Chapter 6

Cellular Screening Methods for the Study of Nanoparticle-Induced Lysosomal Damage

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Additional information is available at the end of the chapter

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Abstract

Nanoparticles (NPs) are included in many products of daily life and present in the environment. Due to the potential of NPs to improve quality and stability of consumer and health and medical products, it is expected that the exposure of humans to engineered NPs will rather increase than decrease in the future. Although NPs did not act acutely cytotoxic on these concentrations, they may cause adverse effects upon chronic exposure. Cytotoxicity testing in long-term cultures and analysis of organelle function could identify such effects. Cells take up NPs mainly via active mechanisms, and these routes deliver their payload predominantly to lysosomes. Acute exposure of cells to NPs can have adverse effects on lysosome morphology and function, but lysosomes are also potential targets for accumulation. The chapter explains the role of lysosomes and describes techniques for labeling and assessment of their function. Examples for co-localization studies and vital dye staining are shown. A variety of techniques are available to characterize effects of NPs on lysosomes, but care has to be taken in the choice of the proper technique because NPs may interfere with the detection.

Keywords: cathepsins, intralysosomal pH, staining, nanoparticles, toxicity, fluorescence microscopy

1. Introduction

Screening for acute cytotoxicity is an established procedure in the evaluation of chemicals, drugs, and medical devices and has also been used for the toxicological assessment of nanoparticles (NPs). Routine cytotoxicity testing detects changes in cell number, DNA, protein content, or metabolic activity of cells exposed to compounds applied in a broad concentration range. Exposure is usually for 4–72 h. In the case of conventional compounds, effects at high concentrations can indicate the toxicological potential of the compound at lower doses.

This prediction is not possible for NPs because particle agglomeration is more prominent at higher particle concentrations and agglomerates usually act less toxic than single NPs [1]. The exposure times of routine cytotoxicity testing are also less representative for NPs. Exposure to NPs occurs by contact with food, by the environment, and by consumer products in low doses but for prolonged time. The lack of good prediction of NP toxicity by acute testing protocols is also due to the fact that NPs in these products are usually poorly biodegradable and may accumulate in cells [2]. To address this problem, culture systems have been developed that allow the evaluation of cellular effects over prolonged time [3, 4]. Another option to identify toxicity upon prolonged contact is the study of organelles that are likely targets for damage by NPs. Active cellular uptake, endocytosis, represents the most common mechanism for cellular entry of NPs. Since the main active uptake routes deliver their payload to lysosomes, these organelles are the most likely targets for NP accumulation, potentially leading to lysosome dysfunction and cell damage.

2. Lysosomes

Lysosomes are cell organelles with an acidic lumen and a single outer membrane consisting of a phospholipid bilayer. They contain acid hydrolases, which enable the cell to process nutrients and destroy itself after death. Lysosomes are integrated in the mechanism of secretion and degradation of macromolecules and linked by vesicle transport to other intracellular structures, such as endosomes, the endoplasmic reticulum, and the Golgi apparatus (**Figure 1**).

Mammalian cells, with exception of erythrocytes, possess lysosomes, and some cell types also contain lysosome-related organelles, namely, melanosomes in melanocytes, lytic granules in cytotoxic T cells, delta granules in platelets, and lamellar bodies in alveolar epithelial cells. Lysosomes have spherical or tubular shape and measure <1 μm in non-phagocytic cells [5]. In phagocytes, which have the ability to ingest particles up to 10 μm, lysosomes can reach several micrometers in size [6]. Lysosomes are transported in the cytoplasm by passive and active mechanisms. While diffusion is size dependent and smaller lysosomes move faster than larger ones, active transport is independent from size [7].

Lysosomes digest macromolecules taken up by endocytosis (heterophagy), degrade intracellular macromolecules and organelles sequestered by autophagy, eliminate pathogens engulfed by phagocytosis, regulate metal ion levels, and sense nutrient availability. Lysosomal exocytosis is an unconventional secretion relevant for plasma membrane repair, immune response, and bone resorption [8]. Antigen processing by lysosomes is essential for the presentation of antigenic proteins to T cells [9, 10]. Lysosomes regulate the metabolic (anabolic or catabolic) state of the cells by sensing the nutritional state of the cell and conveying this information to the nucleus [11]. Transcription factor EB (TFEB), a master regulator of lysosomal biogenesis, co-localizes with mechanistic target of rapamycin complex 1 (mTORC1) on the lysosomal membrane. When nutrients are present, phosphorylation of TFEB by lysosomal surface-bound mTORC1 inhibits TFEB activity. The active mTORC1 promotes biosynthetic pathways and blocks autophagy. Catabolic pathways are switched on upon release (combined with inactivation)

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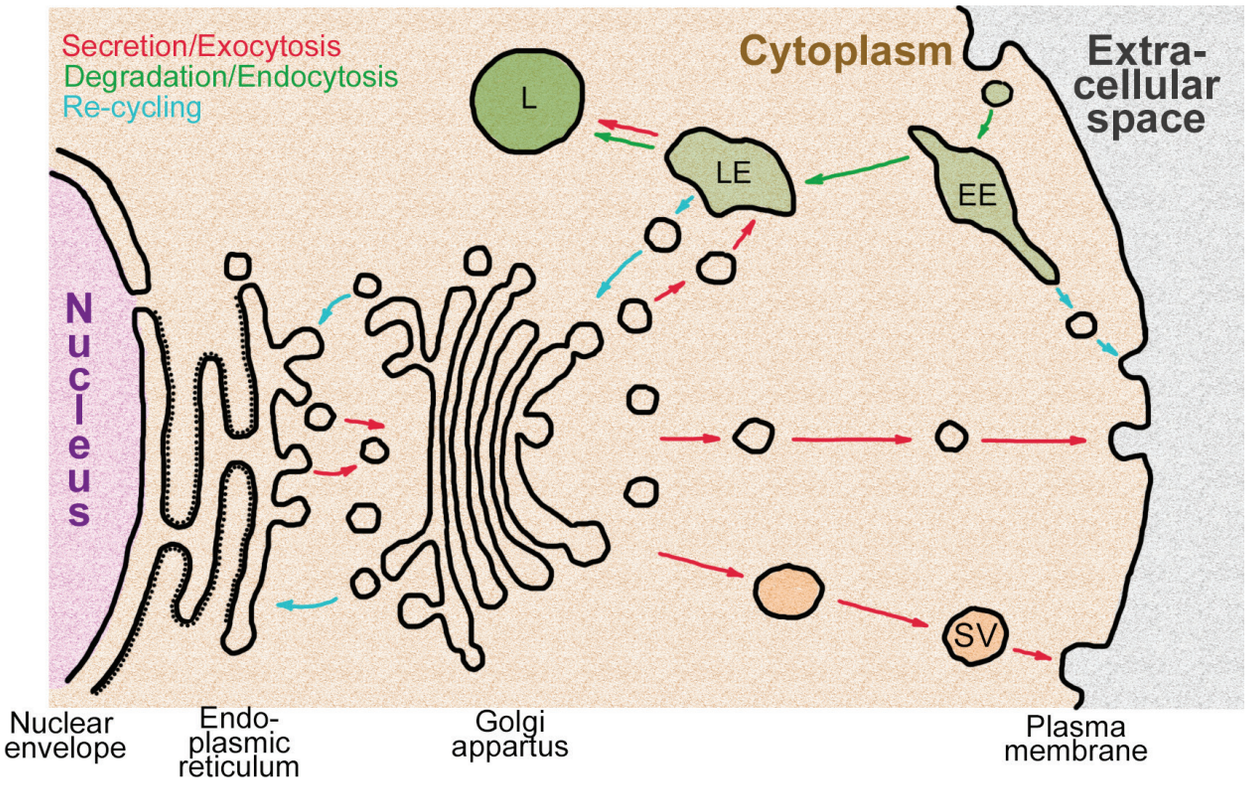

