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Letter

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Phosphole-oxide-based Fluorescent Probe for Super-resolution Stimulated Emission Depletion (STED) Live Imaging of the Lysosome Membrane

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ABSTRACT: Super-resolution imaging techniques have become increasingly important tools to visualize sub-organelle structures and dynamic processes in living cells on the nanoscale. However, the utility of these imaging techniques is currently limited by the availability of advanced fluorescent probes that enable the specific labeling of the organelle of interest and provide the absorption/emission properties required for super-resolution imaging techniques. Herein, LysoPB Yellow is presented as a new small-molecule fluorescent probe that can selectivity stain the lysosomal membrane. Its outstandingly high photostability and long fluorescence lifetime are beneficial for its use in super-resolution stimulated emission depletion (STED) microscopy. A lysosomal membrane stained with LysoPB Yellow was successfully visualized by STED imaging in living cells with a full width at half maximum (FWHM) of 70 nm, which is substantially below the diffraction limit of light. Additionally, LysoPB Yellow displayed excellent retention ability and negligible cytotoxicity. Consequently, long-term time-lapse confocal imaging of living cells was successfully conducted, which demonstrates the practical utility of LysoPB Yellow in fluorescence imaging.

Lysosomes are acidic cytoplasmic membrane-bound organelles in eukaryotic cells. They play pivotal roles in the degradation of macromolecules and cell components in the autophagy process, as well as in other cellular events, including plasma membrane repair, mitochondrial fission, pathogen defense, and cell signaling.[1–4] Methods for the visualization of lysosomes, especially their membrane structure, are thus highly desirable for the study of their various functions. Transmission electron microscopy (TEM) is generally employed for this purpose, as lysosomes are hundreds of nanometers in size, and TEM can provide a spatial resolution of 1–2 nm. However, as TEM can only be used to visualize fixed cells, temporal information on dynamic biological processes cannot be obtained.

In this context, super-resolution imaging techniques have become increasingly important as tools to visualize sub-organelle structures and dynamic processes in living cells on the nanoscale.[5–7] Lysosome-specific small-molecule fluorescent probes for super-resolution techniques such as stimulated emission depletion microscopy (STED),[8,9] structured illumination microscopy (SIM),[10,11] photoactivated localization microscopy (PALM),[12] stochastic optical reconstruction microscopy (STORM),[13] and single-molecule localization microscopy (SMLM)[14] have recently been developed. These probes enable the visualization of lysosomes with a spatial resolution of up to tens of nanometers in living cells. However, direct observations of the membrane structure of lysosomes remain challenging for two main reasons: 1) lysosome probes stain not only the membrane, but also the inner matrix of the lysosomes, and 2) the probes do not fully satisfy the absorption/emission requirements of the corresponding super-resolution techniques. Very recently, the lysosomal membrane structure was successfully visualized with high spatiotemporal resolution via grazing incidence structured illumination (GI-SIM) microscopy using HaloTag-Lamp1 labeled with the ligand JF_{646}, which is a late endosome/lysosome marker.[15,16] However, tag-protein expression is time-consuming and the expression levels and transfection efficiency depend strongly on the cell type. Therefore, the development of new small-molecule fluorescent probes for specific super-resolution microscopy techniques that enable the visualization of the membrane structure of lysosomes in untransformed cells represents a highly attractive research target.

The main advantage of STED microscopy over other super-resolution techniques is that the images of sub-organelle structures can be acquired immediately without post-processing as in the case of confocal imaging. We have recently demonstrated the structural reinforcement of

