. They found that the cell death was prevented by cyclosporine A but not that pan-caspase inhibitor ZVAD (Z-val-Ala-Asp-fluoromethylketone), suggesting that the mitochondrial permeability transition pore was responsible for triggering necrotic cell death. Other studies have shown the importance of clearing damaged mitochondria to reduce ischemia/reperfusion injury. Postconditioning was also shown to restore autophagic flux during reperfusion, whereas inhibition of flux (with chloroquine) abolished the protective effects of sevoflurane postconditioning.

Histone Deacetylase Inhibitors

Histone deacetylases (HDAC) regulate an expanding number of pathways, and substrate specificity is restricted according to the enzyme subtype. In 2011, Cao et al reported that trichostatin A, an inhibitor of class I and II HDACs, reversed cardiac hypertrophy because of pressure overload by down-regulating autophagy. In their study, autophagy measurements were limited to detection of autophagic puncta and LC3 Western blots. No flux measurements or assessment of p62/SQSTM1 were performed on the hearts although they did assess flux in neonatal rat ventricular myocytes treated with trichostatin A. A careful inspection of their results (their
Online Figure I) reveals that autophagic flux was enhanced in trichostatin A–treated cardiomyocytes (lysosomal blockade resulted in a 2.1-fold increase in LC3-II in controls versus a 2.8-fold increase in trichostatin A–treated cells). Confusingly, the authors concluded that because the absolute levels of LC3-II were lower in the trichostatin A group, its effects were because of suppression of autophagy, stating that “suppression of autophagic flux contributes to the salutary effects of HDAC inhibitor therapy.” It should be noted that if autophagic flux was suppressed, LC3-II levels would not increase after lysosomal blockade. The fact that the levels nearly triple after lysosomal blockade suggests that the low level of LC3-II seen in the trichostatin A group (with or without lysosomal blockade) is because of brisk autophagic flux.

In 2013, the same group used a related HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA; vorinostat), to show that HDAC inhibition increased autophagic flux and decreased infarct size in a rabbit model of I/R injury.27 Using mice expressing tandem RFP-GFP-LC3, they observed that SAHA increased autophagic flux. The effect of SAHA on autophagic flux was confirmed in cell culture in which flux was assessed by the gold standard of comparing LC3-II in the presence and absence of lysosomal blockade with bafilomycin. The requirement for autophagy in SAHA’s beneficial effects was further confirmed by downregulating ATG7, which abolished the protective effects of SAHA. These 2 studies again illustrate the challenges in interpreting LC3 results and also support the use of the tandem RFP-GFP-LC3 construct for evaluating autophagic flux. Importantly, they provided further evidence to support the role of autophagy through knockdown of a key factor in the canonical autophagy pathway, ATG7 (we have used dominant negative ATG5 with good effect28). As noted above, BECN1 can have paradoxical effects on autophagy because of its interaction with KIAA0226/Rubicon and suppression of autophagosome–lysosome fusion.

Lessons Learned From Analysis of These Studies
These publications have been widely cited and have influenced our understanding of autophagy. However, the failure to measure flux and the controversy over interpretation of results has resulted in confusion about the role and significance of autophagy. The purpose of our rather detailed dissection of those publications is to point out the challenges associated with analyzing autophagy, to encourage investigators to use proper methodology in assessing autophagy, to call for a thoughtful reevaluation of the conclusions of those studies, and to exhort readers to exercise caution when reading new publications.

Several things emerge from this discussion. Static levels of LC3 or scoring of autophagic puncta is an incomplete assessment of autophagy without the assessment of flux (directly, through lysosomal blockade, or indirectly, inferred from a decrease in p62/SQSTM1). The most definitive way to demonstrate a role for autophagy is to knock down ATG7 or inhibit ATG5. BECN1 has paradoxical effects, and a knockdown of this protein may be confusing when assessing the role of autophagy in a particular organ or disease process. Although not discussed in detail in this review, it should be noted that the transcriptional regulation of autophagy has not been closely correlated with functional autophagic flux: mRNA may be increased when autophagy is impaired, and mRNA levels may be stable even when autophagic flux is active. However, mRNA upregulation is part of the longer term autophagic response. There is a need for additional methods to assess autophagy in tissues and to be able to image autophagy noninvasively.