Figure 1. Integration of lysosomes in the cellular vesicle network. Ingested macromolecules or particles from the extracellular space reach the lysosomes (L) via early endosomes (EE) and late endosomes (LE) and can be degraded there. Macromolecules can be recycled through vesicle transfer to the Golgi apparatus and endoplasmic reticulum. Secretion products are synthesized at the rough endoplasmic reticulum and Golgi apparatus and are exported for constitutive secretion, like transport to the plasma membrane, or are included in secretory vesicles (SV) for regulated secretion into the extracellular space.

of bound mTORC1 from lysosomes in case of starvation or exercise, for instance. TFEB acts as a sensor of lysosomal state, when on the lysosomal surface, and as an effector of lysosomal function when translocated into the nucleus. Nuclear localization of TFEB is prevented when lysosome function is optimal. When TFEB has translocated into the nucleus, it upregulates the expression of genes encoding lysosomal proteins in order to improve lysosome function [12].

Normal lysosome function depends on an acid intralysosomal pH, and vacuolar/vesicular type H^+-ATPase (v-ATPase) is the most important proton pump for the regulation. Variation in pH between lysosomes of the same cell is considerable because lysosomes display functional and structural heterogeneity. By labeling lysosomes with a pH-insensitive and a pH-sensitive dye, Johnson et al. found that peripheral lysosomes had more alkaline pH values than peri-nuclear lysosomes [13]. The group also reported that the increase of the intralysosomal pH was linked to a change in the intracellular localization. The cellular volume of lysosomes is regulated in such a way that accumulation of undigested material induces increase in size and number of lysosomes to compensate reduced lysosome function.

Although lysosomes are involved in synthesis and recycling of macromolecules, their main role is usually seen in degradation. Degradation of extracellular material occurs after active

uptake (**Figure 2**). With the exception of caveolin-dependent uptake, all routes deliver their cargo exclusively to lysosomes. Caveolin-mediated uptake can deliver macromolecules to the Golgi apparatus, endoplasmic reticulum, and lysosomes [14]. Endocytosis is relatively fast, and the maturation of endosomes to lysosomes takes approximately 40 min [5]. Lysosomes are also involved in the degradation of intracellular macromolecules and organelles, which occurs as microautophagy, chaperone-mediated autophagy, and macroautophagy (usually referred to as autophagy). Autophagy includes the following steps: vesicle nucleation (phagophore formation), vesicle expansion (autophagosome formation), maturation (fusion with multivesicular bodies (MVBs) or lysosomes), and degradation [15, 16]. Degradation can only occur when lysosomes are active. The increased presence of autophagosomes may be due to excessive induction of autophagy or to blockade of autosome degradation (autophagy flux).

Macropinocytosis, clathrin-mediated uptake, caveolin-mediated, and clathrin- and caveolin-independent uptake ingest NPs although with different size preferences (for more detail, see, for instance, Ref. [17]). Particles larger than 500 nm are taken up by phagocytosis (**Figure 3**).

