Evaluating Nanoparticle Sensor Design for Intracellular pH Measurements

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Intracellular pH plays a pivotal role in cellular processes and is highly regulated in every organelle. The structural stability and function of proteins are tightly associated with pH; for example, it has been shown that mutations in the Vacuolar-type H⁺-ATPase (V-ATPase), responsible for acidification of the Golgi compartment, results in impaired glycosylation of proteins. Furthermore, cell cycle progression and programmed cell death have both been linked to changes in intracellular pH. Thus, quantification of pH fluctuations in organelles of living cells is essential for increasing our understanding of cellular processes. Another area where an increased understanding of the pH profile in the endosome–lysosome pathway is highly important is related to design of pH-sensitive drug delivery systems. A number of nanoparticle-based pH-sensitive drug delivery systems are being reported every year where various surface ligands, e.g., folate and antibodies, are attached to the surface of nanoparticles for targeting overexpressed receptors. However, there is very limited knowledge on the intracellular trafficking of these systems, particularly regarding the pH that the particles are experiencing after internalization. At present, it is just assumed that the pH-sensitive delivery system ends up in acidic compartments, but this has not been tested, and it is reasonable to hypothesize that the targeting ligands used could have an effect on trafficking. Thus, nanoparticle pH sensors could play an important role in enhancing our knowledge on how different targeting ligands affect trafficking of nanoparticles in cells, which could further improve our understanding of how to design better drug delivery systems that release their cargo in a controlled manner as a response to acidification in the surroundings. Unfortunately, the methodologies for conducting measurements of pH in the endosomes and lysosomes using nanoparticle-based pH sensors are not well developed. Thus, we here focus on developing the necessary methodology and furthermore evaluate nanoparticle sensor design, which will allow us to improve the sensor systems for these types of measurements in the future.

A general limitation with fluorescence-based pH measurements is the concentration range the sensor covers. This range depends on the acid dissociation constant ($K_a$) of the pH-sensitive fluorophore, which gives a sigmoidal calibration curve in a $\text{pH}$ range of the $K_a$ to 1, as a rule of thumb, with a nonlinear relationship between fluorescence ratio and $\text{pH}$. Several investigations have been made on the endosomal–lysosomal system using particle sensors with a maximum range of two $\text{pH}$ units. Thus, it is evident that at some point the actual $\text{pH}$ will fall outside the range of the sensor since the $\text{pH}$ differs by more than 2 $\text{pH}$ units between early endosomes and lysosomes. The calibration curve can in principle provide $\text{pH}$ values that are more than one $\text{pH}$ unit from the $K_a$ value;
however, these measurements are sensitive to even the smallest errors due to sensor fluorophore saturation,11–14 and such measurements can give misleading results. In addition, as we will show in this report, it is problematic to do reliable quantification of the pH close to the sensor’s range limit. The calibration curves have been the Achilles heel in many studies, and even though it is the most important part of a quantitative study of pH in cells, it is often carried out in an inappropriate way or even not presented, thereby precluding the possibility of data evaluation.15–17 Finally, localization of the sensor inside the cell is essential in order to evaluate and understand the measurements,18 and failure to do so can lead to misinterpretation of results. Many of these considerations are also important in the quantification of other metabolites in cells.19–24

During the past two decades numerous optical nanoparticle-based sensors (nanosensors), dual-labeled with a pH-sensitive and an insensitive fluorophore for ratiometric measurements, have been developed and are well designed from a synthetic point of view.25–29 These nanosensors have overcome many of the problems encountered when cells are loaded with free dye;29–31 however, lack of sensor evaluation in a biological setting has precluded the realization of the fact that earlier developed dual-labeled nanosensors are too limited in their pH sensitivity range to be useful in cellular measurements in the endosome–lysosome pathway. In a series of cellular experiments we have found that previous dual-labeled sensors can give misleading results. This problem has actually been pointed out by Downey et al.32 more than 10 years ago, where they studied the pH in phagocytic cells using fluorescent-labeled zymosan and provided important insight into the challenges associated with pH measurements. We show here that recently reported nanometer-sized polymeric particle sensors, triple-labeled with a dynamic measurement range of almost 4 pH units33 that covers the entire physiologically relevant range of the endosome–lysosome pathway, provide reliable results. We demonstrate the application of this sensor in real-time pH measurements in living cells and show the importance of its design relative to earlier reported sensor types.34 Furthermore, we provide an equation describing the calibration curve of this triple-labeled sensor derived from thermodynamic principles, along with an in-depth description of how to analyze and evaluate ratiometric images.