Additional Variables That May Confound Autophagy Results

Effect of Mouse Strain on Autophagy
In addition to the difference in autophagy noted in the mCherry-LC3 transgenics that may be because of strain differences (FVB/N versus C57BL/6), a comparison of cardiac autophagy in C57BL/6 and BALB/c mice conducted by Phyllis Linton’s group revealed substantial differences in autophagy (unpublished data). Although many upstream signals driving autophagy were increased in BALB/c mice when compared with C57BL/6 mice at 3 months, the BALB/c mice had increased aggregate-associated p62/SQSTM1, higher levels of protein carbonylation, and lower levels of LC3-I and -II. Autophagic flux was impaired, evidenced by a failure to increase LC3-II after chloroquine treatment and by the presence of elevated levels of p62/SQSTM1 and ubiquitinated proteins. Strain differences have been noted by others as well.29,30

Circadian Effects
Many investigators have noted a significant diurnal variation in autophagy:11–34: we observed that LC3-II levels increased by 40% between 10 am and 2 pm in hearts of FVB/N mice maintained on a 12-hour light/dark schedule (lights off at 6 pm; B. Ito, R.M. Mentzer, R.A. Gottlieb, unpublished data, 2010). The magnitude of this increase is comparable with that achieved by fasting, thus it is important to design experiments to avoid circadian effects.

Effects of Sex on Autophagy
Examination of autophagy (LC3-II levels) in hearts of C57BL/6 mice revealed that females showed 50% more LC3-I and BECN1 under basal conditions than male animals (M. Gurney and P.-J. Linton, unpublished data, 2012). Sex differences in autophagy in various tissues have been reported by several laboratories.6,35–40 Response to rapamycin also differed according to sex, suggesting sex-related differences in the MTOR signaling pathway.41,42

Methods to Measure Autophagy and Mitophagy

Measurement of Autophagy in Cells and Tissues

Western Blot of LC3 and p62/SQSTM1
Measuring protein expression remains the mainstay method for investigating the process of autophagy. Until recently, the paradigm for measuring autophagy was to examine LC3-II and LC3-I via Western blot. LC3-II is consistently associated with autophagosomes and for that reason is a useful indicator of autophagosome initiation.43–46 Conveniently, lipidation of LC3 alters its conformation such that it has faster mobility in SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Although some groups have used the LC3-II/LC3-I ratio as an indication of autophagic activity, this
is unreliable and has been generally discounted.\textsuperscript{47} Although LC3-I levels can rise in response to an autophagic stimulus, if flux is rapid, levels may remain stable or actually decrease if lipitation outstrips the expression of LC3 or recycling of LC3-II off of autolysosomes by ATG4. Furthermore, LC3-II levels can rise with autophagy, but can rise even more if lysosomal degradation of autophagosomes is impaired. Depending on the particular dynamics of a given cell responding to stress, the ratio can be up, down, or unchanged. LC3-II normalized to a protein loading control is used for flux measurements.

The autophagy receptor protein p62/SQSTM1 can serve as a surrogate marker of increased autophagy when levels are diminished although caveats are appropriate here as well. It is important to understand that p62/SQSTM1 can be present in cells or tissues as a freely soluble form in the cytosol or associated with detergent-insoluble ubiquitinated protein aggregates. The normal response to an autophagic stimulus involves an early increase in p62/SQSTM1 expression in the cytosol, followed by clearance of p62/SQSTM1 associated with aggregates or other cargo. Although not indicated in every instance, assessment of soluble and detergent-insoluble p62/SQSTM1 pools can provide valuable information.\textsuperscript{48} Like LC3, Western blot analysis of p62/SQSTM1 is only a snapshot of a dynamic process. For that reason, measurement of LC3 and p62/SQSTM1 in the presence or absence of lysosomal blockage with chloroquine or bafilomycin A1 provides essential information about the rate of transit of autophagosome cargo through lysosomal degradation (autophagic flux).\textsuperscript{49–52} The Table provides the expected changes in LC3 and p62/SQSTM1 under basal conditions, during initiation of autophagy, at equilibrium upregulated autophagy, when autophagic precursors are limited, and in the setting of impaired flux (acute and chronic).