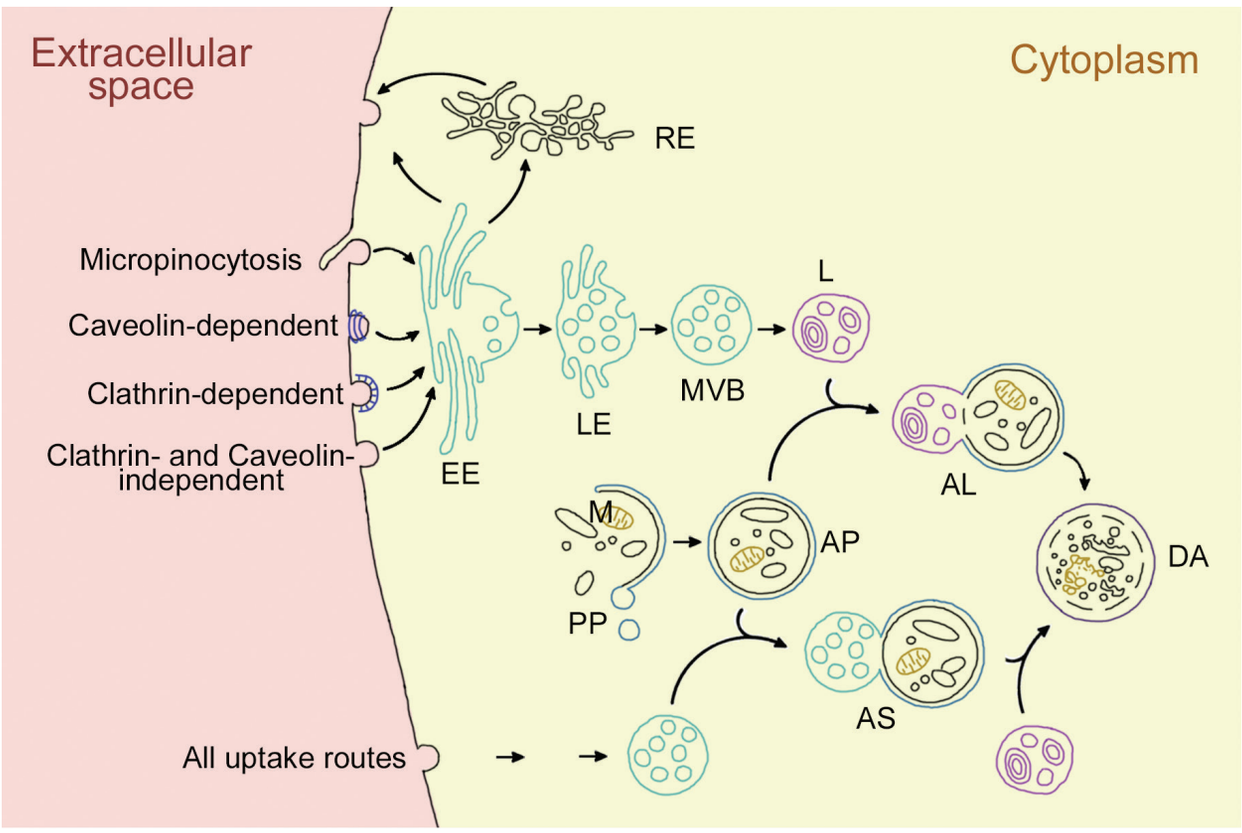

Figure 2. Interaction of lysosomes with uptake routes and autophagy. Payload of the active uptake routes micropinocytosis, caveolin-dependent, clathrin-dependent, and clathrin- and caveolin-independent routes is delivered to early endosomes (EEs), late endosomes (LEs), multivesicular bodies (MVBs), and lysosomes (Ls). EEs can recycle to the plasma membrane as recycling endosomes (REs). Macroautophagy is started by the formation of the phagophore (PP) and forms the autophagosome (AP). PPs can contain parts of cytoplasm and organelles, for instance, mitochondria (M). APs may fuse with MVBs to build amphisomes (ASs) or with Ls to form autolysosomes (ALs). ALs mature to degrading autolysosomes (DAs), which can also arise by the fusion of ASs with Ls.

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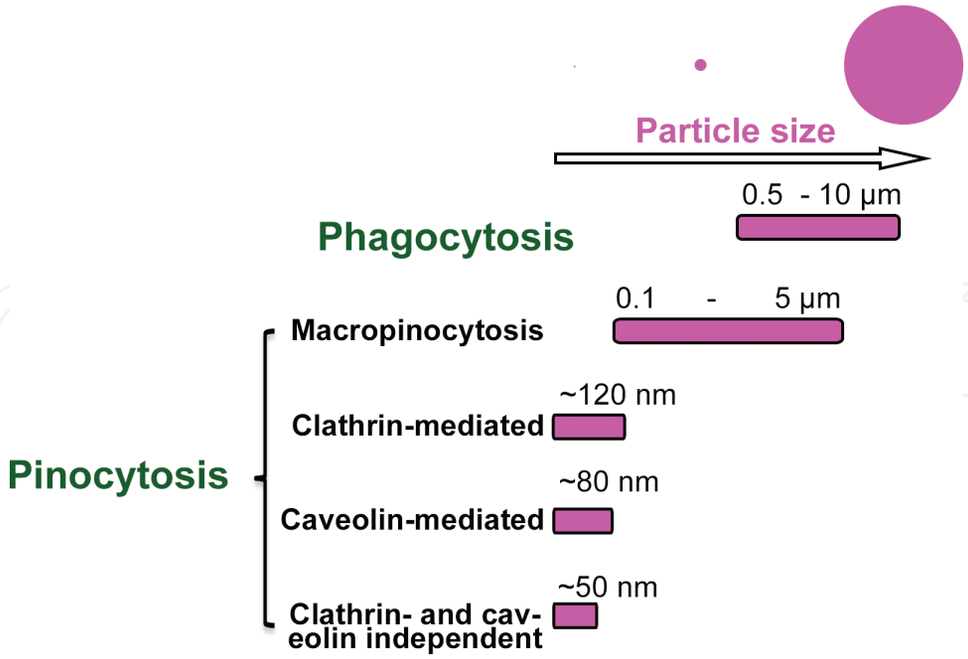

Figure 3. Overview of size preferences of the different active uptake routes for particles.

Silica (SiO_2), gold, iron oxide, polystyrene particles, and quantum dots have been detected in lysosomes in the absence of obvious morphological damage [18–28]. They can reach the lysosomes by different uptake routes, and it appears that particle size, and cell-specific expression of the different uptake routes determines the preferential uptake mechanism [29]. Cellular excretion of NPs is estimated to be low, and therefore, accumulation of NPs in lysosomes and chronic impairment of lysosome function may occur [30].

3. Symptoms and causes of lysosome dysfunction

Garnett and Kallinteri [31] suggested that accumulation of NPs might interfere with lysosomal function and cause similar symptoms as lysosomal storage diseases. The deficiency of specific lysosomal enzymes, mainly lysosomal sulfatases arylsulfatase A, B, and G, causes lysosomal storage diseases [32] with neurological, pulmonary, and cardiac impairment [33]. Lysosomal dysfunction, however, can also be caused by accumulation of pharmaceutical compounds. Several drugs, such as nonsteroidal anti-inflammatory drugs, statins, antidepressants, beta-blockers, tyrosine kinase inhibitors, anti-histamines, and so on, are sequestered in lysosomes and may cause drug-induced lysosomal damage, termed phospholipidosis. Phagocytic cells, alveolar and peritoneal macrophages, are more sensitive than non-phagocytic cells, and damage causes the histopathological image of "foamy macrophages" [34]. The intracellular accumulation of membranous material shows analogies to inherited lysosomal storage disorder [35] and suggests that external factors may also cause analogous symptoms. Molecules, which cause lysosomal damage, are typically organic amines, such as chloroquine, amiodarone, perhexiline, aminoglycosides, and chlorphentermine. These compounds accumulate in endosomes and can cause swelling and disruption of lysosomes with subsequent cell death [36].

Impaired lysosomal function may also lead to abnormal autophagy. The role of autophagy in diseases is not completely clear. Reduced autophagy promotes cell transformation and development of cancer, whereas in later stages, increased autophagy allows the cancer cells to survive regardless of deprivation of nutrients. Disruption of autophagy has also a promoting role in neurodegenerative diseases [37]. Drugs act on autophagy mainly by alteration of lysosomal enzyme activity and of intralysosomal pH [38]. The epoxysuccinyl compound E64d and Pepstatin A inhibit cysteine and aspartic proteases, whereas chloroquine, hydroxychloroquine, Lys05, NH_4Cl, matrine, momensine, and lucanthone increase intralysosomal pH, and bafilomycin A1, azithromycin, and concanamycin A inhibit v-ATPase.

Inactive non-digested material as part of normal biological aging can accumulate in the form of the autofluorescent pigment lipofuscin. This accumulation of lipids, carbohydrates, and aldehyde-crosslinked proteins increases cellular susceptibility to oxidative stress, alters intralysosomal pH and lysosomal membrane permeability, and impairs lysosomal function [39].