RESULTS

Design and Principle of Triple-Labeled Nanosensor. We have investigated a nanoparticle-based optical sensor with two sensor dyes and a reference dye for ratiometric measurements utilizing an acrylamide cross-linked matrix. This matrix constitutes a porous nanoparticle (Figure 1a) that protects the sensor dyes from interferences in the cell as earlier reported,35 with a very fast response time to changes in pH and full control over dye ratios during measurement. The two pH-sensitive fluorophores that are covalently attached to the particle are Oregon Green (OG) and fluorescein (FS), along with the pH-insensitive rhodamine B (RhB) (for synthetic procedure see Supporting Information). When incorporated into the employed particle matrix, the pKₐ values of Oregon Green and fluorescein are 4.1 and 6.0, respectively (found as fitting parameters to the calibration curve). This gives a small overlap in their pH measurable ranges and results in a doubling of the measurable pH range in comparison to sensors with one sensitive fluorophore.34 Oregon Green and fluorescein are both excited at 488 nm and show identical emission spectra, but their intensity dependency on pH is not the same, which is easily realized by their

Figure 1. The triple-labeled nanosensor: design, calibration, and pH measurements. (a) Schematic of the cross-linked polyacrylamide nanoparticle. (b) In vitro calibration of the triple-labeled sensor with both OG and FS, and two dual-labeled sensors with either OG or FS. Normalization of ratios has been performed by subtraction of Rmax and division by (Rmax − Rmin) for all sensors in relation to their respective fitted calibration curve. Mean ± SD between 450 regions of interest (ROIs) are presented. (c) Uptake of the triple-labeled sensor by a HepG2 cell after 24 h and washing and imaged with confocal microscopy. A combined image where the ratios from the intensity images are converted into pH via the calibration curve and color coded on a linear scale according to pH, thereafter overlaid with the differential interference contrast (DIC) image. Scale bar, 10 μm. OG = Oregon Green; FS = fluorescein.
differences in pK_a. Furthermore, when used as covalently bound sensor fluorophores in nanosensors, their ratio has to be optimized to obtain optimal ratiometric curves. Figure 1b shows ratiometric curves, measured on the microscope, of calibration emission spectra between the sensor and reference fluorophores in the nanoparticle sensors, excited at 488 and 561 nm, respectively, as a function of pH. Two dual-labeled sensors with FS or OG and the recently reported triple-labeled sensor (FS and OG in the same nanoparticle) are shown (all with rhodamine B as the reference fluorophore).

The dual-labeled sensors follow a sigmoidal function described by

$$R = \frac{R_1}{10^{pK_a1 - pH + 1}} + R_0$$

where R is the ratio of emission intensities excited at 488 and 561 nm, $R_0 = R_{min}$ (the ratio for the fully protonated form), $(R_1 + R_0) = R_{max}$ (the ratio for the fully deprotonated form), and $pK_a$ is the specific $pK_a$ value for the fluorophore when incorporated into the particle. This equation is in accordance with what has previously been derived for transcribed GFP-based pH indicators. In this field of transcribed pH sensors important considerations on derivation of calibration curves and actual pH measurements have been published.

The triple-labeled sensor follows an extended version of eq 1:

$$R = \frac{R_1}{10^{pK_a1 - pH + 1}} + \frac{R_2}{10^{pK_a2 - pH + 1}} + R_0$$

where $R_0 = R_{min}$, $(R_1 + R_2 + R_0) = R_{max}$ and $pK_{a1}$ and $pK_{a2}$ are the specific $pK_a$ values of the two pH-sensitive fluorophores when incorporated into this particle, here fluorescein and Oregon Green. The calibration curves have been normalized according to their intensities by subtraction of $R_{min}$ and division by $(R_{max} - R_{min})$. Methods for calculating pH as a function of R are presented in the Supporting Information (supporting eqs S1 and S2), along with a more thorough description of the derivation of a more generalized form of eqs 1 and 2 (supporting eq S3).