Also shown are the expected effects of lysosomal blockage in each scenario. Availability of precursors can be limiting if protein synthesis is suppressed (eg, during ischemia). In that case, brisk autophagic flux is reflected by a substantial depletion of available factors.\textsuperscript{53} Besides LC3 and p62/SQSTM1, other autophagy-related factors, including ATG16L1, BECN1, and ATG5–ATG12 complex, commonly rise in parallel with LC3-II and provide supportive evidence, particularly if LC3 blots are of poor quality (see Technical Concerns Specific to LC3 section of this article).

**Immunostaining of LC3 Puncta**

Detection of autophagosomes by immunostaining of LC3 in fixed cells or tissues is technically challenging because of the high background from LC3-I unassociated with autophagosomes; this can be improved by gentle permeabilization of cells (eg, with digitonin or saponin) to extract free LC3-I without affecting membrane-associated LC3-II.\textsuperscript{54} This method is technically challenging and has not been widely accepted, however.

**Technical Concerns Specific to LC3**

LC3 is a fragile low-abundance protein that can be lost from frozen tissue samples with repetitive freeze–thawing, a concern that diminishes for samples stored in SDS-PAGE sample buffer. Heart tissue should be snap-frozen in liquid nitrogen and stored at −80°C until it can be processed. We have compared radioimmunoprecipitation assay buffer (50 mmol/L Tris; 150 mmol/L NaCl; 0.1% SDS; 0.5% sodium deoxycholate; 1% NP-40), a Triton X-100–based detergent extraction buffer (50 mmol/L Tris–HCl; pH, 7.4; 150 mmol/L NaCl; 1 mmol/L EGTA; 1 mmol/L EDTA; 1% Triton-X 100), and radioimmunoprecipitation assay buffer solubilization of tissue ground to a powder at liquid nitrogen temperatures, and have concluded that the most reliable protocol for solubilization of LC3 from frozen heart tissue is with radioimmunoprecipitation assay buffer followed quickly by centrifugation at 1000g to remove nuclei. After removing an aliquot for protein determination, the solubilized sample should be promptly boiled in sample buffer with fresh 2-mercaptoethanol and stored frozen at −80°C until used for gel loading (fresh 2-mercaptoethanol may be added back). LC3 binds more avidly to polyvinylidene difluoride than nitrocellulose (this differential binding is particularly notable for human heart samples) and is readily lost from membranes that are stripped and reprobed. For that reason, LC3 should be the first protein probed for on a membrane. Because of its low molecular weight, transfer time should be kept short (eg, 150 mA for 2 hours). Antibodies to LC3 are available from several sources and vary with respect to isoforms recognized, species specificity, and avidity for LC3-I versus LC3-II. We have found the antibody against LC3A/B (cat 4108; Cell Signaling Technology) to be reliable for rodent heart tissue (rat and mouse) and for porcine and human heart samples.

**Cell Signaling Markers of Autophagy Induction**

Autophagy initiation is controlled by 2 major cell signaling pathways. The AKT/MTOR signaling axis is a well-recognized negative regulator of autophagy, whereas AMPK is known to promote initiation. These opposing signaling pathways converge on ULK1 to dictate the fate of autophagy. ULK1 is important for the nucleation of autophagosomes. Phosphorylation of ULK1 by AMPK on Ser317, 555, and 777 promotes autophagy.\textsuperscript{55,56} In contrast, phosphorylation of ULK1 at Ser757 by MTOR inhibits autophagy initiation.\textsuperscript{56} Markers of MTOR activity include MTOR phosphorylation at Ser2448, phosphorylation of ribosomal subunit RPS6 at Ser235/236, and phosphorylation of the translation repressor protein EIF4E-BP1 at Ser65 and Thr70.

**mRNA Markers of Autophagy**

Measurement of mRNA for autophagy proteins can provide additional insights, but the presence of upregulation at the mRNA level does not necessarily correlate with protein expression or functional autophagy. It can, however, reflect an intact signaling pathway for the induction of autophagy.

**Measuring Mitophagy**

The specific targeting of mitochondria for autophagic disposal during I/R insult is an important element of cardioprotection.\textsuperscript{7,22} Because mitochondrial autophagy is a major pathway for turnover of mitochondria, it is important to be able to monitor mitophagy. Examining mitochondrial content via Western blot or mitochondrial DNA:nuclear DNA ratio must be paired with the same measurement in the presence of lysosomal blockage with chloroquine or bafilomycin A\textsubscript{1}, in order to infer mitophagy. It is important to assess markers in outer mitochondrial membrane (TOMM70 or VDAC) and...
inner membrane/matrix (COX4I1/COX IV or ACO2/aconitase). This is because outer mitochondrial membrane proteins are also subject to degradation by the ubiquitin proteasome system. An observed decrease in mitochondrial mass that is prevented by lysosomal blockade is indicative of mitophagy.