Accumulation in lysosomes has a prominent influence on the elimination of drugs from the body [40]. Based on the assumption that drugs that are trapped in lysosomes (lysosomotropic drugs) and non-lysosomotropic drugs have a plasma concentration of 1mg/L and no physical binding to blood or tissues occurs, the distribution volume of non-lysosomotropic drugs is 42L for the average 70kg person. To determine the distribution volume of lysosomotropic drugs, the lysosome volume has to be added. It was calculated as 0.5 L based on the assumption that the body contains 15 trillion cells with a volume of 3.4×10^{-9} cm^3 (average hepatocyte volume) and 1% of which represent lysosomes. To be factored into the total body volume, drug concentration must be the same as in plasma. The apparent lysosome volume, therefore, is 500L, which means ~10 times greater than the distribution volume of a non-lysosomotropic drug. This estimation appears not unrealistic since accumulation of chloroquine in rat tissues at typical therapeutic concentrations was 800 higher than values in plasma, and cells were able to accumulate propranolol 1000-fold compared to extracellular concentrations. Based on these calculations, a 10 times longer half-life for lysosomotropic compared to non-lysosomotropic drugs is expected. A long persistence in tissues has also been reported for NPs in several studies (e.g., in Refs. [41, 42]).

4. Lysosomal damage by nanoparticles

In contrast to nuclear membrane and plasma membrane, lysosomes possess only a single phospholipid bilayer for resistance against the around 60 different intralysosomal hydrolases. More than 50% of the lysosomal membrane proteins of late endosomes and lysosomes consist of lysosome-associated membrane proteins (LAMPs). LAMPs are the most densely glycosylated proteins (>60% of total mass) and form the inner lining of the lysosomal membrane. It is postulated that the composition with high glycosylation and low content of cholesterol represents a better protection against the action of lysosomal hydrolases than the composition of conventional membranes [9].

Despite the good protection against hydrolases, lysosomal membranes can be acutely damaged by NPs [43, 44]. Depending on the extent of the damage, cell death via different mechanisms

ensues [45]. Partial permeabilization, in general, results in reactive oxygen species (ROS) generation and apoptotic cell death, whereas massive permeabilization induces cytosolic acidification and necrosis. Iron oxide NPs induced cytotoxicity through generation of ROS by lysosomes. Toxic ions released by partial degradation of particles in lysosomes were the main toxic mechanism of zinc oxide (ZnO) and copper oxide (CuO) NPs [46]. Quantum dots in lysosomes caused swelling of lysosomes linked to morphological alterations [47], and cationic cerium oxide (CeO_2) NPs and polystyrene particles induced disruption of lysosomes [48]. As toxic mechanism of cationic NPs, buffering of H^+ with increased lysosomal pH is assumed [49]. Multiwalled carbon nanotubes (CNTs) increased lysosomal permeability by direct action on lysosomal membranes [50]. The increase in autophagosomes is a common finding in the cellular action of NPs, such as fullerenes, gold NPs, iron oxide NPs, rare-earth oxide NPs, quantum dots, CNTs, titanium dioxide (TiO_2) NPs, and SiO_2 NPs [51–61]. In addition to decreased phagosome degradation, impaired lysosomal trafficking by disruption of the actin cytoskeleton may be a reason for the increased presence of autophagosomes [37]. Since all NPs, despite different compositions and surface functionalities, increased cellular autophagosome content, it is suspected that increase in autophagy is inherently linked to the small size of these particles.

Subtle changes in lysosome physiology without obvious morphological alterations have also been reported. Exposure to 15–200 nm TiO_2 and 10 nm ZnO NPs induced changes in pH and enzyme activities in epithelial cells [43, 62, 63], while polystyrene and TiO_2 NPs increased intralysosomal pH of macrophages [62, 64]. In addition to increasing intralysosomal pH, polystyrene particles interfered with lysosomal enzyme activity [65]. By increasing the intralysosomal pH, silver NPs decreased the pH-dependent uptake of fluorescent indicator dyes [66]. Increased expression of CatB protein was observed in SiO_2 NP-treated cells [51]. Based on the finding that accumulation of undigested material leads to upregulation of lysosome size and number [5, 67, 68], the increase of lysosomal activity may represent a compensation mechanism for impaired lysosome function caused by SiO_2 NPs.

5. Marker for interaction with lysosomes and its morphology, integrity, and function

A panel of methods is available to assess lysosome morphology and function. Area measurements can be performed by image analysis of (fluorescence-labeled) lysosomes. Morphological changes can be identified by transmission electron microscopy (TEM). Lysosomal function can be determined by changes in the expression of lysosome-related genes or proteins, while fluorescent substrates or fluorescent dyes indicate changes in enzyme activities or pH. Not all assays, however, are suitable for the assessment of NPs because colorimetric, fluorescent, and luminescent assays are prone to interference with NPs [69]. Interference can cause false-positive and false-negative results. In cytotoxicity testing with assays, where enzymatic activity is determined by absorbance of a colored product, colored NPs can mask cell loss because the absorbance caused by NPs leads to an overestimation of viable cells. Examples for overestimation of cell damage are membrane permeability assays with fluorescence-based assays. The increase of the signal by NPs with inherent fluorescence indicates more cell damage than

actually present. High concentrations of NPs, on the other hand, may quench the fluorescent signal, and it is often appropriate to compare reader data with microscopical observation (**Figure 4**). In general, the parallel assessment by several techniques (plate reader and microscopy) and the switch to another detection method (fluorescence instead of absorbance) help to avoid false conclusions due to interference.

Microscopic techniques are frequently used for the assessment of NPs because they allow the correlation of intracellular localization and cellular effects. Examination by TEM cannot only reveal organelle damage but can also be used for co-localization studies because NPs contained in food, consumer products, and cosmetics can generally be visualized by TEM. Fluorescence microscopy using life stains and immunocytochemistry serves for area measurements. In combination with fluorescent particles, co-localization studies and intracellular tracking can be performed. Cells transduced/transfected with fluorescent protein-LAMP-1 constructs can also be used for these studies. The commercially available technology Organelle Lights™ uses a targeted fluorescent protein with viral delivery (http://web.mit.edu/rkarimi/www/Special/Other/Protocol/Organelle%20Lights_%20Intracellular%20Targeted%20Fluorescent%20Proteins.pdf) for transduction of mammalian cells. By using LAMP-1 transfected cells, it was found that small 20 nm carboxyl-functionalized polystyrene particles were preferentially located in the perinuclear region, whereas 200 nm particles were detected to a greater extent in the cellular periphery (**Figure 5**). Given the fact that perinuclear lysosomes have a more acidic pH than peripheral lysosomes [13], the uptake in different types of lysosomes may cause different cellular effects. For co-localization with lysosomes also immunocytochemical detection with antibodies against LAMP-1, LAMP-2, and LAMP-3 can be used.

