Review

Optical Imaging Paves the Way for Autophagy Research

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Autophagy is a degradation process in eukaryotic cells that recycles cellular components for nutrition supply under environmental stress and plays a double-edged role in development of major human diseases. Noninvasive optical imaging enables us to clearly visualize various classes of structures involved in autophagy at macroscopic and microscopic dynamic levels. In this review, we discuss important trends of emerging optical imaging technologies used to explore autophagy and provide insights into the mechanistic investigation and structural study of autophagy in mammalian cells. Some exciting new prospects and future research directions regarding optical imaging techniques in this field are also highlighted.

The Rise of Optical Imaging

In the last few decades, optical imaging has greatly enhanced our ability to view the world microscopically [1]. Optical bioimaging and biophotonics, which provide visible detailed information about the target sample, have been used widely in advancing biomedicine at scales ranging from a single molecule to tissues [2]. The huge demands of biological specimen detection have strongly promoted the development of bioimaging instrumentation. According to the desired spatial resolution (see more details in Table 1), optical bioimaging can be achieved mainly through wide-field microscopy, confocal microcopy, multiphoton microcopy, and super-resolution microcopy. For example, organic and inorganic nanoparticles, small-molecule fluorophores, and colorful fluorescent proteins can be used alone or conjugated with other bioactive compounds to image the cell or living tissues [2]. In addition, in biomaterial and biomedicine sciences, near-infrared fluorophores are emerging for imaging and can penetrate tumor tissue or other human organs [3].

Autophagy, a degradation process of cellular contents and pathogens, occurs under starvation or intracellular stress in eukaryotes to recycle nutrients and maintain cell homeostasis. This balanced condition refers to the cytoplasm or other unwanted cellular components such as endoplasmic reticulum (ER), mitochondria, nucleus, peroxisomes, ribosomes, or invading pathogens being converted into amino acids, glucose, and lipids by lysosome/vacuole hydrolysis and then returning back to the cytoplasm for energy supply and biomacromolecule synthesis (Figure 1). This autophagic process is key to maintaining cellular environment homeostasis and acts as a stress response to the hard environment. Autophagy-related research has been extended to multiple organisms from yeasts to humans because autophagy has been found to have critical roles in almost all kinds of major human diseases known. Some of the most important discoveries of autophagy have been revealed using electron microscopy (EM; Box 1). However, EM can only show cellular structures at selected time points and requires professional skills. The interpretation of an EM image also usually requires expertise. Optical bioimaging is widely acknowledged as a promising tool in autophagy research. Under a light microscope, the dynamic activities of an autophagosome were observed *in vivo* with weak

Trends

Various fluorescent bioprobes have removed challenges in monitoring living organisms and cellular and subcellular structures with optical bioimaging instrumentation.

Super-resolution imaging technology at the individual biomacromolecule scale could quantitatively monitor autophagy-related components in living systems in real time.

To directly answer questions about structure, a super-resolution imaging method is needed to reveal more detailed information about autophagy, such as the core autophagic machinery, concerning the initiation of the isolation membrane; the interaction of Atg proteins and the ER; the formation of the autophagosome; and the dynamic process of autophagic cargo degradation.

More and more chemical fluorescent compounds with fluorophores, and nanomaterials with special fluorescent characteristics, will be employed for precisely microimaging autophagic compartments.

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fluorescence background signals in transgenic mice [4]. However, optical bioimaging using traditional fluorescence microscopes faces a resolution limit due to optical diffraction.

Ultrastructural characterization (at a scale of 10–100 nm) of live cells used to be difficult in the 20th century. The dimensions of intracellular proteins, membrane structures, and some biological organisms, such as viruses, actin cytoskeletons, ribosomes, microtubulin, and actin, are smaller than the light diffraction limit. Therefore, characterizing these structures calls for fluorescence imaging of super-resolution technology [5,6]. Thanks to breaking the optical diffraction limit, super-resolution microscopy (Box 2) has been used to provide new insights into autophagy research, such as demonstrating the origin of the autophagosomal vesicles [6]. There are several reports in which super-resolution bioimaging methods have been employed to image autophagy-related structures (Figure 2). In this review, we summarize the newly emerging progress on optical imaging (mainly referring here to optical fluorescence bioimaging), describe the optical bioimaging methods that have emerged during the past decades, which are now used in autophagy research, and look ahead to the possible exciting areas that demonstrate the core machinery of **selective autophagy** (see Glossary) emphasized in mammalian cells using high- or super-resolution bioimaging technologies. This primer can be referred as a guide for researchers to explore autophagy in the post-Nobel Prize era in the autophagy field.

Classification: Optical Imaging Technologies for Autophagy

Optical imaging, which mainly involves fluorescence imaging, bioluminescence imaging, chemiluminescence imaging, and Raman imaging, can obtain noninvasive, 2D or multidimensional image data at both the micro and macro scales [7]. Compared with biochemical assessment methods, such as western blotting, flow cytometry, and immunohistochemistry, optical bioimaging, which mainly refers to a fluorescence bioimaging method, is more widely accepted by researchers. This is in part because the fluorescence imaging results are intuitive, less time consuming, and more easily interpreted than those of biochemical methods, especially for a beginner. So far, optical bioimaging techniques have been used in cellular function investigations in physiological and biochemical processes in cell biology, developmental neurobiology, medical diagnosis and treatment, pharmacological research and discovery, and analysis of animal models [8]. In addition, systematic studies on programmed cell death and apoptosis have been promoted by optical clinical imaging techniques [9]. Nanoparticle probes using **quantum dots (QDs)** have been developed for cancer tissue imaging in living animals [10]. Finally, imaging probes and near-infrared optical imaging techniques for the *in vivo* diagnosis of neurodegenerative diseases and breast cancer have been well described in recent reviews [11,12].

Fluorescence microcopy makes it convincing to visualize biological samples via fluorescence colocalization. In autophagy research, the marker of the autophagosome, a protein called microtubule-associated protein light chain 3 (LC3), is usually labeled with one or more exogenous fluorescently labeled proteins [GFP or red fluorescent protein (RFP)] to monitor the amount of autophagic membrane [13]. Those bioimaging technologies shown in Table 1, usually integrated with specific optical probes, all of which are commercially available or currently being marketed, broaden the scales of biological structures and activity ranging from single molecules to the whole cell. Therefore, remarkable new insights into diverse areas depend on advances in bioimaging for its qualitative and quantitative data [14].