The triple-labeled sensor has a very large dynamic range, with a 13.5-fold increase in ratio from pH 3.2 to 7.0. This makes it very suitable for measurements in the endosomal–lysosomal pathway. The developed sensor is spontaneously taken up by HepG2 cells via endocytosis due to the surface chemistry of the sensor (being weakly cationic), and after 24 h they reside in compartments with a pH of 4.5 ± 0.4 (mean ± standard deviation (SD)) (Figure 1c). With the new sensor even large changes in pH in both directions can now be reliably measured. All measurements of pH were performed after subtraction of background in each channel. The background of every image series was determined from a histogram of all pixels (Supporting Figure S1). Further image analysis was performed by two methods, either based on regions of interest (ROIs) utilizing the Fiji processing package based on ImageJ or with a pixel by pixel method based on custom-made software, both described in the Methods section. The two methods give identical results, and a comparison can be found in Supporting Figure S2. The pixel-based method allows us to generate corresponding pH images by mapping the ratio of each pixel onto a linear pH color scale, which was combined with differential interference contrast (DIC) images to produce overlay images (e.g., Figure 1c).

**pH-Sensing Capabilities of the Triple-Labeled Sensor.** During maturation of endosomes to lysosomes there is a characteristic decrease in pH, which is driven by the V-ATPase proton pump. Bafilomycin A1 is a specific inhibitor of V-ATPases and has been shown to inhibit the acidification of phagosomes, but also a study on the accumulation of the acidotrophic weak base acridine-orange in acidic compartments has indicated that it increases the pH of endosomes and lysosomes. Figure 2a (top panel) shows the uptake of the triple-labeled sensor after 24 h, before and after treatment with bafilomycin A1. This sensor shows a profound shift in pH, which is illustrated as a color change from yellow-green to a more blue color after 30 min. The histogram in Figure 2b also reveals that the pH does indeed increase more than 1 pH unit from having a maximum at pH 4.3 to 5.6. Inhibition as a function of time with cell measurements at 5 min intervals shows a steady increase in pH up to 30 min (Supporting Figure S3). This pH range lies well within the boundaries of the pH sensitivity of the triple-labeled sensor, thereby giving reliable and accurate measurements. In order to exclude that the change in pH was caused by differential photobleaching of the fluorophores, control experiments were performed; cells with internalized nanosensor were imaged continuously for more than seven images. This experiment showed no alterations in pH between the seven images, showing that it is the treatment with bafilomycin A1 that is causing the increase in pH. Furthermore, as it is the pH-sensitive fluorescein derivatives that are least photostable (easily observed by increasing laser power), a decrease in ratio would be the consequence, hence a decrease in pH.

**Erroneous pH Measurements with a Conventional Dual-Labeled Sensor.** Unreliable results are obtained when making the same measurements as described above with a conventional dual-labeled sensor with a $pK_a$ value of 4.3. Figure 2a (middle panel) shows the uptake of the dual-labeled sensor with Oregon Green as the pH-sensitive fluorophore after 24 h. Images were taken before and after treatment with bafilomycin A1 for 30 min, and the color bar used for the pH scale is the same as applied for the triple-labeled sensor (Figure 2a top panel). The corresponding pH histograms are shown in Figure 2c. With a $pK_a$ value of 4.3 the measurable range
of this sensor covers the interval 3.4 - 5.2. If the actual pH exceeds these limits by a small margin, the sensor will still return pH values; however, these values will be outside the range where quantitative measurements are possible due to the sigmoidal shape of the calibration curve, i.e., at the plateaus where small changes in the ratios correspond to large changes in pH. Thus, the measurements are sensitive to even the smallest errors and are unreliable. For the dual-labeled sensor, the total amount of pH values measured that exceed pH 5.2 and thus fall outside the range where measurements are reliable is approximately 34% and 70% before and after treatment with bafilomycin A₁ treatment. The pH axis of the histograms corresponds to the sensitivity range of the triple-labeled sensor. Bottom histogram is a magnification of the middle histogram excluding measurements with a ratio larger than Rₚ. Mean ± standard error of the mean (SEM) (n = 8 and 5 images for triple and dual-labeled nanosensors, respectively) are presented, and at least 1400 ROIs were analyzed for each treatment. The presented data are representative of six and three independent experiments for the triple and dual-labeled nanosensors, respectively. OG = Oregon Green; FS = fluorescein; Baf = bafilomycin A₁.