Lysosome markers, such as gold-coupled albumin and fluorescence-labeled dextran, use active uptake for the labeling [70]. Since NPs may interfere with active uptake routes, these markers are less suitable for NP studies. Information on lysosome function can be obtained by detection of enzymatic activity or pH-dependent dyes.

Activity measurement of acid phosphatase, β-glucuronidase, and β-hexosaminidase, which have been released from lysosomes, can be used as marker for lysosome function but needs isolation of the organelles. NPs that were located outside the lysosomes can get access to the assay compounds during the isolation procedure and cause artificial effects. Such interference occurred when cathepsin B (CatB) activity of cells exposed to polystyrene particles was detected in homogenates [65]. In situ assays, where cells are not homogenized, can avoid this problem because only NPs located inside lysosomes get access to the substrate. For quantification of enzyme activity, in situ substrates for sulfatases and cathepsins are available. The substrate SulfGreen is metabolized by all lysosomal sulfatases (http://www.markergene.com/product_sheets/pis1377.pdf) and fluorescent substrates, for instance, CV-$(RR)_2$ for CatB and MR-$(FR)_2$ for cathepsin L indicate protease activity [65, 71].

Changes in intralysosomal pH value can be studied using pH-dependent dyes (acridine orange and neutral red retention). Acridine orange has been used for many years to visualize organelles with acidic pH. The dye stains lysosomes in green and red fluorescence at low extracellular concentrations (2.6 μM). When the concentration of the dye in the staining solution is higher (26–37 μM), stacks can be formed and lysosomes show red fluorescence. Unfortunately, the red stacks bleach very fast and only the monomeric (green) form

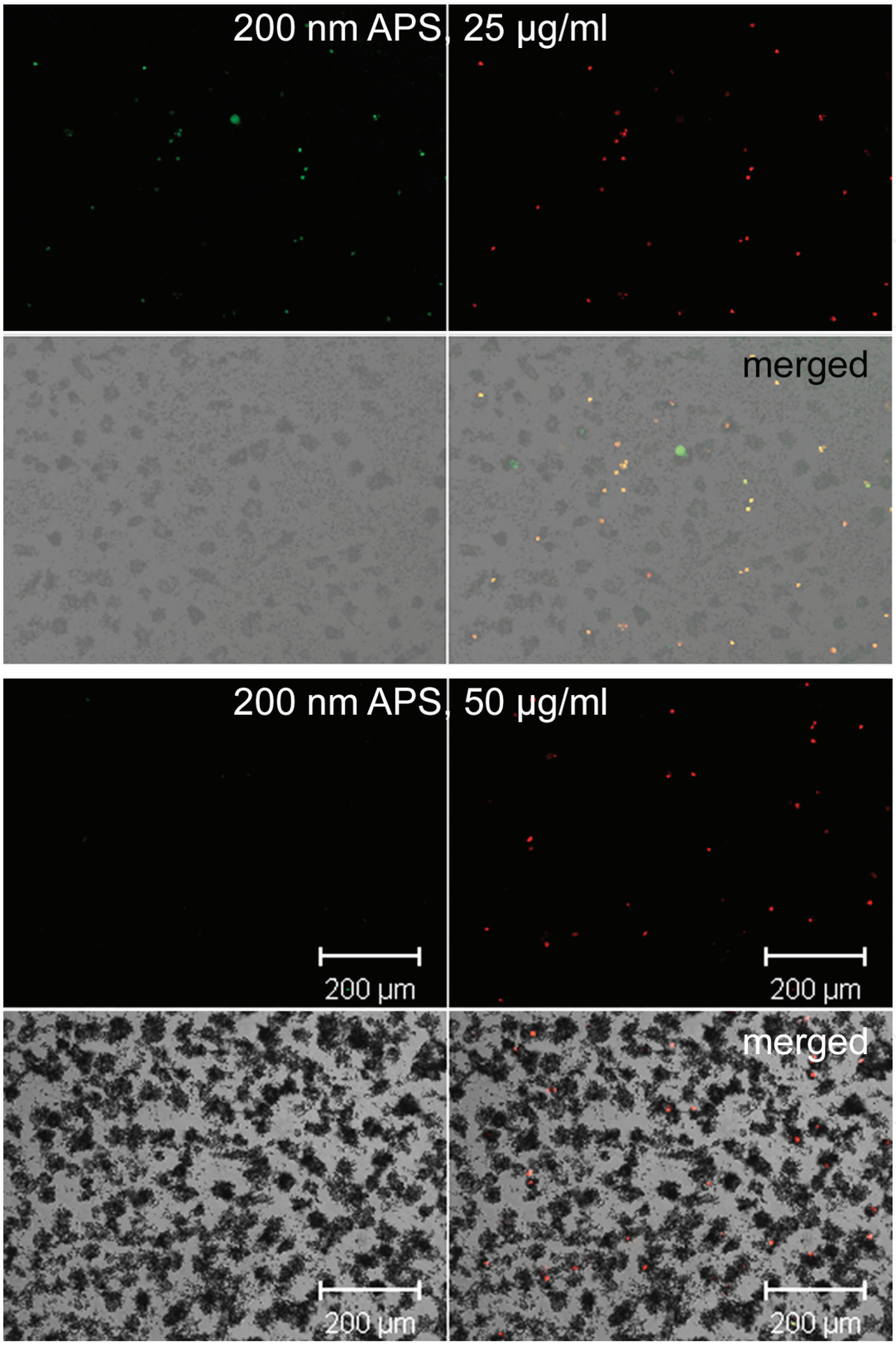

Figure 4. Detection of apoptosis and necrosis caused by 200 nm amidine-functionalized polystyrene (APS) particles in EAhy926 endothelial cells Staining in the different channels (green, upper left; red, upper right; bright field, lower left) and overlay (merged, lower right) is shown. Despite particle sedimentation (dark precipitates), the staining with YoPro-1 (green channel, upper left) for apoptosis and propidium iodide (red channel, upper right) for necrosis is visible. Lower concentrations (25 µg/mL) of the particles caused apoptosis and necrosis, whereas the higher concentration (50 µg/mL) induced only necrotic cell death.