The Union of Light Microscopy and Electron Microscopy

The good news is that a newly emerging imaging technique, **correlated light and electron microscopy (CLEM)**, strikes a balance between light microscopy and EM. CLEM makes it possible to simultaneously observe the cellular contents labeled with a fluorescent probe and

Glossary

Aggregation-induced emission: An uncommon photophysical phenomenon in which luminogens are non-emissive in the dispersive state but become excited and luminescent when they are concentrated as aggregates.

Autophagic flux: a comprehensive index that evaluates the total development of autophagy.

Correlated light and electron microscopy (CLEM): a new imaging method that combines fluorescence microscopy and electron microscopy, which can be used to analyze the proteins of interest both in a biological context and in a cellular ultrastructure scale.

Endoplasmic reticulum exit sites (ERESs): the transitional or ribosome-free endoplasmic reticulum exit sites where the coat protein complex II (COPII) vesicle complex assembles, mediating the accumulation of secretory vesicles or cargoes on the ER.

Imaging flow cytometry (IFC): a newly developed technology that combines multiparameter and high-throughput analysis of the conventional flow cytometry and the cellular location information of fluorescence microscopy.

Omegasome: membrane compartments originating from the ER that form at the initial stage of autophagy, mediating the origin of the autophagosome.

Point spread function (PSF): the dimensional distribution pattern of a single fluorescent object that forms a focal but blurry spot with a finite size under an optical microscope, which results from light diffraction.

Quantum dots (QDs): fluorescent semiconductor materials with dimensions of a few nanometers, which are widely used in imaging of living cell, tissue, and medical diagnostics.

Reversible saturable optical fluorescence transitions: a nonlinear optical imaging technique where the resolution is not limited by the diffraction limit of light. It relies on the photoswitching of proteins and can provide a 3D structure of the specimen.

Saturated structured illumination microscopy: a super-resolution imaging method based on spatially patterned excitation, using strong excitation light to quickly change the

obtain the ultra-high resolution structural information of the same targets. The idea of the CLEM technique was first demonstrated two decades ago, while the autophagy-related work in cell biology using this technique emerged during the recent decade partially due to the newly developed QDs [15] and fluorescent proteins [16]. Hanson and coworkers [17] described a standardized method of CLEM to image the autophagosome using GFP-tagged LC3 and performed control experiments with laser scanning confocal microscopy (LSCM) and differential interference contrast microscopy. Meanwhile, LSCM was also used to investigate the autophagosome formation [18] and the role of the Atg9 protein in the initial stage of autophagy [19]. However, the applications of CLEM in the life sciences field are still limited by extensive sample-preparation procedures and the inability to develop compatible hardware and software that connects the light microscope with an electron microscope. Moreover, the spatial resolution of CLEM under fluorescent imaging mode is still confined to the diffraction limit of the optical imaging instrument integrated in this combinational imaging system.

The traditional wide-field fluorescence microscopy (WFM) now faces two limitations (Box 3). First, its spatial resolution is limited to the optical diffraction limit. Second, the objective lens of a WFM collects the emission light from the whole depth of the target specimen of interest, producing confusing and uncertain imaging results [20]. Recently, compared with the 250-nm resolution of LSCM, deconvolution algorithms and nonlinear structured illumination have been developed to improve the 2D imaging resolution to 50 nm [21]. In addition, combining two opposite objective lenses in a WFM, namely, the I^5M high-resolution fluorescence microscope, Gustafsson and colleagues [22] improved the axial resolution of wide-field light microscopy from 500 (for LSCM) to 100 nm. In another way, the wide-field fluorescence microscope can also be integrated with other functional modules of electronic devices and optical units to extend its applications in cell biology, such as for real-time live-cell imaging or animal tissue imaging.

Confocal Imaging

A fluorescence microscope equipped with laser beams, which can excite the emission light with a specific wavelength to present the image, is called 4Pi confocal microscopy [23] or LSCM [24]. This imaging method can provide images at various depths. Ultimately, a 3D image of the target sample at a lateral resolution down to 0.1–0.5 μm can be obtained [23,25], which is able to identify the smallest organelle in eukaryotic cells. In LSCM imaging, the whole processes of mitochondrial remodeling and mitochondrial autophagy (mitophagy), the fusion of the autophagosome and lysosome, and the degradation of the sequestered cellular contents at a high resolution were successfully tracked in Chinese hamster ovary (CHO) cells [26,27]. The marker proteins of autophagy, LC3, p62 (sequestosome-1, *SQSTM1*), and *NBR1* (neighbor of *BRCA1*), can be imaged by LSCM. In selective autophagy, the cytosolic cargos were recognized by receptors and then enclosed by the double membrane of autophagosomes, whose sizes (1–5000 nm) depend on the autophagic substrates [28]. Therefore, during the degradation process in the autolysosome, the autophagic vacuole can be quantitatively analyzed by immunofluorescence with the high resolution of LSCM [29]. The advantage of LSCM is that it allows scanning targets layer by layer to obtain a high-resolution and 3D reconstruction imaging.

Imaging Flow Cytometry

In the last decade, research work on analyzing autophagy by flow cytometry has continued to grow. Traditional flow cytometry can simultaneously measure multiple parameters of individual cell in biophysics and biochemistry studies and provide reliable, high-throughput, and statistically robust data about many cells. However, information about spatial resolution, morphological differences between single cells, and the subcellular colocalizations of specific microscopic structures such as the autophagosome and lysosome is out of reach. These shortcomings

fluorophore from excited state to ground state, and finally breaking the resolution limit of traditional light microscopy.

Selective autophagy: A cellular physiological process, mediated by some specific autophagy receptor proteins, in which unwanted components are removed and degraded in eukaryotic cells for nutrition supply.

Stochastic optical reconstruction microscopy (STORM): a super-resolution microscopy based on the single-molecule localization method. Activating and deactivating a single fluorescent molecule can obtain a super-resolution image.

UNC-51-like kinase 1 (ULK1): a mammalian serine/threonine protein kinase and a key autophagy-related protein complex that mediates the initiation of autophagy.