Figure 2. Measurements of pH changes of more than 1.1 pH units. (a) Nanosensor internalized during 24 h by HepG2 cells imaged by confocal microscopy before and after treatment with bafilomycin A₁ for 30 min. The ratio of the pH-sensitive and reference fluorophores was converted into pH via the respective calibration curve and color coded on a common linear scale according to pH. Top: triple-labeled sensor. Middle: Dual-labeled nanosensor with pKₐ value of 4.3, including pixels yielding ratios larger than Rₚ in blue. Bottom: Same as middle, without pixels with ratios larger than Rₚ. Scale bars, 10 μm. (b, c, and d) Histograms showing pH distribution of nanosensor-containing vesicles of the cells in (a) respectively, before and after bafilomycin A₁ treatment. The pH axis of the histograms corresponds to the sensitivity range of the triple-labeled sensor. Bottom histogram is a magnification of the middle histogram excluding measurements with a ratio larger than Rₚ. Mean ± standard error of the mean (SEM) (n = 8 and 5 images for triple and dual-labeled nanosensors, respectively) are presented, and at least 1400 ROIs were analyzed for each treatment. The presented data are representative of six and three independent experiments for the triple and dual-labeled nanosensors, respectively. OG = Oregon Green; FS = fluorescein; Baf = bafilomycin A₁.
Distribution of Ratios within a Measurement. The distribution of pH measured in a cell is determined by the distribution of ratios. Figure 3 shows the distribution of ratios corresponding to the pH histograms presented in Figure 2 for the triple and dual-labeled sensors before and after treatment with bafilomycin A₁. The ratio is normalized in order for the full range of the sensor to cover the interval 0 to 1, where $R_{\text{max}} = 1$. From the distribution of ratios it can be seen that the change in the main ratio is larger for the dual-labeled sensor than for the triple-labeled sensor. However, as the pH interval covered by the triple-labeled sensor is larger, the ratio interval from 0 to 1 also represents a larger pH interval for the triple-labeled sensor than for the dual-labeled; hence the same change in ratio corresponds to a larger pH change for the triple-labeled sensor. What is also evident from the distributions of ratios for the dual-labeled sensor is that both distributions exceed $R_{\text{max}} (=1)$, and it is therefore not possible to determine the exact pH of these measurements. This is especially pronounced after treatment with bafilomycin A₁, where the maximum of the distribution resides at 1, leaving about 50% of the measurements above $R_{\text{max}}$ in the presented data set, indicating that the true pH is larger than the upper limit of the dual-labeled sensor.

The true pH, as measured with the triple-labeled sensor seen in Figure 2b, indeed has a maximum around pH 5.6 and covers a broad range from 4.1 to 7.1. As the majority of this distribution of pH exceeds the measurement range of the dual-labeled sensor (3.4–5.2), it is evident that the distribution of the ratios will be situated around $R_{\text{max}}$, and measurements exceeding this cannot not be reported as a specific pH but rather as being larger than a certain pH, as illustrated in Figure 2c. To circumvent this problem, one should utilize sensors that have a measurement range wide enough to measure all pH values in the cell, such as the triple-labeled sensor evaluated in this article.

Other sensors, such as magnesium, calcium, and zinc sensors, which also rely on the binding of the metabolite the sensor is designed for, could potentially encounter the same problems.

pH Measurements in Time and Space. The triple-labeled sensor spans a pH interval that covers the whole physiologically relevant interval with respect to the endosomal–lysosomal system, and its surface chemistry furthermore ensures that it targets the lysosomes after endocytosis. It is therefore possible to study the kinetics of compartmental acidification as the endosomes mature into lysosomes. HepG2 cells were treated with the nanoparticle sensor for 1.5, 2.0, and 24 h. Images presented in Figure 4a show DIC images to the left, images with pH represented by a color bar in the middle, and to the right overlays of the two. After 1.5 h it is evident that the nanosensor particles are taken up by endocytosis, showing a distinct punctuate pattern throughout the cytoplasm of the cell. A histogram of the pH after 1.5 h of treatment shows a broad peak with a mean pH of 5.1 ± 0.6 (mean ± SD) (Figure 4b). Many particle sensors reside in endosomes, some have just been taken up and experience a high pH > 6.5, and some have already reached more acidic compartments. After 2 h the pH histogram reveals a shift toward lower pH with a mean pH of 4.9 ± 0.6 (mean ± SD). A long tail toward higher pH can still be observed, indicating that not all particles have reached an acidic compartment. After 24 h of treatment the pattern has changed to a more perinuclear location, and more particles have been taken up. At this point the pH shows a narrow peak around 4.5 ± 0.4 (mean ± SD), indicating that all nanoparticle sensors have reached a highly acidic compartment. These kinetic measurements of the acidification process reveal that the endocytosis event and successive transport to acidic compartments is a fast process, taking less than 1.5 h. Furthermore, judging from the kinetics of acidification from 1.5 to 2 h, most particles have probably reached...
the acidic compartments before 24 h. The pH does not decrease any further over time, indicating that the sensors have reached their final destination, potentially the lysosomes. Importantly, we still see a strong signal from both rhodamine B and the pH-sensitive fluorophores co-localizing with each other, indicating that both the fluorophores and the particles are intact at this low pH, which has also been confirmed by chemical degradation studies.