**Use of Fluorescent Reporters to Assess Mitophagy**

The coral-derived fluorescent protein Keima changes its fluorescence properties over the pH range of 4 to 8; when targeted to the mitochondrial matrix (mitoKeima), it serves to report on delivery of mitochondria to the lysosome. MitoKeima holds great promise for monitoring mitochondrial autophagy in cells, and a transgenic reporter mouse would have a considerable use. A somewhat different approach was pioneered by our laboratory: we targeted fluorescent Timer protein to the mitochondrial matrix (MitoTimer). Newly synthesized protein fluoresces green, but the conformation matures >24 to 48 hours to a more stable red fluorescent conformation. This allows detection of mitochondrial biogenesis (green MitoTimer) and mitophagy (decrease in red MitoTimer if new protein is not being made). Using constitutive expression of MitoTimer electroported into mouse skeletal muscle, Lake et al showed that exercise increases turnover of mitochondria (biogenesis and mitophagy), whereas a high-fat diet slows turnover. The advent of a transgenic MitoTimer mouse will doubtless provide additional insights.

**Methods to Assess Autophagy in Intact Animals**

**Measuring Autophagic Flux In Vivo With Lysosomal Blockade**

This method to measure flux introduces a pharmacological blockade that prevents lysosomal/autophagosomal fusion or prevents lysosomal-mediated enzymatic degradation. Several agents are suitable to achieve lysosomal blockade: bafilomycin A, chloroquine, and ammonium chloride raise the intralysosomal pH, preventing autophagosome fusion with the lysosome, whereas protease inhibitors such as pepstatin A, E-64d, and leupeptin inhibit lysosomal proteases and microtubule inhibitors such as vinblastine prevent trafficking of autophagosomes and lysosomes. For in vivo studies, chloroquine (10–50 mg/kg intraperitoneal [IP]), leupeptin (20–40 mg/kg IP), or bafilomycin A (2.5 mg/kg IP every 12 hours) have been used to block autophagic flux. Mice subjected to lysosomal blockade and untreated comparators are euthanized for tissue harvest at a specified time (usually 2–4 hours) after blockade. Lysosomal inhibition results in the accumulation of autophagosomes that would have progressed through the pathway during that period. Differential accumulation of autophagosomes is assessed by microscopy, or autophagosomal LC3-II is measured by Western blot.

Intact autophagic flux is indicated by an increase in autophagosomal puncta or in LC3-II on Western blot when compared with paired animals without lysosomal blockade and is calculated as a ratio rather than the difference (according to Tanida et al). Little work has been done to establish whether the magnitude of increase is proportional to the rate of autophagic flux. We have found that the receptor protein p62/SQSTM1 is a fairly reliable surrogate marker of autophagic flux: when flux is intact, p62/SQSTM1 levels should decrease, whereas when flux is absent, p62/SQSTM1 levels will rise. This is helpful when interpreting results of samples obtained before and after an intervention, such as atrial biopsies obtained at the beginning and end of aortic cross-clamp.

**Measuring Autophagic Flux Ex Vivo in Tissue Samples**

Kaushik and Cuervo have developed a method to measure autophagic flux in small chunks of liver tissue that are minced finely and then divided between 2 wells (with or without lysosomal inhibitors) and then incubated at 37°C for 1 to 2 hours with occasional swirling. The minced tissue is then recovered, homogenized, and processed for Western blotting. We have begun preliminary work to adapt this to heart tissue and are optimistic that this ex vivo flux assay can provide reliable information about the level of autophagy and the presence/absence of autophagic flux in small tissue samples. This approach will eliminate the need for paired animals treated ± lysosomal inhibitors and will also make it possible to monitor flux in freshly obtained biopsies from human tissues.