Table 1. High/Super-Resolution Imaging in Autophagy Research[a]

Imaging technologies	Fluorescent probes (fluorescent proteins or dyes)	Related autophagic protein markers	Lateral resolution	Location of autophagic structures	Cell line models	Disease models	Refs
LSCM	mCherry, eGFP, RFP, Alexa 488, FITC	LC3B, CD63, FAM134b	250 nm	Autophagosomal membranes, lysosome, ER	U2OS, A549	Sensory neuropathy	[49]
TIRFM	Alexa Fluor 647, CFP, GFP, dsRed, eGFP	LC3B, paxillin	~60 nm	Endosome, autophagosome, omegasome, ER	Neutrophils, HEK293, 4T1	Human sepsis Human tumor cell metastasis	[74–76]
SIM	dsRed, DyLight 488 and DyLight 594, GFP, mCherry	Atg8, LC3 and VWF, Atg13, Atg9A, and VMP1	80 nm	Phagophore, autophagosome, ER	Yeast, HUVECs, HEK293, MEFs	Von Willebrand disease	[35,72, 77,78]
STED	GFP, Alexa Fluor 532, and Alexa 488	LC3, and ORF-9b, Rab7 and CD40	50–70 nm	Mitochondria	A549, mouse B cells	SARS-CoV infection	[79,80]
dSTORM	Alexa 647, CF568, GFP	Atg9, Atg13, Atg16, LC3	20–40 nm	ER, autophagosome	HEK293	Not addressed	[5,37]
CLEM	GFP, mRFP, Alexa 647	LC3, Atg9	0.1–10 nm (depends on the integrated EM)	IM, autophagosome, vesicles	Hela, zebrafish larvae, HEK293	MTB infection	[18,19,42]

[a]Abbreviations: 4T1, murine mammary carcinoma cell line; CFP, cyan fluorescent protein (max excitation/emission, 435/485 nm); dsRed, a red fluorescent protein variant from coral (max excitation/emission, 558/583 nm); dSTORM, direct stochastic optical reconstruction microscopy; eGFP, enhanced green fluorescent protein (max excitation/emission, 484/507 nm); FITC, fluorescein isothiocyanate (max excitation/emission, 488/525 nm); GFP, green fluorescent protein (max excitation/emission, 488/510 nm); HEK293, human embryonic kidney (HEK) 293 cell line; HUVECs, primary human umbilical vein endothelial cells; IM, isolation membrane; mCherry, a fluorophore with max excitation/emission, 587/610 nm; MEFs, mouse embryonic fibroblasts; mRFP, monomeric RFP (max excitation/emission, 584/607 nm); MTB, *Mycobacterium tuberculosis*; ORF-9b, a protein encoded by open reading frame-9b of SARS-CoV; RFP, red fluorescent protein (max excitation/emission, 555/584 nm); SARS-CoV, severe acute respiratory syndrome coronavirus; STED, stimulated emission depletion microscopy; TIRFM, total internal reflection fluorescence microscopy; VMP1, vacuole membrane protein 1; VWF, von Willebrand factor.

have been overcome by a newly developed instrument with more comprehensive functionality, **imaging flow cytometry (IFC)**, which is also known as multispectral IFC. Integrated with the capabilities of conventional flow cytometry, IFC can image a cell similar to a fluorescence microscope in a high-throughput manner (over 1000 cells/s under the imaging mode) and was first applied to quantification of autophagosomes by Lee and coworkers [30]. Reports from two research groups further extended the applications of IFC in the autophagy field. First, a statistically robust method that evaluated the **autophagic flux** in human primary cells was presented [31], and a technique that assesses the level of autophagy and apoptosis has also been established [32]. The discrimination of the autophagosome from the autolysosome, and autolysosome formation, can also be measured by IFC, while the precise location information of autophagic compartments still cannot be obtained [33]. With unprecedented perspectives in the autophagy field, IFC will be one of the most powerful methods combining the advantages of traditional flow cytometry and fluorescence microscopy (FM).