A co-localization study after 24 h between the lysosomal marker, lysosome-associated membrane protein 1 (LAMP-1), and rhodamine B-labeled nanoparticles recognizes these acidic compartments as lysosomes (Figure 5a). Transient expression of LAMP-1 fused to green fluorescent protein (GFP) was obtained in HepG2 cells using BacMam viruses (recombinant baculoviruses with mammalian expression cassettes). Significantly, co-localization was observed with an overlap coefficient of 71% and a Pearson’s correlation coefficient of 0.69, whereas co-localization with the early endosomal marker Rab5 fused to GFP showed no co-localization with coefficients of 26% and 0.18, respectively (Figure 5b). Similar results were obtained with a neutral nanoparticle (Supporting Figure S4), demonstrating that localization to lysosomes does not depend on the nanosensor surface charge.

Finally, we show that the nanoparticle has a very low cytotoxicity at the concentrations (10 μg/mL) utilized for these measurements, as assayed by the XTT viability assay (Supporting Figure S5).

**DISCUSSION**

We have carried out the first biological evaluation of a new principle in sensor design, i.e., using triple-labeled pH nanosensors incorporating two pH-sensitive fluorophores and a reference fluorophore for ratiometric measurements of up to 4 pH units within one nanoparticle-based sensor. Furthermore, we have compared this sensor to conventional dual-labeled...
sensors. The triple-labeled sensor covers the physiologically relevant pH interval from 3.2 to 7.0 of the endosome/lysosome system. With this sensor, the pH was measured in the lysosomes, and the sensor can follow the rapid increase in pH up to 5.6 after treatment with the V-ATPase inhibitor bafilomycin A1. The data obtained lie well within the sensitivity range of the triple-labeled sensor, ensuring reliable measurements. We demonstrate how these same measurements done with dual-labeled sensors have limitations, and their use can result in erroneous conclusions.

In fact, a number of critical factors should be addressed before continuation to actual pH measurements with a nanosensor in cells. These factors include consideration of the measurable range of the sensor, appropriate performance of calibration (which should always be presented), appropriate background subtraction, localization of the sensor in the cells, and finally cytotoxicity of the sensor.

Furthermore, we emphasize that the width of the distributions of ratios in a measurement is highly important. As seen in Figure 3, a measurement will always return a distribution of ratios around the mean value even for a homogeneous sample. This behavior has important implications. When the mean of the distribution approaches the $R_{\text{max}}$ part of the distribution actually exceeds the $R_{\text{max}}$ and is thus not available for measurements of the pH. Ultimately, this renders the sensitive range of the sensor even smaller than the rule of thumb, $pK_a$ as ratios start to fall out of the measurable range before the mean of the distributions is close to $R_{\text{max}}$. The width of a distribution of ratios is influenced by the natural distribution of pH in the cell; however, other factors also contribute. First, the amount of different fluorophores within the particles has a distribution; that is, the ratios of the amount of sensitive and reference fluorophores in the particles are distributed around a mean value, adding to the width of the overall distribution measured. More uniform particles will contribute less to this effect. Also, the scan speed necessary to obtain sufficient signal allows a small degree of particle diffusion and, in the worst case, a one-pixel movement between scanning at the two wavelengths, which ultimately changes the

![Figure 5. Localization of the nanosensor. Co-localization of (a) RhB-labeled nanoparticle with lysosomal marker GFP-LAMP-1 and (b) early endosomal marker GFP-Rab5a. HepG2 cells were transduced with plasmids encoding GFP-tagged marker and incubated with nanoparticle for 24 h. Top left image: GFP tagged lysosomal/endoosomal marker; top right: nanoparticle; bottom left: overlay; and bottom right: scattergram of all pixels in overlay relating green intensity to red intensity of the same pixel. Scale bar, 10 μm. Representative of three independent experiments.](image)

