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Edited by

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ISBN: 978-1-58829-853-9

e-ISBN: 978-1-59745-157-4

Library of Congress Control Number: 2007940755

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Background: An electron micrograph of a nascent autophagosome in a normal rat kidney cell. The autophagosome limiting membrane is visible as a dark line. Endoplasmic reticulum cisternae with attached ribosomes surround the autophagosome on two sides. Endoplasmic reticulum is also among the contents captured by the autophagosome. Golgi ribbon is seen on the bottom right. Prepared by Eeva-Liisa Eskelinen, University of Helsinki.

Inset: A novel autophagy reporter, tandem monomeric RFP-GFP-tagged LC3 (tfLC3) can be used to distinguish between autophagosomes and autolysosomes. The GFP signal is sensitive to the acidic and/or proteolytic conditions of the autolysosomal lumen, and thus GFP fluorescence is lost in autolysosomes, whereas mRFP is more stable and its fluorescence persists. Therefore, colocalization of GFP (green) and RFP (magenta) fluorescence indicates a compartment, such as the phagophore or an autophagosome, that has not yet fused with a lysosome. In contrast, an mRFP signal without GFP fluorescence corresponds to an autolysosome, as evidenced by colocalization with Lamp1 (cyan). Prepared by Shunsuke Kimura, Takeshi Noda, and Tamotsu Yoshimori, Osaka University.

Printed on acid-free paper

9 8 7 6 5 4 3 2 1

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Preface

The intent of this volume is to provide a comprehensive resource with detailed methods for study of two distinct but partially morphologically similar processes of autophagy and phagocytosis. Autophagy is a rapidly growing field, and there is a need for standards of assessment in identification of autophagosomal organelles and for monitoring various aspects of autophagic functions. Phagocytosis is a relatively mature field that has established methods but can benefit from an update on the current trends. Finally, cross-pollination between the two fields is of interest. Although cross-cutting studies between phagocytosis (which could be viewed as a special case of autophagy of a cell's exterior) are presently few and far between, it is possible that a merger of methods in both fields will prompt further explorations of similarities and differences.

The collection of methods described in this book should allow the reader to find appropriate techniques to identify, monitor, and quantify autophagic processes in cellular and animal models of autophagy. Since the basic autophagic machinery is highly conserved, these methods can be applied nearly universally—of course with appropriate and judicious modifications. Among the core battery of assays are: (1) GFP-LC3 (Atg8) puncta formation, monitored by fluorescence microscopy; (2) lipidation of LC3 and associated electrophoretic mobility shift, monitored by immunoblotting; (3) ultrastructural analysis by electron microscopy; and (4) proteolysis of stable proteins by monitoring radioactive amino acid release during autophagic turnover. These techniques can be complemented by less specific but relatively quick methods of staining with acidotropic dyes (lysotracker and monodansylcadaverine) and more importantly mechanistic studies using pharmacological agonists and antagonists and, very importantly, siRNA knockdowns of key autophagic proteins (e.g., Beclin 1, Atg5, Atg7). Somewhat less accessible, but very important, are Atg knockout cell lines and transgenic animals, including murine and fly models. The core methodologies and approaches are applicable whether the objective is to study cell survival, cell death, cancer, neurodegeneration (Huntington's, Alzheimer's, Parkinson's diseases), development, aging, intrinsic (cell-autonomous) resistance to infection, innate and adaptive immunity, antigen processing, T- or B-cell homeostasis, and numerous other

health and disease states. The chapters in this volume, from a number of authorities in the field of autophagy, should facilitate work in laboratories with or without prior experience in autophagy research.

There are many important questions to be answered regarding fundamental and applied aspects of phagosomal biology, apart from the partial overlaps with the autophagic pathway highlighted here. The methods described in this volume should allow researchers to find in one place several modern techniques for in vitro and in vivo studies of phagosomal organelles.

While this book was in its production stages, several autophagy methods and biological relationships of high relevance have been published, attesting to the impressive speed at which this field is moving. These are: (i) A work in *Nature* (**1**), directly linking autophagy and phagocytosis, along the lines anticipated in this book and touched upon in Chapter 1. (ii) A multi-author comprehensive discussion on the use and limitations of various autophagy assays (**2**). The interested reader is advised to consult this text. (iii) An important methodological refinement on how to monitor and quantify LC3-I-to-II conversion by immunoblotting (**3**). This method calls for comparison of samples from cells treated with a putative inducer of autophagy in the presence and absence of Bafilomycin A (an inhibitor of acidification and maturation of autophagosomes into degradative organelles). The intensity of the LC3-II band is compared to the intensity of actin (unlike comparisons to LC3-I, as often done in the past). A suspected inducer of autophagy under examination is expected to increase the intensity of the LC3-II band (relative to actin) in the sample treated with both the putative inducer and Bafilomycin A when compared to the intensity seen in a parallel control (without the putative inducer) treated only with Bafilomycin A. (iii) Another significant advance is the use of a tandem RFP-GFP-LC3 fusion (instead of single GFP-LC3 fusion) to monitor LC3 puncta by imaging (see book cover) (**4**). This assay is based on differential sensitivity of GFP and RFP to luminal pH in autophagic organelles: GFP is pH-sensitive but RFP is not. Here, doubly positive puncta (green⁺red⁺; or yellow when green and red images are merged) represent newly induced autophagosomes, while singly positive (green⁻red⁺) puncta represent autophagic organelles that have acidified and matured into degradative organelles. By consulting these new methodological developments and using the detailed, step-by-step protocols in this volume, the researchers entering or already working in this field will have a full panel of methods at their fingertips.

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January 2008, Placitas,
New Mexico, USA

Vojo Deretic

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