Super-Resolution Fluorescence Imaging

During the past decade, some new super-resolution imaging methods have been established in cell biology (Figure 2). Although only a few research groups have considered the applications of super-resolution methods in the autophagy area, an increasing number of important challenges have been resolved [34]. In 2013, the interaction between the autophagosome and ER during the prolongation of the phagophore was demonstrated through the use of super-resolution fluorescence imaging [35]. In both yeasts and mammalian cells, a 3D structured illumination microscope (3D-SIM) was used to observe the autophagosome being captured into the vacuole and ER. The contact point of these two organelles provides the autophagosome with
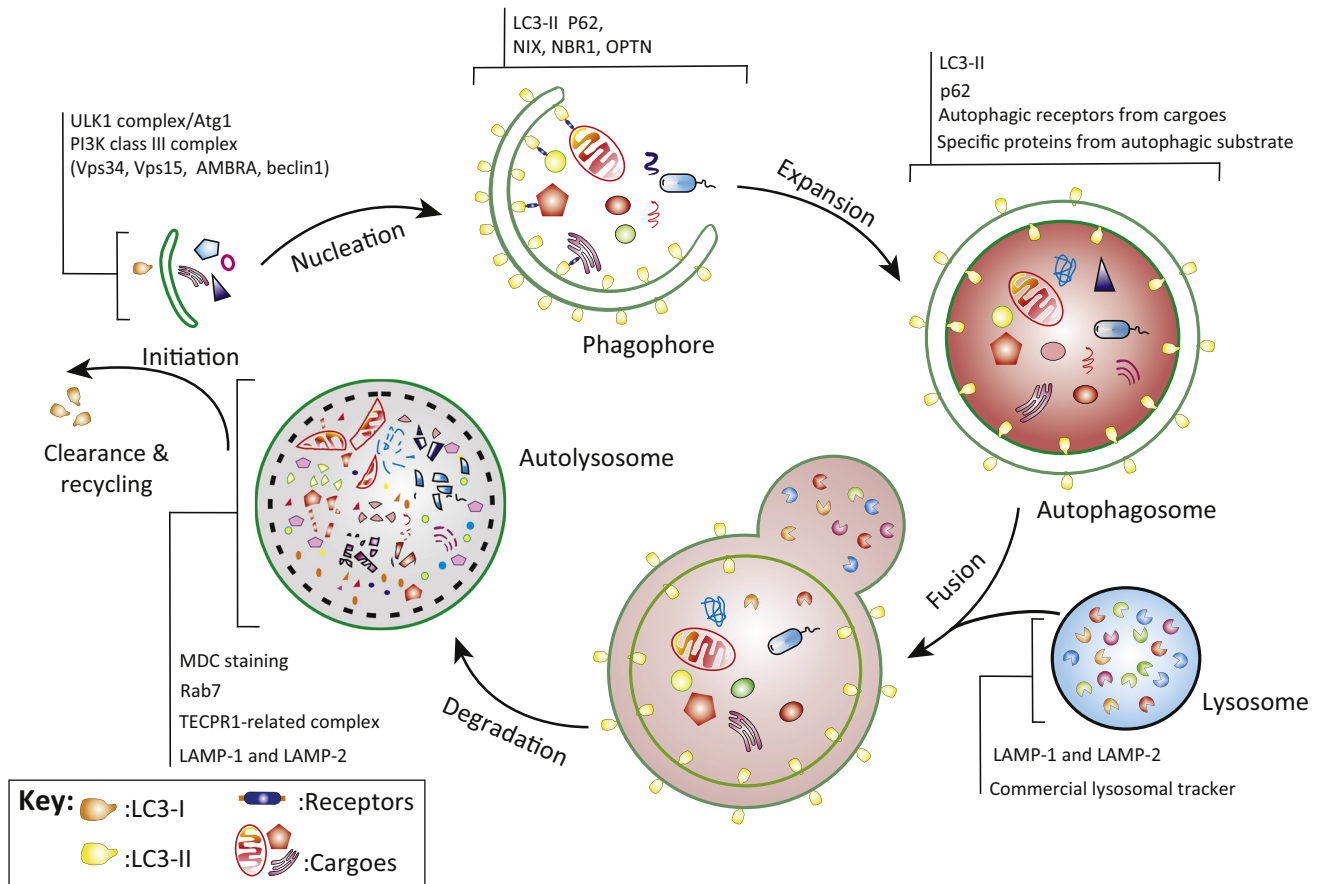
Figure 1. Autophagy-Related Marker Proteins. A few protein complexes that specially accumulate in different stages of autophagy can be employed in optical imaging to indicate the autophagic flux. At the beginning of autophagy, a phagophore originating from the phagophore assembly site (PAS) with initial complexes containing ULK and PI$_3$K Class III (also known as omegasomes) elongates and seals itself around the selected cargoes to form a double-membrane structure called an autophagosome. The autophagosome then fuses with the lysosome individually, or alternatively, sequentially with the late endosomes and lysosome (not shown), to generate an autolysosome. After a degradation process, cytoplasm or other various unwanted cellular components and invading microbes can be recycled and hydrolyzed into amino acids, glucose, and lipids, which then return back to the cytoplasm for energy supply and biomacromolecule synthesis to maintain cellular homeostasis. The marker proteins at the different stages of autophagy can be labeled by bioprobes as potential indicators of autophagic intensity or the whole autophagic flux. LAMP, lysosome-associated membrane protein; MDC, monodansylcadaverine; PI3K, phosphoinositide 3-kinase; ULK, Unc-51-like kinase.

the assembly site, suggesting a new initiation mechanism of autophagy. The maturation process of autophagosome and the recruitment events of LC3 to autophagosome in infectious pathogen-induced autophagy (also termed xenophagy) can also be imaged on an SIM [36]. By contrast, direct stochastic optical reconstruction microscopy has been applied to image the autophagic machinery-related compartments at a resolution higher than the diffraction limit in another related work, which shows that the autophagosome initiates with the **UNC-51-like kinase 1** (**ULK1**) complex at the **endoplasmic reticulum exit sites (ERESs)** [37] (Table 1). It is worth mentioning that in super-resolution imaging of cell autophagy, to obtain an objective and comprehensive evaluation of the autophagy at the population level, an artificial selection of the location in the specimen always should be avoided.

Live-Cell and *In Vivo* Tissue Fluorescence Imaging
The explosive growth of demand to study live cells or intravital animal models in biomedicine and drug development, with better spatial and temporal resolution, has facilitated the emerging

Box 1. Electron Microscopy in Autophagy

The EM technique can be widely exploited for providing information about cells at nano-structural resolution to reveal elaborate details, thus giving new insights into the biogenesis of autophagic structures. EM provides ultrastructural morphological information about proteins and cell organelles at a nanoscale resolution that reveals specific cytoplasmic inclusions such as the ribosome, mitochondrial membrane, and double-membrane structure of the autophagosome [57]. Over the past 60 years, EM has been an indispensable method to clarify almost all of the milestone events in the autophagy field. The discovery of autophagy in the 1950s, the degradation behavior of vacuoles and the discovery of autophagic vacuoles in the 1960s, the autophagy-lysosomal degradation in the 1970s, the initiation of autophagosome membranes in the 1980s, the phagophore–ER interaction, and the discovery of various kinds of selective autophagy occurring from yeasts to mammalian cells in the recent decade have all been verified by transmission electron microscopy [58].

A landmark breakthrough in the autophagy field was the use of EM. Although there is a huge gap in resolution levels between fluorescence microscopes and electron microscopes in cell biology, in some research fields such as autophagy, they must be complementary to each other. Assessing the autophagosome number by EM requires considerable specialized expertise and is time consuming compared with the optical microscopy and biochemical methods, which are more widely accessible to researchers in different fields. Furthermore, to reveal the autophagic compartments in live cells or animal tissues, fluorescence imaging exhibits more feasible solution projects than the EM [59]; for instance, the dynamic conversion processes of specific protein-enriched structures and organelles in live cells [60]. Although a series of experiments have been performed in yeast and mammalian cells to demonstrate the prevalent applications in autophagy-related cellular processes with FM, the advantages and applications of EM and optical imaging in autophagy research cannot be replaced by each other.

practice of live-sample FM. Live-cell FM, the most commonly used instrument for observing dynamic processes in tumor cells, has accompanied almost all of the landmark breakthroughs in cell biology. Through live-cell imaging, the molecular mechanism by which telomerase is recruited to telomeres and then maintains genomic stability and integrity has been uncovered [38]. In other areas, Park and colleagues [39] summarized the advances of living-cell imaging methods used in stem cell regulation.