![Figure 6. In vitro and in situ calibration of the triple-labeled nanosensor. Calibration was done in buffer, artificial cytoplasm, and inside cells. Ratiometric measurements of the nanosensor in different buffers are related to pH and fitted to eq 2 for triple-labeled nanosensors. The artificial cell cytoplasm was prepared by sonication of HeLa cells and mixed with buffers with controlled pH. In situ calibration was obtained by treatment of nanosensor-containing cells with nigericin in K⁺-rich buffers. Normalization of ratios has been performed by subtraction of $R_{\text{min}}$ and division by $(R_{\text{max}} - R_{\text{min}})$. Mean ± SD between three images are presented.](image)
ratio obtained in that pixel. This will contribute to a broadening of the distribution of measured ratios, but the effect can be reduced by averaging by using regions of interest or using pixel binning. Faster scanning leads to reduced signal intensity to noise ratios; hence, a compromise between the two has to be met for every experiment. In addition, the background noise also has a distribution, and even though a background subtraction is performed, this is subtracted as an average value, leaving the width of the distribution unchanged. Furthermore, the distribution of light from a point source (a particle), i.e., the point spread function, contributes with different intensities of light to neighboring pixels, adding to the width of the distribution of measured ratios. Finally, nonsynchronized fluctuations of the lasers will also result in alterations of intensities and therefore contribute to a broadening of the distribution of ratios. Correction of laser intensity for each image can overcome this issue.

Despite the aforementioned challenges, the triple-labeled sensor extends the sensitive range considerably compared to a dual-labeled sensor and proves itself as a valuable tool for pH measurements of the endosomal—lysosomal system, where the measurements will reside well within the pH-sensitive range, ensuring easily interpretable and reliable measurements. The principle can even be expanded to include more sensor dyes, giving an even broader measurement range.

**METHODS**

**Materials.** Nigericin and bafilomycin A₁ were purchased from Sigma-Aldrich. For the BacMam virus transduction the Organelle Lights Lysosome-GFP and Organelle Lights Endosome-GFP were purchased from Invtrogen. Images were captured by a Leica TCS SPS AOBS confocal microscope with a 63× water-immersed objective (Leica Microsystems, Germany). The microscope was equipped with an incubator box and CO₂ supply for optimal growth conditions during imaging (Life Imaging Services GmbH, Switzerland).

**Characteristics of Employed Nanoparticles.** Synthesis of cross-linked polycrylamide nanoparticles is described in the Supporting Information. Characteristics of the employed nanoparticles are presented as their size and zeta potential as assayed by dynamic light scattering and phase analysis light scattering, respectively (ZetaPALS, Brookhaven, SE). Triple-labeled nanosensor: 57 nm and 4.6 mV. Dual-labeled nanosensor with Oregon Green: 61 nm and 7.8 mV. Fluorescein: 68 nm and 7.4 mV. Nanosensor for co-localization: 110 nm and 30 mV.

**Cell Culture.** The HepG2 and HeLa cell lines were originally obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 100 U/mL penicillin and streptomycin (Lonza). Cell cultures were incubated in a 5% CO₂ humidified incubator at 37 °C.

**Calibration.** In vitro calibration curves were generated from fluorescence images of the nanosensor at 12.5 or 25 mg/mL in 60 mM buffers (20 mM HEPES/20 mM MES/20 mM acetate/100 mM NaCl) from pH 2.1 to 8.2. For calibration in buffers with artificial cytoplasm, 10⁶ HeLa cells per mL of Milli-Q water was sonicated for 1/2 h and mixed 1:2 with 120 mM buffers, pH was measured, and the solution was finally mixed with sensors in a 3:1 ratio for a final concentration of 60 mM buffer and 12.5 mg/mL nanosensor. The microscope was focused in a plane within the image was thresholded to produce a binary image, and all regions larger than 0.15 μm² were recognized as ROIs. This mask was generated by addition of the two corresponding intensity levels for both colors (Supporting Figure S1). The calibration was utilized, including (i) binning of neighboring pixels (in order to eliminate artifacts caused by sensor diffusion in between scanning the two color channels), (ii) detection of sensor domains by comparison of the pixel intensity (of the reference dye) to the average intensity of the neighborhood (eliminate single pixels domains), and (iii) removal of pixels with reference dye intensity lower than a cutoff. The intensities of the included pixels were converted to pH via the calibration curve. The second method was based on custom-made software where image preprocessing was utilized, including (i) binning of neighboring pixels (in order to eliminate artifacts caused by sensor diffusion in between scanning the two color channels), (ii) detection of sensor domains by comparison of the pixel intensity (of the reference dye) to the average intensity of the neighborhood (eliminate single pixels domains), and (iii) removal of pixels with reference dye intensity lower than a cutoff. The intensities of the included pixels were converted to pH via the calibration curve. pH histograms obtained from both methods were determined as averages based on 5–9 images.