**Imaging of Autophagic Puncta in Transgenic Mice**

Transgenic mice expressing a fluorescent protein fused to LC3 have been used to monitor autophagy in many studies, although as noted above, it is essential to monitor flux either through comparison with mice in which lysosomal function has been inhibited or inferred from changes in p62/SQSTM1. Cryosections are preferable for preserving fluorescence of the fusion proteins. Puncta can be scored in various ways: as the number per unit area, or as the percentage of area occupied by fluorescent puncta (useful when puncta are so numerous that it is difficult to count individual units). Because autophagosomes are typically more numerous in the perinuclear zone, scoring will be more consistent in sections where myocytes are longitudinally arrayed. Normalizing puncta number (or area) to nuclei may be preferable to normalizing to area. It should be noted that transgensics expressing LC3 fusion proteins may also develop protein aggregates, which can easily be mistaken for puncta. It is important to select a line in which expression is relatively low to reduce the occurrence of aggregates. Although the transgene is not under control of a physiological promoter, the abundance of the fusion protein changes rapidly in response to physiological cues, such as exercise, fasting, or ischemic preconditioning, suggesting that post-translational regulation dominates. Proteasomal inhibition increased the abundance of mCherry-LC3 (C. Perry, unpublished observations, 2008), consistent with findings reported in astrocytes. Several additional caveats should be noted when using LC3 fusion proteins for assessing autophagy. LC3B, which is preferentially detected by one of the commonly used antibodies (rabbit-anti-LC3B antibody; Cell Signaling [4108S]), and which was used for the fusion proteins, is a member of the ATG8 gene family, which has 7 members in humans: MAP1LC3A, B, B2, C, GABARAP, GABARAPL1, and GABARAPL2/GATE-16. There are tissue-specific differences in the expression of ATG8 family members: GABARAPL2 and GABARAPL1 are expressed predominantly in the central nervous system, whereas GABARAP is more heavily expressed in endocrine glands, and LC3C is predominantly expressed in the lung. Different
family members may be responsible for specific roles in protein trafficking and selective target engulfment, and they may be upregulated in response to different cues. It has been suggested that GABARAPL1 interacts with starch-binding domain 1 and therefore may play a role in autophagic degradation of glycogen granules (glycophagy). Thus, monitoring LC3B (including LC3B fusion proteins) may be only part of the story. Noncanonical autophagy (independent of LC3B and ATG5) shares some autophagy machinery and often responds to similar cues.

LC3 fusion proteins can also be monitored by Western blotting; conveniently, the initial degradation of GFP-LC3 in the lysosome gives rise to a fragment that can be readily detected with antibody to GFP.

Use of Tandem GFP-RFP-LC3 to Assess Flux

This approach uses a tandem fluorescent–tagged LC3 protein, monomeric RFP (or mCherry) coupled to GFP-LC3 (mRFP/mCherry-GFP-LC3), to report autophagy induction and flux. A similar approach can be achieved by crossing GFP-LC3 and mCherry-LC3 transgenic mice although theoretical disadvantages of the double-transgenic have been noted. GFP fused to LC3 loses fluorescence rapidly in the acidic environment of the lysosome, whereas mRFP or mCherry retains fluorescence somewhat longer in the lysosome. Thus, autophagosomes fluoresce in both green and red channels, but after fusion with lysosomes, the green fluorescence is lost, leaving only red fluorescence. Samples may be fixed; however, it has been suggested that some fixation protocols may artifactually restore green fluorescence by neutralizing the acidic autolysosome. The group of Sadoshima embedded heart slices in Tissue-Tek OCT (optimum cutting temperature) compound, and 10 micron cryosections were air-dried and fixed in ethanol before counterstaining with DAPI (4’6-diamidino-2-phenylindole) and mounting. Because this fails to preserve lysosomal pH, differences in red versus green fluorescence may either be a function of proteolytic degradation of GFP in the lysosome, which must proceed at a faster rate than degradation of mRFP or mCherry or may be lost on neutralization. To determine flux, the relative abundance of red-only versus red+green puncta are compared. The presence of red-only puncta indicates intact autophagic flux, although here again, there is little information as to whether the relative ratio of red-only to red+green puncta is a quantitative index of flux. A key advantage of this method is that it allows an estimation of flux in a single animal, rather than having to use paired animals (±chloroquine) for each data point. This was used to study autophagic flux after ischemia/reperfusion. The disadvantage is that it is limited to the transgenic mouse line and as such is not available for concurrent use in other mouse lines or other species.