Advances in autophagy research strongly depend on the tools of living-cell or tissue imaging. To date, the dynamic processes of autophagosome formation, autophagosome–lysosome fusion, the degradation of autophagic substrate, or even the activity of **omegasomes** in early stage of autophagy were all demonstrated via living-cell imaging [40,41]. Recently, intravital imaging was also introduced to autophagy research in live animal models. In the early days,

Box 2. Super-Resolution Fluorescence Imaging

A great deal of effort has been made to improve the resolution of the light microscope in the last century. However, the lateral resolution of the traditional optical microscope is limited to approximately 250 nm because of the diffraction limit [61]. The super-resolution imaging techniques can be divided into two categories, when trying to overcome this limit. One, the single-molecule approach, which includes photo-activated localization microscopy (PALM) and **stochastic optical reconstruction microscopy (STORM)**. The other approach is the patterned illumination technique. Based on the accurate single-molecule imaging technique, the lateral resolution can reach as low as 1.5 nm *in vitro* [62]. Furthermore, the achievements of genetic engineering manipulation of fluorescent proteins have revolutionized super-resolution imaging. By taking advantage of single-molecule imaging and the photoactivable GFPs, PALM can achieve a resolution of 20 nm [63]. In addition to PALM, STORM can also reach a resolution of 20–40 nm [64]. STORM is similar to PALM, in theory, when it uses Cy3 and Cy5 as the photoactivable fluorescence probes. In comparison with PALM, STORM can observe endogenous proteins, while PALM can only observe exogenous proteins. Both STORM and PALM are limited in temporal resolution because for both of these imaging methods, the fluorescence probes need to be activated and quenched over and over again.

Patterned illumination techniques mainly include stimulated emission depletion microscopy (STED), **saturated structured illumination microscopy**, and the **reversible saturable optical fluorescence transitions** technique [65]. In optical physics, a point light source will form a fuzzy spot when focused on by the objective lens. The profile intensity of the fuzzy spot is called **point spread function (PSF)**. The resolution will be improved as the PSF 'slims'. Because of the high time resolution, STED has become the most widely used super-resolution imaging approach in cell biology among all these patterned illumination techniques. For instance, in living cells, STED has a video rate imaging up to 28 frames/s with a focal spot size of 62 nm [66]. Dual-label STED nanoscopy, using photochromic GFP as markers, displayed how the Map2 and connexin37 proteins distribute in the live PtK2 cells [67].
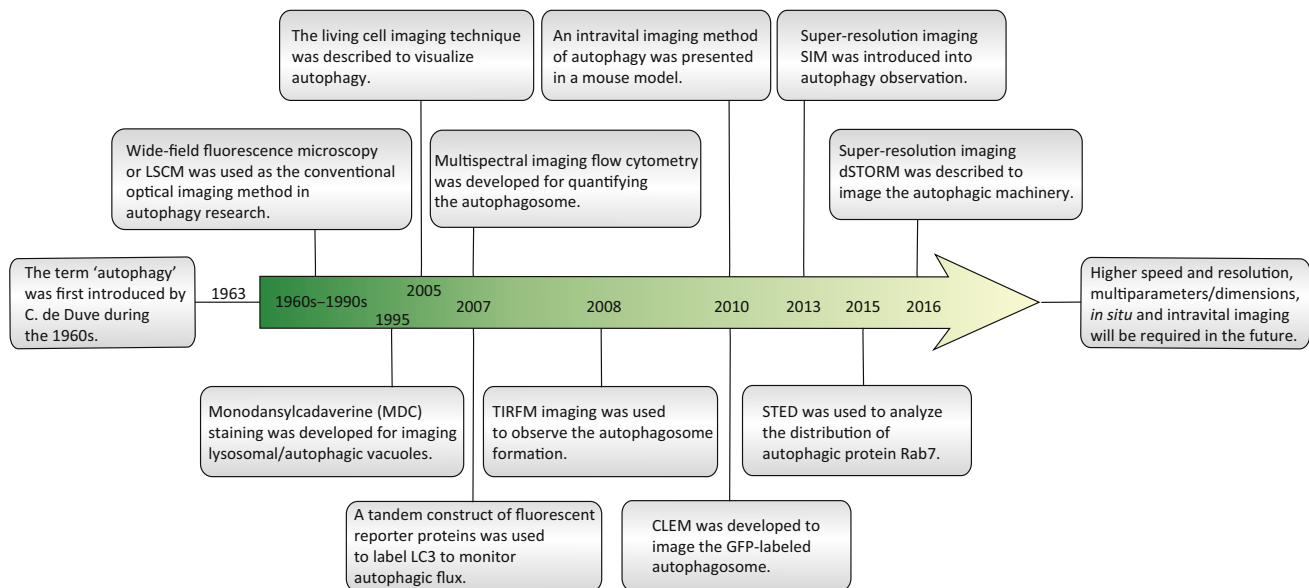
Figure 2. Milestones of Optical Imaging in Autophagy Research. These events involve specific fluorescence probing methods that localize the selected autophagic components and optical imaging platforms. Relative to the half-century history in the autophagy field, most of the new investigative methods and advanced imaging equipment were developed during the past decade, during which some super-resolution optical imaging technologies have emerged. CLEM, correlated light and electron microscopy; dSTORM, direct stochastic optical reconstruction microscopy; LC3; light chain 3; LSCM, laser scanning confocal microscopy; SIM, structured illumination microscope; STED, stimulated emission depletion microscopy; TIRFM; total internal reflection fluorescence microscopy.

Tian and colleagues [4] developed a technology that can monitor autophagic behaviors *in vivo* using a mouse model [4]. In addition, a zebrafish model showed that *Mycobacterium marinum* infection was rescued by autophagy [42]. Furthermore, using live-cell imaging, researchers tracked the temporal and spatial events of autophagy activities in living *Caenorhabditis elegans* using QDs [43]. Each image acquired from the aforementioned living targets is usually combined with a highly specific bioprobe, which is minimally invasive for the living sample, such as fluorescent proteins or biocompatible nanoprobes.

