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Structured illumination microscopy and correlative microscopy to study autophagy

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ABSTRACT

Autophagy is a predominant eukaryotic mechanism for the engulfment of "portions" of cytoplasm allowing their degradation to recycle metabolites. The autophagy is ubiquitous among the life kingdom revealing the importance of this pathway that appears more complex than previously thought. Several reviews have already addressed how to monitor this pathway and have highlighted the existence of new routes such as the LC3-associated phagocytosis (LAP) and the non-canonical autophagy. The principal difference between autophagosomes and LAP vacuoles is that the former has two limiting membranes positives for LC3 whereas the latter has one. Herein, we propose to emphasize the use of correlative light electron microscopy (CLEM) to answer some autophagy's related questions. The structured illumination microscopy (SIM) relatively easy to implement allows to better observe the Atg proteins recruitment and localization during the autophagy process. While LC3 recruitment is performed using light microscopy the ultrastructural morphological analysis of LC3-vacuoles is ascertained by electron microscopy. Hence, these combined and correlated approaches allow to tackle the LAP *vs.* autophagosome issue.

1. Introduction

The autophagy pathway is usually defining a self-eating mechanism, allowing the sequestration of cytoplasmic components in double-membrane vesicles, called autophagosomes, that ultimately fuse with lysosomes (defining the macroautophagy pathway). The formation of functional autophagosomes depends on a hierarchically ordered activity of over 30 Autophagy related proteins (Atg), which defines the canonical autophagy pathway [1–2]. The yeast Atg8 protein, whose the mammalian homologue is the microtubule-associated protein light chain 3 (MAP1LC3; called LC3 in the text) is generally considered as the hallmark of autophagosomes [1,3]. In fact, the soluble form, LC3-I, is conjugated to phosphatidylethanolamine (PE) to generate the LC3-II form, which is incorporated in the autophagosome membrane [4].

These past recent years, a non-canonical autophagy mechanism was reported in the literature. This pathway has the same structure and function but does not require the intervention of all of the Atg proteins to form a functional autophagosome [2,5–7]. Moreover, it has also been demonstrated that a set of Atg proteins could be recruited directly to preexisting membranes [8]. At variance with classical morphologically defined autophagosomes, an autophagy pathway was described through a direct LC3 association with single limiting-membrane vacuoles also able to convey the luminal content towards lysosomal degradation [8]. This unconventional pathway is known as LC3-associated phagocytosis or LAP described for the first time by Sanjuan and collaborators [9]. The Toll-like receptor phagocytosis in murine macrophages triggers the LC3-II recruitment to autophagosome with a single-limiting membrane

Abbreviations: Atg, Autophagy related proteins; CLEM, correlative light-electron microscopy; CLSM, confocal light scanning microscopy; LAP, LC3-associated phagocytosis; LC3, Light chain3; PALM, photo activated localization microscopy; SIM, structured illumination microscopy; STED, stimulated emission depletion; STORM, stochastic optical reconstruction microscopy; TEM, transmission electron microscopy; YCV, *Yersinia*-containing-vacuoles.

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under the dependence of Atg5 and Atg7 [9–11]. The LAP pathway was described playing a role in antigen presentation by MCH class II molecules [12], and also during bacterial infection [13–15]. Variation in the theme exists, as for instance, in epithelial cells *Yersinia pseudotuberculosis* can indeed replicate inside a single-membrane vacuole displaying LC3 [16]. The fusion events occurring during the autophagy (canonical and LAP) pathway are mediated by soluble NSF attachment protein receptor (SNARE) as VAMP7 and VAMP3 [16–17]. VAMP7 was shown involved in autophagosome formation and the fusion with lysosome [17–19]. This SNARE is also involved in the recruitment of LC3 to the *Yersinia*-containing-vacuoles [16].

Last years, some guidelines articles have been published with advices for monitoring the autophagy pathway [3,20–21]. In fact, these articles overview the different techniques developed for assessing autophagy. The autophagy pathway was mostly historically described using the transmission electron microscopy (TEM) by the observation of double membrane limiting autophagosomes. The TEM-observation allowed to show different autophagy structures as phagophores, autophagosomes, autolysosomes based on morphological analysis. Further analysis was based on LC3-immunogold studies that allowed deeper organelle characterization. The autophagy structure can be determined by the observation of electron microscopy sections and/or using multi-slices reconstruction to obtain 3-D autophagosome structures [20]. Quantification is performed by counting directly the number of gold-labeled antibodies per organelle or per cell. TEM-observation is thus a qualitative and a quantitative method for monitoring autophagy. To this aim, TEM observation can be associated with and correlated to other approaches such as biochemistry or light microscopy approaches. In routine assays, the processing of LC3-I to LC3-II is appreciated using western blot that allows recording autophagy in population of cells. An alternative imaging approach relies on monitoring autophagy using photonic microscopy. By this method, LC3 dots (either after immunocytochemistry or upon expression of GFP-tagged LC3) formed upon autophagy activation are quantified indicating autophagy flux variation. In parallel, co-distribution studies are performed either between two Atg proteins or between Atg proteins and organelle markers during the autophagy process. The expression in the cell of the mRFP-GFP-LC3 probe permits to follow the autophagosome maturation by light microscopy. The mRFP-GFP-LC3 probe gives an indication about the stage of autophagosome maturation thanks to the pH level inside the compartment. In fact, during maturation, autophagosomes present increasing level of acidic pH. The observation of both mRFP and GFP signals indicates a non-acidic membrane compartment, whereas the disappearance of the GFP signal upon protonation enables acidic compartments to be identified [22].