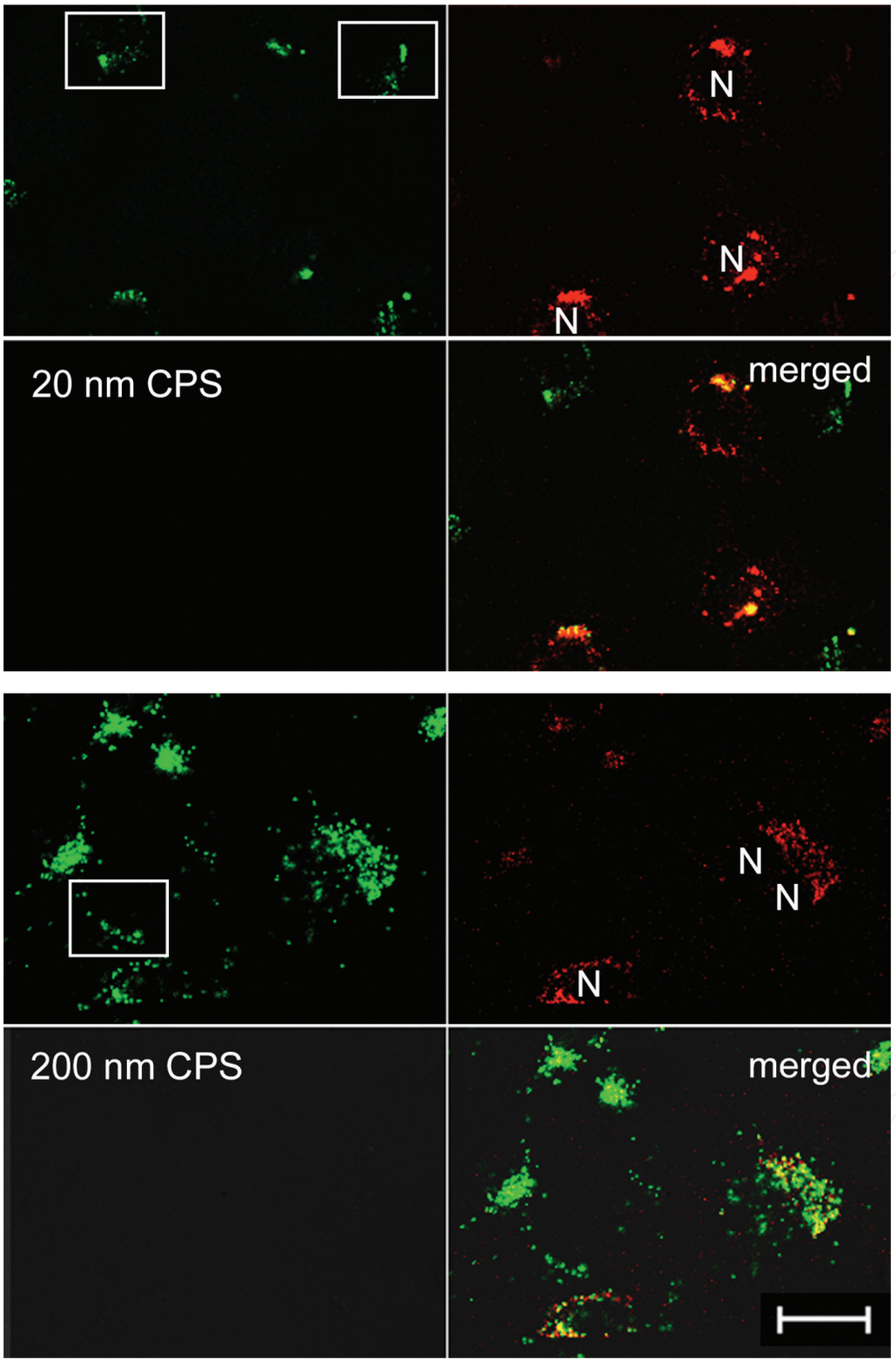

Figure 5. Confocal fluorescence images of 20 nm and 200 nm green-yellow fluorescently labeled carboxyl-functionalized (green) polystyrene NPs in RFP-LAMP-1 transfected EAhy926 endothelial cells (red). Staining in the different channels (green, upper left; red, upper right) and overlay (merged, lower right) is shown. Lysosomes are seen throughout the cells with exclusion of the nucleus (N). Uptake in non-transfected cells (indicated with squares in the green channel) was not obviously different from transfected cells. Small polystyrene particles (a) co-localized to a greater extent with perinuclear lysosomes than larger (b) particles. Abbreviation: RFP, red fluorescent protein. Scale bar, 20 μm.

remains [72]. In addition to that, acridine orange is phototoxic and induces burst of dye-loaded vesicles. Quinacrine also accumulates in lysosomes with acid pH, but the loading is accompanied with morphological alterations (swelling), which makes the staining less suitable for physiological studies. The probe LysoTracker™ Red DND-99, a lipophilic amine with logP 2.10 and pK_a 7.5, is another marker for accumulation in lysosomes [73]. Although it acts not markedly phototoxic, its usefulness as lysosome marker is limited by bleaching. Neutral red can be used as indicator for functional lysosomes [74] but is less sensitive than fluorescent dyes and more often used as viability screening test. Membrane permeant dyes, such as LysoTracker™ and LysoSensor™ probes, label lysosomes in living cells [75]. They are more selective than the classical neutral red and acridine orange dyes. The compound commercialized as Lyso-ID® is a cationic amphiphilic tracer that accumulates in acidic organelles. It can be used as indication for lysosome size and number and is used in the drug screening for lysosomal damage [76] (**Figure 6a, b**). Increased staining indicates swelling of lysosomes and increase in lysosome number. This increase is seen as adaptation to insufficient intracellular degradation capacity.