Ideally, a noninvasive or nearly noninvasive excitation light source for imaging studies should be employed to support the physiological conditions of intravital samples [44]. Accordingly, the limitation of live-cell imaging is that the phototoxicity of long exposures to the excitation light may reduce the physiological activity of the cell. To overcome this barrier, more biocompatible and specific exogenous proteins have been selected as autophagic markers, and the imaging conditions of the instrumentation (e.g., the wavelength of excitation, the working frequency of the shutter, and the external environment maintained around the living sample) should be optimized (Figure 3, Key Figure).

Imaging Autophagic Compartments
Bioprobes

The most conventional experimental scheme in autophagy research is to track or monitor the autophagic membrane, autophagosome/autolysosomes, autophagic flux, and accumulation of autophagy-related proteins. The development routes of fluorescent imaging probes and standardizing patterns of optical imaging experiments for autophagy evaluation share some essential procedures (Figure 3). Bioprobes for imaging autophagic compartments can be categorized into three groups: an exogenous expression tag of a fluorescent protein conjugated with an autophagic marker protein, chemical fluorescent compounds with fluorophores,

Box 3. Wide-Field Fluorescence Microscopy

Fluorescence microscopy is a traditional technique that has been applied in biological sample imaging for more than a century [68]. Under an excitation spectrum, the compartment of interest can be localized by the emission spectra by attaching a suitable external fluorophore or a bioprobe with high specificity and signal-to-noise ratios to the sample. WFM is one kind of traditional fluorescence microscopy. In WFM, a continuous light, such as from a mercury or xenon lamp, was used as the light source. Combined with a series of filters before the continuous light source, the emission fluorescence with corresponding different wavelengths can be acquired using a beam splitter mirror [20]. Hence, for living-cell imaging, the fast speed of imaging acquisition makes the wide-field fluorescence microscope more suitable than a confocal microscope, thanks to a lower photobleaching effect on living cells [69]. As one of the basic imaging technologies used in cell biology, WFM has become a widely used tool for autophagy research. In yeast and eukaryote cells, the significant role of FM in studying autophagy has been demonstrated [70]. With specific bioprobes and a strict control group, the signal intensity detected by FM is positively correlated with the amount of autophagic proteins. Furthermore, a general qualitative and quantitative analysis method of autophagy-related proteins using FM has been developed [71].

and nanomaterials specifically located in autophagic structures. For example, **aggregation-induced emission** fluorogen and luminogen have been developed as bioprobes for tracking the mitochondria and lysosome, respectively, in the autophagy process with good specificity, biocompatibility, and photostability [45,46].

Imaging Autophagy-Related Key Proteins

By immunofluorescence staining or other fluorescence imaging methods, the phagophore, autophagosome, and autolysosome, which represent the different stages of autophagic flux, can be characterized by imaging and tracking the corresponding marker proteins (Figure 1). Immunofluorescence imaging and living-cell imaging can also monitor the degradation of corresponding receptor proteins in selective autophagy; therefore, the selective autophagic progress could be indirectly evaluated. Recently, some multiplexed fluorescence imaging technologies have been applied in basic biological field. Using chemical cleavable fluorescent linker, a great number of marker proteins (more than 100 kinds) in individual cells can be profiled *in situ*, and the spectral overlap of traditional fluorescent dyes can be overcome [47]. By contrast, a multiplexed FM based on the chemical inactivation of organic fluorophores may remove the limitations of analyzing multiple key proteins in individual cells and tissue compartments [48], thus providing a new possibility to distinguish between the different types of selective autophagy.

The degradation of the ER membrane in mammalian cells was selectively mediated by FAM134b (family with sequence similarity 134, member B), thus the expression level of this protein indicates ER autophagy [49]. The p62 protein acts as the degradation substrate of autophagic membranes in ubiquitin-dependent autophagy, so the downregulation level of p62 expression can be interpreted as the autophagy induction [50]. Because of this fact, the fusion protein GFP–p62 has also been constructed as a fluorescent probe for detection of autophagy in live-cell imaging [51].

However, there are still some sophisticated mechanisms regarding cargo recognition and degradation in selective autophagy that remain unknown [52]. Discovering new autophagy-related marker proteins will further facilitate the application of optical imaging in autophagy research *in vitro* and *in vivo*.

Imaging the Autophagic Flux

The initiation of isolation membrane (IM) and the fusion of the autophagosome with lysosome are dynamic and depend on diverse conditions, thus the autophagic flux would be misunderstood by only detecting the expression level of one kind of autophagic marker protein at a single time point (Box 4). Currently, the most widely accepted trend that overcomes this confusion is