Optical Imaging of Fluorescent Proteins

We hoped to use cardiac-restricted mCherry-LC3 to image autophagy in the heart. mCherry was chosen because its fluorescence properties were better suited to spectral imaging than GFP. We used the Caliper Life Sciences Spectrum In Vivo Imaging System to detect an increase in photon flux from the baseline of 3.19±0.72 to a mean of 3.93±1.10 at 4 hours after administration of rapamycin (2 mg/kg IP) and chloroquine (10 mg/kg IP; P<0.01; n=14; Figure 2). This approach raised hope that it would be possible to image autophagy in vivo.

Figure 2. Optical imaging of autophagy in hearts of mCherry-light chain 3 (LC3) mice. A, Mice were imaged at baseline (Pre) and 4 hours after rapamycin and chloroquine administration (Post), using a protocol of 3 acquisitions of 15 s each. Representative images from a single mouse are shown. B, Graph shows the change in fluorescence from baseline to 4 hours after rapamycin and chloroquine administration (n=14 mice; each point represents the average of 3 acquisitions). C, Cryosections of typical hearts of mCherry-LC3 mice under fed (left) and fasted (right) conditions, showing the typical increase in total fluorescence and the increase in number of fluorescent red puncta (autophagosomes). Nuclei are stained blue with DAPI (4’6-diamidino-2-phenylindole; images from A and B are reprinted from Circulation Research with permission of the publisher [copyright ©2011, Wolters Kluwer Health]. Abstract P066: Imaging Autophagy in Living Mice v109:AP066, 2011. Unpublished images from C provided by Dr Chengqun Huang and Dr Roberta A. Gottlieb, 2010).
A cathepsin-activated near-infrared fluorochrome (CAF-680) was used to demonstrate an 8-fold increase in fluorescence 2 hours after rapamycin administration followed by ischemia/reperfusion. The signal was localized to lysosomes as documented by colocalization with LAMP2 in immunostained cryosections. Interestingly, Feraheme (Ferumoxytol) nanoparticles that are fluorescently tagged with CyAl5.5 are taken up via endocytosis at a much faster rate when autophagy is stimulated with rapamycin, suggesting that it may be possible to infer autophagic activity from retention of such nanoparticles, which can be readily imaged by MRI.

Delayed enhancement after injection of chelated Gd MRI contrast agents (late gadolinium enhancement [LGE]) has been noted in a variety of conditions and has been attributed to slower clearance from tissue because of fibrosis or the presence of macrophages that take up the agent. However, many of the pathological processes that give rise to fibrosis and LGE are also associated with increased autophagy, including scleroderma, heart failure, radiation-induced myocardial injury, Friedreich ataxia, and Fabry disease. It is possible that some instances of LGE are because of upregulation of autophagy rather than fibrosis or macrophage activity, but this will need to be formally tested.

### Assessment of Autophagy in Human Subjects

Detection of autophagy in humans is restricted to biochemical or microscopy analysis of biopsies of the tissue of interest or analysis of peripheral blood, thus severely limiting our ability to understand its role in human disease or to develop effective therapies. Kassiotis et al reported a decrease in autophagic markers in hearts of patients who received a left ventricular assist device; they inferred that autophagy was an adaptive response in the failing heart, and that mechanical unloading relieved the stress responsible for triggering autophagy. Garcia et al studied autophagy markers by western blot and electron microscopy of atrial appendage tissue obtained during coronary artery bypass graft surgery; they noted that postoperative atrial fibrillation was more common in patients with evidence of impaired autophagic flux. Jahania et al detected autophagic flux in atrial tissue in patients undergoing cardiopulmonary bypass based on changes in p62/SQSTM1 and other autophagy proteins, and found that the magnitude of p62/SQSTM1 clearance was inversely related to mortality and morbidity risk scores. However, Gedik et al examined autophagy markers (by Western blot) in left ventricle of patients who underwent remote ischemic preconditioning before coronary artery bypass graft surgery and saw no evidence of enhanced autophagy compared with nonpreconditioned patients, or did they detect evidence of an autophagic response during early reperfusion in either group. A study conducted by Singh et al examined autophagy gene expression by polymerase chain reaction and Western blotting for autophagy proteins and regulators AMPK and MTOR. They identified upregulation of mRNAs for autophagy components, including LC3B, ATG4A, ATG4C, and ATG4D, as well as WIPI1, GABARAP, and GABARAPL2; they also observed an increase in LC3-II from beginning to end of cardiopulmonary bypass and an increase in phospho-AMPK and a drop in protein levels of MTOR. Interestingly, they did not see a drop in p62/SQSTM1 levels. The results differ widely between these various studies, making it difficult to establish specific guidelines to apply to interpreting results, or even to generalize about the findings. Although the studies of atrial tissue noted changes in autophagy, this was not the case for ventricular samples, although because no study compared atrial and ventricular samples, it is not possible to know whether the human left ventricle is unresponsive when compared with the right atrium or whether the procedural differences in that study accounted for the lack of change in autophagy markers. More work will be needed to resolve these questions, but the difficulties in obtaining human heart tissue pose substantial obstacles to a comprehensive study.