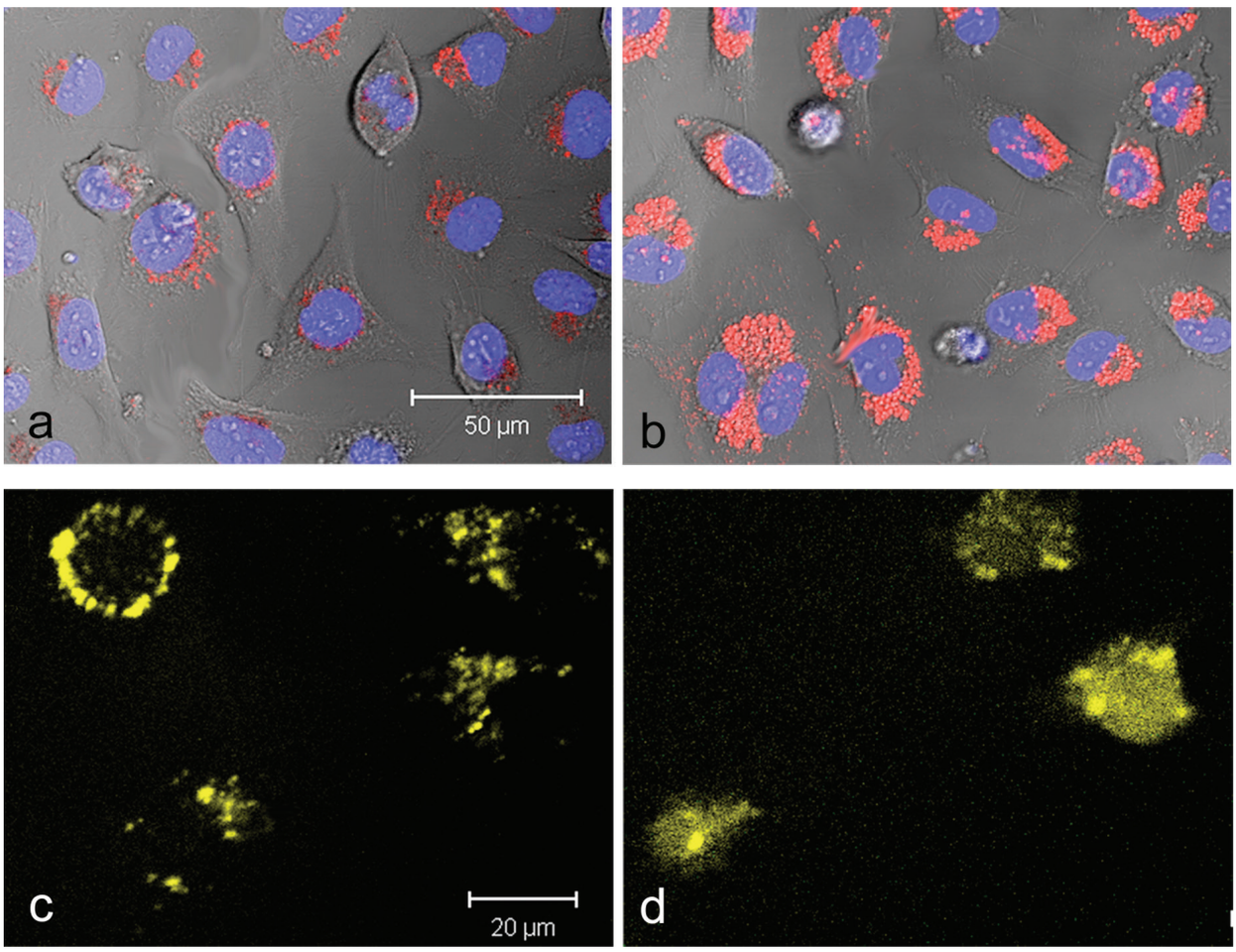

Figure 6. Lyso-ID staining in the cytoplasm (red channel) of untreated EAhy926 cells (a) is low but increased when cells are exposed to 25 µM chloroquine (b). Nuclei are stained with Hoechst 33342 (blue channel). Lysosomes also increased in size upon chloroquine treatment. Massive lysosome damage changed the staining pattern with Lucifer yellow in the cytoplasm from punctuate in untreated cells (c) to diffuse in chloroquine-treated cells (d).