Key Figure

Developmental Routines of Fluorescent Imaging
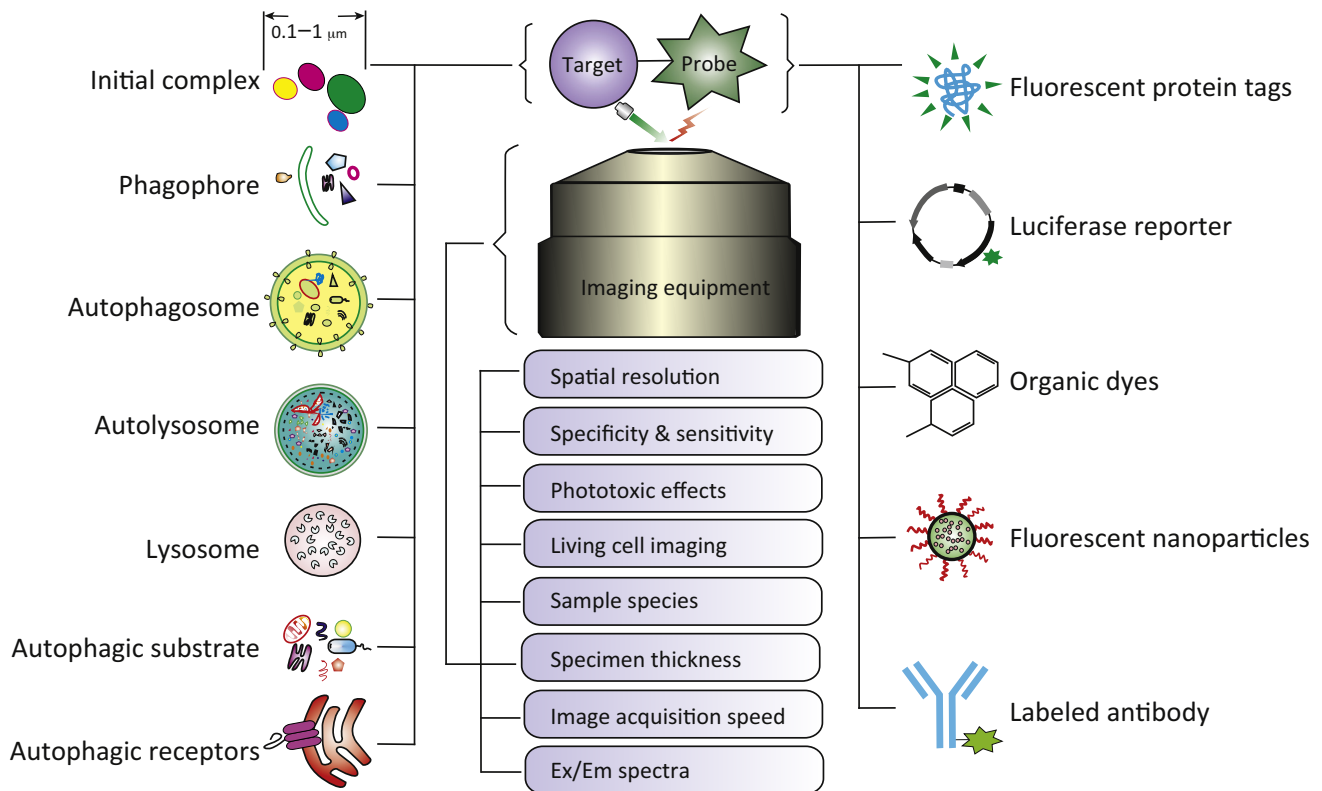
Figure 3. Probes and standardizing patterns of optical imaging procedures for assessment of autophagy were schematized for researchers. To investigate the target of interest (purple ring), an elaborately designed fluorescence probe (green star) is used to tag the targets, which involve initial complexes, phagophore, autophagosome, autolysosome, lysosome, and other related autophagic cargoes in an ordinarily defined autophagic machine. Five mature fluorescent probing methods – fluorescent protein tags (e.g., reporter gene transfection and expression), luciferase reporter system (bioluminescence), organic dyes (e.g., small-molecule fluorophores), fluorescent nanoparticles (e.g., AIE and quantum dot nanomaterials), and fluorescently labeled antibodies (e.g., direct/indirect immunofluorescence assay) – are candidates to observe these structures by optical imaging. Obviously, the scales of the different autophagic compartments (usually ranging from 0.1 to 1 μm), the specificity of the probing methods, and the characteristics of specimen (species, thickness, and live), as well as the experimental objective that one intends to achieve, may impact the selection of a suitable optical imaging instrumentation for a researcher to visualize the different stages of autophagy or the whole autophagic flux. AIE, aggregation-induced emission.

to construct a multicolor fluorescence protein reporter for colocalization analysis (Figure 4A). A tandem fluorescent-tagged LC3 (RFP–GFP–LC3) has been developed as an autophagosome and autolysosome indicator by fluorescence imaging [53]. The conversion of LC3 from LC3-I to LC3-II in RFP–GFP–LC3 has now been widely imaged on a fluorescence microscope to observe the whole autophagic flux (Figure 4B). Recently, a modified tandem fluorescent protein marker called GFP–LC3–RFP–LC3ΔG was newly developed to monitor autophagic flux in living animal models such as a zebrafish and a mouse, which displayed a promising application perspective in detecting the basal autophagic level *in vivo* [54].

To explore the process of autophagy at various stages, different corresponding bioimaging techniques have been established. For example, the autophagic events of biogenesis and

Box 4. The Initiation of Autophagy

Growing evidence has verified that the initiation of autophagy is a dynamic physiological process that involves a large number of specific proteins, including ULK1 [72].

In mammalian cells, the autophagy machinery is initiated from the isolation membrane (IM), which originates from mitochondria-associated ER membranes [52]. The phosphatidylinositol 3-kinase () complex PI3K recruits the ULK1 complex to facilitate the initiation of phagophore [73]. Then, the expansion of the phagophore membrane results in the formation of autophagosome. Thus, the early stage of autophagy can be evaluated by imaging the expression level of the aforementioned proteins.