**Co-localization.** HepG2 cells were seeded in 24-well plates on 9 mm cover glass slides for 24 h. They were then transduced

**Nanosensor Treatment and Image Acquisition.** HepG2 cells were seeded in 35 mm culture dishes with a 10 mm microwell glass bottom for 24 h. Cells were incubated with 10 μg/mL nanosensor for 24 h (and 1.5 and 2 h for the kinetic study), washed three times with ice-cold phosphate-buffered saline (PBS) supplemented with heparin (20 units/mL), washed once with PBS, and kept in growth medium without phenol-red for observation by confocal microscopy. Images were collected by sequential line scanning, with excitation at 488 and 561 nm. Emission was collected by photomultiplier tubes in the ranges 498–570 and 571–735 nm, respectively, obtained by tunable high-reflection mirrors. The microscope was equipped with an incubator box and CO₂ supply to ensure optimal growth conditions during microscopy. Cells were imaged sequentially (by line) with excitation at 488 and 561 nm along with a DIC image. For bafilomycin A₁ analysis cells were first imaged in media and then, while on the microscope stage, supplemented with 50 nM bafilomycin A₁. The same cells were then imaged after 30 to 35 min.

**Image Analysis.** The background of every image series was determined by plotting a histogram with number of pixels per intensity level for both colors (Supporting Figure S1). The background level was identified as the main peak at low intensities, and the top of this peak was used as a measure of the background level for each color. This value was subtracted from all images in a series and the corresponding calibration curve. Two methods have been employed for the measurements of pH. The first method utilized the Fiji processing package based on ImageJ for the generation of a mask with ROIs localizing the nanosensor-containing vesicles. The mask was generated by addition of two corresponding intensity images (in order to include all nanosensor-containing vesicles), the image was thresholded to produce a binary image, and all regions larger than 0.15 μm² were recognized as ROIs. This mask was superimposed onto the original intensity images, and mean intensity for each ROI for each color was calculated. After background subtraction the intensity ratio of each ROI was converted to pH via the calibration curve. The second method was based on custom-made software where image preprocessing was utilized, including (i) binning of neighboring pixels (in order to eliminate artifacts caused by sensor diffusion in between scanning the two color channels), (ii) detection of sensor domains by comparison of the pixel intensity (of the reference dye) to the average intensity of the neighborhood (eliminate single pixels domains), and (iii) removal of pixels with reference dye intensity lower than a cutoff. The intensities of the included pixels were converted to pH via the calibration curve. pH histograms obtained from both methods were determined as averages based on 5–9 images.
with Organelle Lights reagents according to the manufacturer. Briefly, cells were washed in PBS and incubated with baculovirus (containing either GFP-Rab5a or GFP-LAMP-1 plasmids) diluted 1:1 to 1:6 in PBS for 4 h at room temperature with gentle shaking. Virus-containing solution were then aspirated and replaced with full growth medium supplemented with 0.1% BacMam enhancer. Cells were incubated at optimal growth conditions for 2 h, and medium was replaced with normal growth medium containing 10 μg/mL RHB-labeled nanoparticles and incubated overnight. This nanoparticle resembles the nanosensor without pH-sensitive fluorophores in order for co-localization with GFP-tagged markers. Cells were then washed as described for nanosensor treatment and imaged by confocal microscopy with excitation at 488 and 561 nm. After appropriate background subtraction the correlation coefficients—Pearson’s correlation coefficient and an overlap coefficient—were calculated.

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Supporting Information Available: Detailed description of synthetic procedure of nanosensor preparation and derivations of pH equations. Furthermore, determination of background levels of images, comparison of image processing methods, temporal pH measurements following bafilomycin A1 treatment, distributions of measurements, co-localization of a neutral nanoparticle with lysosomes, cytotoxicity of nanosensor, and calibration of the triple-labeled nanosensor in buffer. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES AND NOTES