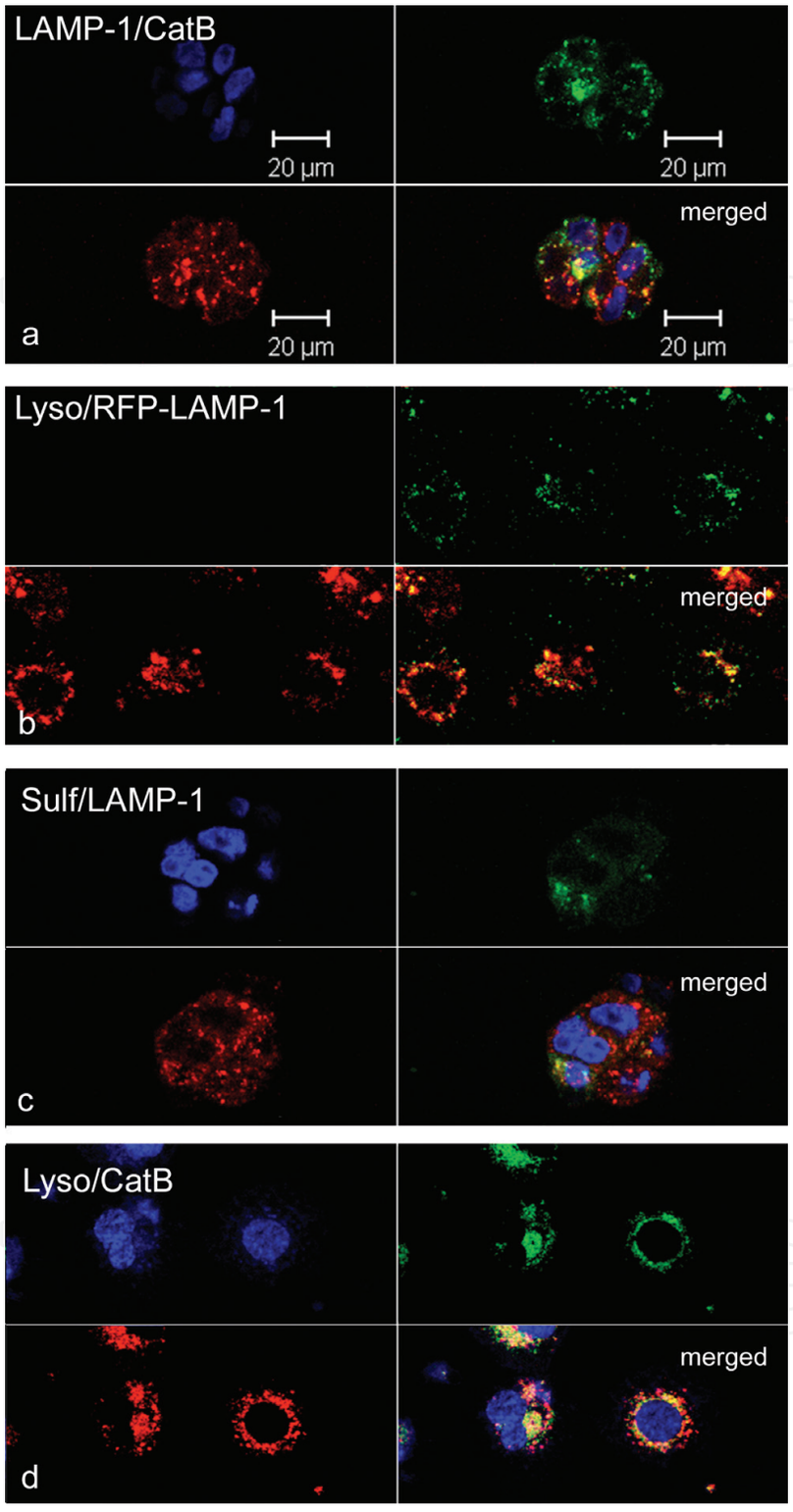

Figure 7. Co-staining with markers for enzyme activity and pH demonstrates interlysosomal heterogeneity of untreated EAhy926 endothelial cells. Lysosomes irrespective of their functional activity are identified by immunocytochemical staining with anti-lysosome-associated membrane protein 1 (LAMP-1) antibody or transduction with RFP-LAMP-1. Staining in the different channels (blue, upper left; green, upper right; red, lower left) and overlay (merged, lower right) is shown. The nuclear stain with Hoechst 33342 is seen in the blue channel. (a) LAMP-1-immunoreactivity (green channel)/CatB staining (red channel), (b) LysoSensor (Lyso) staining (green channel)/RFP-LAMP-1 (red channel), No nuclear staining has been performed. (c) Sulf staining (green channel)/LAMP-1-immunoreactivity (red channel), and (d) Lyso (green channel)/CatB (red channel) staining. Abbreviations: CatB, cathepsin B; LAMP-1, lysosome-associated membrane protein 1; Lyso, LysoSensor dye; RFP, red fluorescent protein; and Sulf, lysosomal sulfatases.

Finally, alterations of the staining pattern with Lucifer yellow from punctuate to diffuse staining can identify lysosome damage. The dye is accumulated and retained in healthy lysosomes but leaks out when the integrity of lysosome membranes is lost (**Figure 6c, d**).

Co-staining with combinations of different lysosomal proteins, pH, and enzyme activity can reveal the heterogeneity of lysosomes and may identify changes in amount and quality of lysosomes. In EAhy926 endothelial cells, LAMP-1-immunoreactive (ir)/CatB-negative lysosomes were seen more frequently than CatB-positive/LAMP-1-not ir structures (**Figure 7a**) and RFP-LAMP-1 positive/Lyso-negative more frequent than Lyso-positive/RFP-LAMP-1-negative structures (**Figure 7b**). Similarly, LAMP-1-ir/Sulf-negative structures were more often seen than Sulf-positive/LAMP-1-not ir structures (**Figure 7c**). This findings can be explained by the fact that RFP-LAMP-1 and LAMP-1 antibodies label all late endosomes and lysosomes, but low pH and prominent enzyme activity are only present in a particular subgroup. LysoSensor and CatB activity stain did also not completely co-localize. CatB-positive vesicles were located more at the cell periphery, while LysoSensor-positive structures were preferentially located in the perinuclear region (**Figure 7d**). This corresponds to the finding that lysosomes with low pH are preferentially located in the perinuclear region [13]. Activity of cathepsin L is also mainly seen in the perinuclear region, but CatB is active over a broader range of pH [77] and can be detected also in peripheral lysosomes.

Lysosomal activity is important for the execution of autophagy, and cellular increase of autophagosomes may indicate impaired lysosomal function. Microtubule-associated protein 1A/1B light chain 3 (LC3) is a cytosolic protein, which, during formation of autophagosomes, is conjugated to phosphatidylethanolamine. The conjugate is first recruited to autophagosomal membranes and, after fusion of autophagosomes with lysosomes, degraded by lysosomal proteases [78]. Immunoblotting or immunocytochemical detection of LC3 has become the most common screening marker for autophagy. Increase of LC3 immunoreactivity indicates an increased content of autophagosomes. Despite the strong link to lysosomes, LC3 and LAMP-1 are not co-localized (**Figure 8**).

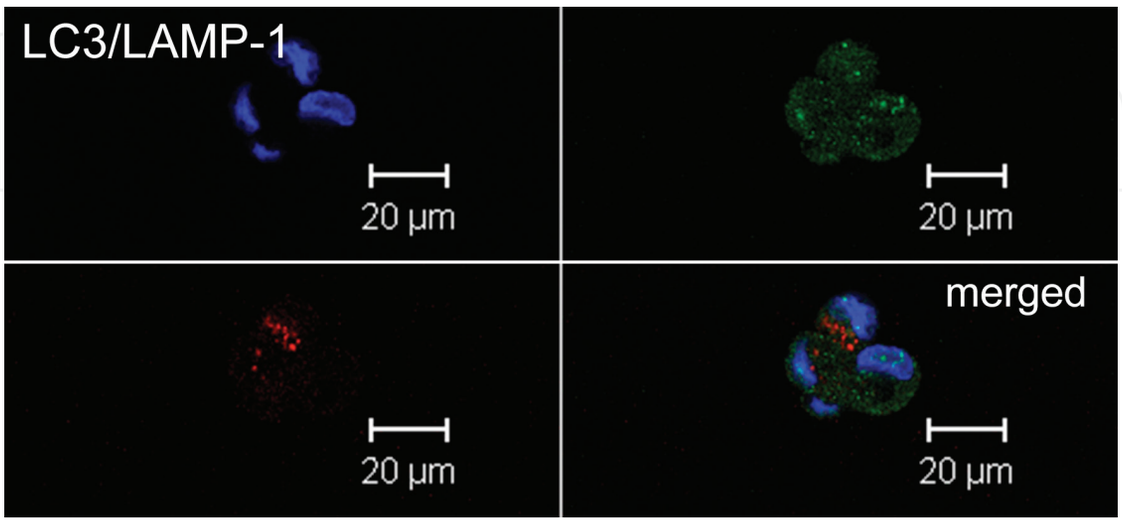

Figure 8. Co-localization of LC3-ir and LAMP-1-ir structures in EAhy926 endothelial cells. Staining in the different channels (blue, nuclei, upper left; green, LC3- ir, upper right; red, LAMP-1-ir, lower left) and overlay (merged, lower right) is shown. Abbreviations: LC3, microtubule-associated protein 1A/1B light chain 3 and LAMP-1, lysosome-associated membrane protein 1.

6. Conclusions

Based on the existing literature, lysosomes may be acutely damaged by high concentrations of NPs. Due to the accumulation in the endosomal-lysosomal system, it is supposed that NPs can damage lysosomes upon prolonged exposure. TEM can identify morphological changes, and a panel of vital stains allows the determination of intralysosomal pH and activity of lysosomal enzymes. While TEM analysis is time-consuming and lacks physiological information, immunocytochemical staining combined with cellular life stains is a good option to study lysosome function. In combination with fluorescent (labeled) NPs, uptake and localization in different parts of the endosomal-lysosomal system can be shown. Given the heterogeneity of lysosomes, particle localization in peripheral or perinuclear lysosomes may cause different cellular effects.

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