Systemic conditions that affect autophagy, such as drug exposure, fasting, or inflammation, might be expected to have parallel effects in multiple tissues. That rationale has led some investigators to examine autophagy in peripheral blood leukocytes. Autophagy markers were assessed in CD4 lymphocytes of men subjected to different exercise regimens or sedentary...
controls after 5 weeks of training; they found that exercise conditioning (especially high-intensity interval training) triggers autophagy and suppresses apoptosis in lymphocytes when the subjects undergo a hypoxic exercise challenge.93 The authors did not go so far as to suggest that the autophagic response in lymphocytes might reliably report on autophagy in other organs, but 2 studies by another group found a significant reduction in autophagy markers (by Western blotting) in leukocytes of patients with coronary artery disease94 or acute myocardial infarction95 when compared with healthy controls. However, none of these studies correlated autophagy markers in leukocytes with those in heart tissue, so it remains to be seen whether peripheral blood cells represent a reliable reflection of autophagic activity in the heart. Dysregulation of autophagy was also noted by the same group in peripheral leukocytes of patients with sporadic Parkinson disease,96 suggesting that underlying abnormalities that affect autophagy in the brain may be paralleled in leukocytes.

Recent advances in flow cytometry-based approaches to measuring autophagy hold promise for broader use of leukocyte autophagy as an index of organism-wide autophagic competence.97,98 These approaches are based on flow cytometric image analysis of LC3-decorated autophagosomes; however, given the parallel upregulation of lysosomal activity, cathepsin-based fluorogenic reagents or lysosomotropic dyes may provide an adequate readout if appropriate controls and validation studies are included.

Conclusions

Autophagy is increasingly recognized to play an important role in mitigating or exacerbating various cardiovascular diseases; hence, it is important to be able to measure it accurately and to understand the significance of the findings. Given the highly dynamic nature of autophagy, it is essential to be able to account for autophagic flux. Current methods are imperfect, but newer technologies are emerging and there is hope that noninvasive imaging modalities may be developed for in vivo animal and human investigations.

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References

24. Zhang YL, Yao YT, Fang NX, Zhou CH, Gong JS, Li LH. Restoration of autophagic flux in myocardial tissues is required for cardioprotection of...


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by contrast-enhanced cardiovascular magnetic resonance. Radiol Med associated with subsequent hospitalization for heart failure. late gadolinium enhancement cardiovascular magnetic resonance is asso-
Codogno P, Mehrpour M, Priolkas-Cezanne T. Canonical and non-canoni-
Kimura S, Noda T, Yoshimori T. Dissection of the autophagosome matura-
Choi KM, Nam HY, Na JH, Kim SW, Kim SY, Kim K, Kwon IC, Ahn HJ. A monitoring method for Atg4 activation in living cells using peptide-
conjugated polymeric nanoparticles. HJ. A monitoring method for Atg4 activation in living cells using peptide-
Frech T, De Domenico I, Murthaugh MA, Reveleo MP, Li DY, Sawitzke AD, Drakos S. Autophagy is a key feature in the pathogenesis of systemic scler-
Wong TC, Pfehler KM, Zareba KM, et al. Myocardial damage detected by late gadolinium enhancement cardiovascular magnetic resonance is asso-
Umezawa R, Ota H, Takamaki I, Ichinose A, Matsushita H, Saitoh K, Takase K, Jinga M. MRI findings of radiation-induced myocardial dam-
Untangling Autophagy Measurements: All Fluxed Up
Roberta A. Gottlieb, Allen M. Andres, Jon Sin and David P.J. Taylor

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