The conventional light microscopy gives information with hundreds nm resolution (200 nm [*x*,*y*] and 600 nm [*z*] in confocal microscopy for instance). To obtain more detailed information of intracellular structures, TEM offers still the best resolution power, but needs a specific and time-consuming preparation of samples (chemical fixation, contrasting methods, sectioning...). Thus, improving photonic microscopy resolution represents a challenging and interesting step. In the last years, different techniques have been developed to overcome the diffraction limits of fluorescence microscopy [23–25]. Among them, three important methods: SIM (structured illumination microscopy) [26–28], STED (stimulated emission depletion) Microscopy [29] and PALM (photo activated localization microscopy) [30]/STORM (stochastic optical reconstruction microscopy) [31–32]. The STED and PALM techniques led to the "nanoscopy" for which E. Betzig, S. Hell and W.E. Moerner won the 2014 Nobel Prize in chemistry. Herein, we will describe mainly the easiest technique to implement in a laboratory: the SIM microscopy that can be correlated with TEM.

SIM enables to double the resolution that could be obtained by confocal microscopy (for an exhaustive overview, see [33]). The principle is based on patterned illumination of the sample: a grid (sinusoidal striped pattern) is superimposed on the specimen while capturing images and recordings are performed using several positions (shifts and rotations of the grid), leading to a Moiré effect (Moiré fringes). The final image is thus composed by the sum of the original information (low frequency) and the information contained in the Moiré fringes (high frequencies). Arithmetic process enables to separate the different contributions and to replace them at their right position. Generally, lateral resolution of about 100 nm and axial resolution approaching 300 nm could be achieved.

SIM presents the advantage to be compatible with many standard dyes (assuming their stability over time) and staining protocols, contrary to other techniques. One of the main advantages of fluorescence microscopy is the capacity to label and observe simultaneously multiple structures inside a cell. Multi-color imaging is easy to implement in SIM and importantly, live-imaging has been shown for this super resolution method [34–35].

Conventional and super-resolution microscopy images display autophagy vacuoles but only the electron microscopy allows to discriminate autophagosomes from LAP, i.e. double-from single-limiting membrane of LC3-positive compartments. Correlative microscopy combines the use of two or more microscopy techniques to study dynamics in a complementary approach allowing analysis of structures and functions of a single sample, such as a cell, a population of cells, a tissue or an organism. While correlative microscopy has been first described a half century ago [36], this technique has been marginally used until recently. Since a decade, it undergoes a rapid growth with numerous different techniques published (reviewed in Caplan, 2011 [37] and [38–39]). In one of the most used correlative microcopy approach, the correlative light electron microscopy (CLEM), the study starts with light and fluorescence microscopy observation and then continues with transmission electron microscopy. The use of GFP and time laps microscopy coupled to TEM rapidly boosted CLEM as it became possible to observe cellular events at the ultrastructural level at any time [40]. CLEM can be further improved by the ability to directly observe fluorescent proteins, such as eosin or the biarsenical derivative of the resorufin ReAsH, in the TEM through the high efficiency photo-oxidation of 3,3′-diaminobenzidine into electron dense precipitates by bleaching of the fluorescence [41,42]. Another improvement came with the use of fast high-pressure cryo-fixation and cryo-substitution techniques, which keep the ultrastructure close to the one present in the living cell [43]. Finally, 3-D-CLEM and Cryo-CLEM are now powering the potential of this technique [44–47]. CLEM has been used in numerous biological fields including autophagy. The earliest use of CLEM came with video-microscopy associated with TEM to observe the retrieval of autophagosomes from the axons and targeted to the cell body of neurons [48]. CLEM has also been used to show the role of p62/SQSTM1 in the degradation of protein aggregates by the autophagy pathway [49]; to study the induction of autophagy around polystyrene beads [50]; to show that Atg9 was present on tubulovesicular membranes surrounding autophagosomes [51]. Also, CLEM has been used in a whole organism as zebrafish to observe the autophagy processes during *Mycobacterium marinum* infection [52]. Electron microscopy is the only technique so far able to distinguish multiple membrane layers as those formed in autophagosomes. Lam and collaborators used CLEM to show that the *Listeria monocytogenes* early stages of infection in RAW264.7 macrophages occurred in vacuoles with only a single membrane despite the presence of LC3 [53]. Using CLEM, we showed a vacuolar compartment positive for LC3 but lacking double membranes, during *Y. pseudotuberculosis* infection of HeLa cells [16].

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