AUTOPHAGY: A CORE CELLULAR PROCESS WITH EMERGING LINKS TO PULMONARY DISEASE

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Online Data Supplement

SUPPLEMENTARY TABLE 1

Methodology	Rationale	Advantages	Limitations
EM quantification of autophagosomes (81)	In starved hepatocytes, the number of autophagosomes per cell correlates with macroautophagic flux.	Unambiguously verifies that autophagosomes are being formed.	Labor intensive and expensive. Elevated autophagosome number does not always correlate with macroautophagic flux in all models.
GFP-LC3 clustering (82, 165)	In starvation models, relocalization of GFP-LC3 from a diffuse to punctate pattern of staining correlates with autophagosome formation.	Applicable to both tissue culture systems and mice. Can indentify cell-specific regulation of macroautophagy in heterogeneous tissues or samples.	When used as a static measurement, does not reliably correlate with autophagic flux. GFP-LC3 clusters can precipitate as aggregates in the cytosol, so not all punctuate GFP-LC3 structures are autophagosomes (166, 167).
GFP-RFP-LC3 clustering (84)	Similar to GFP-LC3	Can differentiate between early ($green^+$, red^+) and late ($green^-$, red^+) autophagosomes. As a result the rate of autophagosome-lysosome fusion can be inferred.	Assumes RFP-LC3 half-life is similar in all circumstances. Currently unclear how this readout correlates with dynamic measurements of macroautophagic flux.
GFP-LC3 cluster life span (time lapse video microscopy). (72)	LC3b-GFP clusters are identified as they appear and then are followed using time-lapse live	Dynamic measurement of macroautophagic flux.	Requires live imaging microscopy setup. Time intensive.

	microscopy until they disappear. Macroutophagic flux can then be expressed in terms of the average life-span of GFP-LC3 puncta or, alternatively, the rate at which GFP-LC3 puncta disappear per cell.	Quantitative.	Applicable to tissue culture models only. Not all GFP-LC3 puncta are autophagosomes.

Table S1: Microscopy-based methods for monitoring macroautophagy.

SUPPLEMENTARY TABLE 2

Methodology	Rationale	Advantages	Limitations
LC3-II abundance on western blot (87)	LC3b-II levels on western blot correlate with autophagosome number.	Easy to perform Can be applied both to cells and tissue samples.	Does not reliably correlate with macroautophagic flux.
p62/SQSTM1 abundance on western blot (89)	p62 is incorporated into autophagosomes by binding to LC3b-II during formation. Assuming p62 synthesis is constant, a decline in static p62 levels should correlate with increased turnover via macroautophagy.	p62 ferries protein aggregates for degradation and therefore is a marker of selective or quality control macroautophagy.	Interpretation requires additional analysis of mRNA levels to exclude transcriptional regulation as a cause of changes in p62 protein abundance.

Table S2: Static biochemical measurements for monitoring macroautophagy

SUPLEMENTARY TABLE 3:

Methodology	Rationale	Advantages	Limitations
Bulk protein turnover assay (26, 37, 41, 168)	Cells or tissues are loaded with radio-isotope labeled amino acids. The rate of protein turnover is then measured in terms of the amount of radioactivity liberated from TCA insoluble protein stores over time.	Gold standard for macroautophagic flux assays. High assay sensitivity and precision Wide linear range of measurement	Technically cumbersome. Requires concomitant use of 3-MA to determine the component of bulk protein turnover degraded by autophagy. Does not distinguish between selective and non-selective macroautophagic activity.
LC3b-II turnover assays (95-98, 169, 170)	LC3b-II inserted into the luminal autophagosome membrane surface is normally degraded. By administering an inhibitor of Cathepsins luminal LC3b-II can be rescued. Macroautophagic flux can then be expressed as the difference in LC3b-II signal on western blot in the presence versus the absence of inhibitor.	Technically simple Can be applied *in vitro* and *in vivo*. Easily quantifiable readout.	Measures autophagosome membrane turnover but the quantitative relationship between LC3b-II turnover and bulk protein turnover is undefined. Makes no distinction between selective and non-selective forms of macroautophagy.
p62 turnover assays (89, 90, 95)	Similar to LC3b-II turnover assays above.	Can measure rate of p62-driven selective macroautophagy.	Does not measure general macroautophagic flux.
Polyglutamine repeat-luciferase	Luciferase fusion proteins containing	Provides information on the	Does not measure the turnover of

protein turnover assay. (98)	long (80-residue) repeats of polyglutamine form protein aggregates that are cleared by autophagy while fusion proteins containing shorter length (19-residue) repeats do not. These constructs are individually expressed in cells and luminescence is determined. A lower ratio of polyQ80:polyQ19-luciferase signal reflects increased clearance of the larger aggregated protein by macro autophagy. A higher ratio of polyQ80:polyQ19-luciferase signal implies decreased macroautophagic flux.	capacity of macroautophagy to clear an exogenous load of protein aggregates Includes an internal negative control protein to provide normalization of the readout. Simple quantification via luciferase luminescence. Can be utilized in vitro and in vivo.	endogenous maacroautophagy substrates. Validated for *in vivo* use only in skeletal muscle so far.
Cytosolic protein sequestration assays. (100, 171)	Cells or tissues are incubated with leupeptin to inhibit lysosomal proteolysis and autophagosomes are purified via electrodisruption or mechanical disruption followed by density centrifugation. Flux is expressed in terms of the amount of cytosolic	Quantitative measurement of bulk macroautophagic flux.	Technically cumbersome to perform on multiple samples. Best suited for tissue culture models.

	protein, such as LDH or BHMT, recovered in the autophagosome fraction.		
LC3b, p62 and NBR1 half-life measurements. (85, 101)	LC3b, p62 or NBR1 proteins that are fused either to GFP or luciferase are transiently expressed. The rate of decay in these proteins is then monitored by fluorescence or luminescence. Macroautophagic flux is expressed in terms of the half-lives of these fusion proteins.	Direct, quantitative determination of macroautophagic flux.	The rate of decay of GFP-LC3 is markedly longer than endogenous LC3b, implying GFP may potentially impose artifacts on the measurement of flux. Applicable to tissue culture systems only.
ATG4 activity assay. (172)	ATG4 proteases are responsible for processing LC3b from its pro-form to LC3b-I and also for recovering LC3b-II from autophagosome membranes. The rate of ATG4 activity should therefore correlate with the rate of autophagosome membrane processing and hence macroautophagic flux.	Simple quantitative measurement.	Applicable to tissue culture cells only. Requires administering nanoparticles containing fluorescent ATG4 substrates ,which may trigger innate inflammatory responses in some cells.

Table S3: Dynamic biochemical measurements for measuring macroautophagy.