nucleation of IM, expansion and cyclization of phagophore, cellular contents engulfed by the autophagosome, and autolysosome formation all can be imaged and tracked under the optical microscope with a proper fluorescent tag. Omegasomes labeled with GFP can be used to track the initiation of early stage autophagy in HEK-293T cells [41]. The Cyto-ID kit can also specifically stain the autophagic vacuole, making it possible for the high-throughput quantification of autophagic flux [55]. In another research work, the recombinant lysosome-associated membrane protein 1 (LAMP1)–Cherry protein has been constructed in the NRK cell line to localize the autolysosomes during the lysosome reformation at late stage of autophagy [56]. In
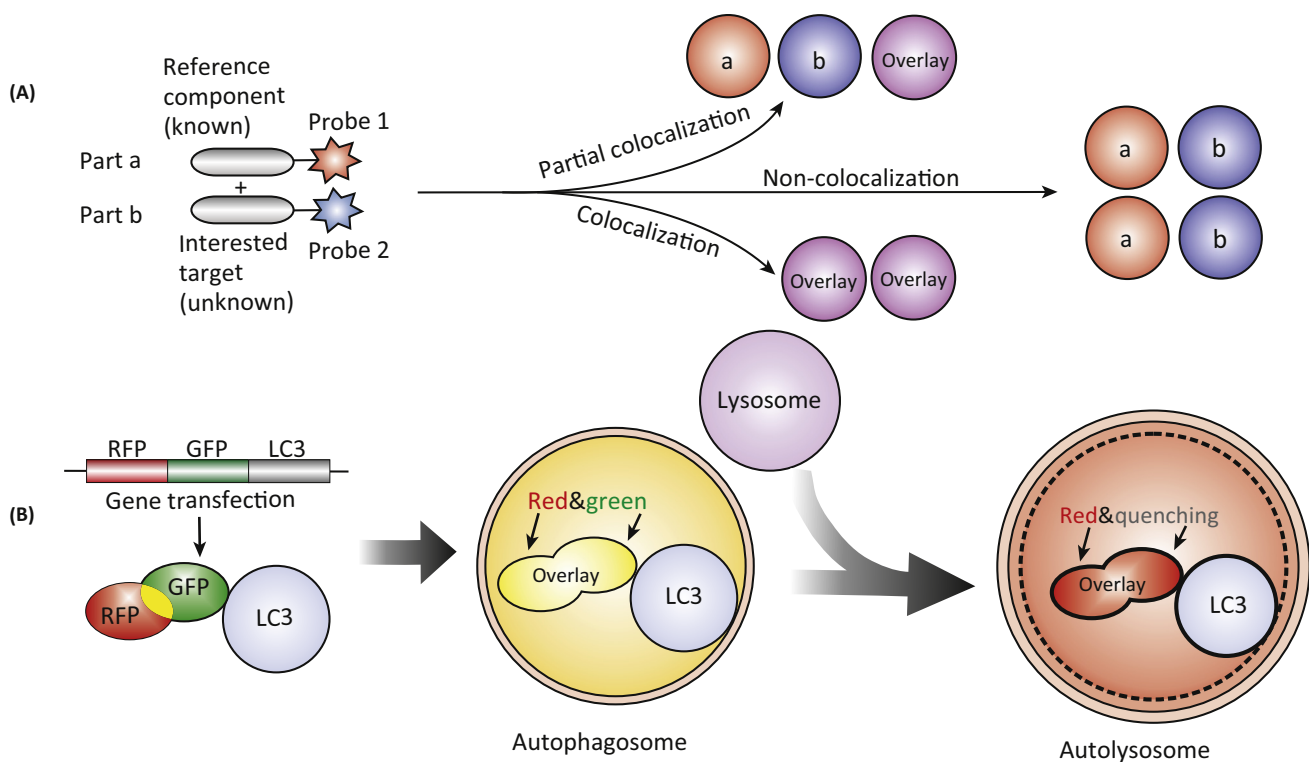
Trends in Biotechnology

Figure 4. Multicolor Fluorescence Imaging (A) and an Autophagy Reporter (B). In a representative fluorescence colocalization imaging experiment, the reference component (Part a) is known for the label of Probe 1, and the target of interest (Part b) is unknown but is labeled with Probe 2. When these two parts encounter each other, three possible situations including colocalization, non-colocalization, or partial colocalization may occur, which generates the merged color of Probe 1 and Probe 2 (pink); individual color of Probe 1 (red) and Probe 2 (blue); or mixed colors of red, blue, and pink, respectively. The working process interpretation of a tandem LC3 fluorescence protein reporter gene delivery system (RFP–GFP–LC3) for autophagic flux evaluation is shown in (B). In the tandem double RFP–GFP–LC3 system, the two colors of GFP and RFP overlay and exhibit yellow in autophagosome. However, GFP is acid sensitive and is fluorescently quenched by the acid hydrolases of the lysosome, which join in the autophagosome to form an autolysosome, resulting in a monochromatic fluorescence of RFP. Thus, the autophagosome and autolysosome can be specifically labeled with yellow and red, respectively. Using this novel fluorescence protein probe, the autophagic flux can be easily traced under different kinds of fluorescence microscopes. LC3, light chain 3; RFP, red fluorescent protein.

brief, a bright fluorophore combined with a protein that specially resides in autophagy-related membrane structure can be used to evaluate the autophagic flux.

Quantification of Autophagy

The quantification of autophagic flux refers to determining the amount of autophagosome and autolysosome, or the autophagic substrates, to be degraded. Compared with the relative quantification methods (EM and flow cytometry), optical imaging offers a noninvasive (or minimally invasive), time-dependent, and *in situ* analysis of autophagy-related proteins and structures. And most importantly, fluorescence imaging can monitor the dynamic autophagic activities in a whole living cell in both space and time. In general, an autophagy marker protein can be used to track the target cellular components at specific time points using fluorescent proteins with different excitation/emission spectra. Qualitative and quantitative fluorescence methods include immunofluorescence-based optical microscopy and flow cytometry, respectively, which depend on the specificity of the primary antibody. A number of selective autophagy-related marker proteins can be also imaged with immunofluorescence staining techniques. However, because the process of autophagy is dynamic and complicated, it is necessary to combine the relative quantification results of fluorescence imaging with other aforementioned methods to obtain a convincing conclusion.

Concluding Remarks

Advances in optical imaging have evidently impacted the development direction of the life sciences field, and have pushed for most of the research progress in cell biology. Since 'autophagy' was first described early in the 1960s, optical imaging technologies have evolved constantly. With the newly established super-resolution fluorescence imaging methods and the high specificity of living-cell fluorophores being applied in the autophagy field, the mechanism as to how autophagy plays a critical role in the development and therapy of human diseases such as cancers, diabetes, and cardiovascular and neurodegenerative diseases will be uncovered in the future years. Higher speed and resolution, multiple parameters/dimensions, and *in situ* and intravital imaging will be required in the future. Here, we have reviewed how optical imaging substantially accelerates autophagy research with a collection of interdisciplinary examples, including examples in biophysics and molecular cell biology studies. One of the questions left unanswered in optical imaging for autophagy exploration, however, is how to develop different categories of indicators and biomarkers to specifically probe the autophagic structures associated with normal physiological cell process and pathologic responses to the external environment (see Outstanding Questions). We believe that this is the most important question to answer for biologists who seek a more powerful imaging tool in observing autophagy.

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Outstanding Questions

Is there one endogenous protein that specially matches with one selective autophagy intensity? If yes, then every kind of selective autophagy can be correctly evaluated by fluorescence imaging.

Can the background signal of basic autophagic level in different cell lines be automatically removed by advanced software for optical imaging instrumentation?

Does a higher-resolution optical imaging approach ensure a more reasonable evaluation of autophagy?

Is there an optical method that can distinguish between physiological autophagic activities and the pathologic autophagic response to the external environment in the human body?

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