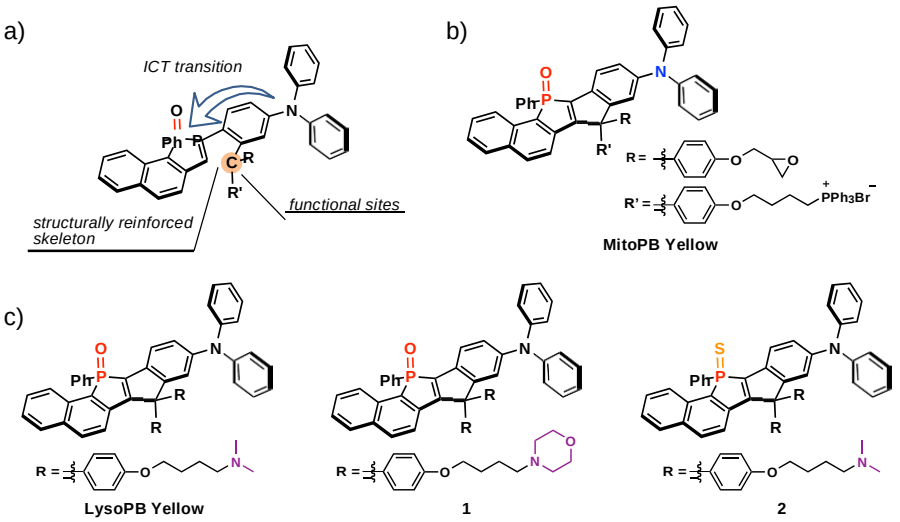

Figure 1. (a) Our molecular design strategy for super-photostable fluorescent probes based on a fused phosphole-oxide skeleton for STED super-resolution live imaging of organelle membranes; chemical structures of (b) the mitochondrial inner membrane probe MitoPB Yellow, and (c) the lysosomal membrane probe LysoPB Yellow and its derivatives **1** and **2**.

electron-deficient phosphole-oxide-based skeletons as an effective design strategy for the development of super-photostable fluorophores that allow the continuous acquisition of STED images in the same area (Figure 1a).[17–19] The introduction of an electron-donating diphenylamino group onto this skeleton produces a donor-π-acceptor-type fluorophore that exhibits environment-sensitive emission due to its intramolecular-charge-transfer (ICT) character in the excited state. Namely, its fluorescence is turned ON in nonpolar environments, such as lipid bilayer membranes, and turned OFF in aqueous media, such as the mitochondrial or lysosomal matrix. This feature allows the membrane morphology to be observed in high contrast. Based on this molecular design, we previously developed the mitochondrial probe MitoPB Yellow, in which the bridging carbon atom is functionalized with mitochondrial targeting and protein labeling groups (Figure 1b).[19] MitoPB Yellow exhibited outstandingly high photostability, a long fluorescence lifetime (~8 ns) in nonpolar environments, and sufficient membrane permeability, which allowed us to conduct not only time-gated STED imaging of the mitochondrial inner membrane in living cells with an unprecedented resolution (45 ± 5 nm), but also time-lapse STED imaging of cristae dynamics.

The design strategy for MitoPB yellow (Figure 1a) should be applicable to the development of various fluorescent probes for other organelles. Herein, we report LysoPB Yellow as a promising membrane probe for acidic organelles (Figure 1c), in which two dimethylamino groups are attached onto the super-photostable fluorophore. LysoPB Yellow exhibits high selectivity towards lysosomes (and late endosomes) while retaining the advantageous characteristics of MitoPB Yellow, including high photostability, long fluorescence lifetime, and membrane-selective staining. Using LysoPB Yellow, clear visualization of the membrane structures of lysosomes was achieved in untransformed wild-type cells with a full width at half maximum (FWHM) of 70 nm via STED microscopy. Notably, LysoPB Yellow showed superior retention ability in the membrane compared to commercially available lysosome markers, as well as negligible cytotoxicity. These features enabled us to conduct long-term time-lapse confocal imaging of living cells for up to 24 h with an interval of 2 min (721 frames), demonstrating the high practical utility of the developed probe in fluorescence imaging.

In this study, we designed and synthesized LysoPB Yellow and its analogues **1** and **2**. Morpholine was introduced in **1** in order to investigate the effect of the targeting group, while a phosphole sulfide skeleton was employed in **2** to examine the influence of the skeletal structure on the cell-staining properties (Figure 1c). The details of the synthesis of these compounds are given in the Supporting Information. Their photophysical properties were investigated in solution using solvents of varying polarity. Given that these three compounds employ the same fluorophore skeleton, they exhibited essentially identical absorption and emission properties (Figures S1 and S2, Table S1 in the

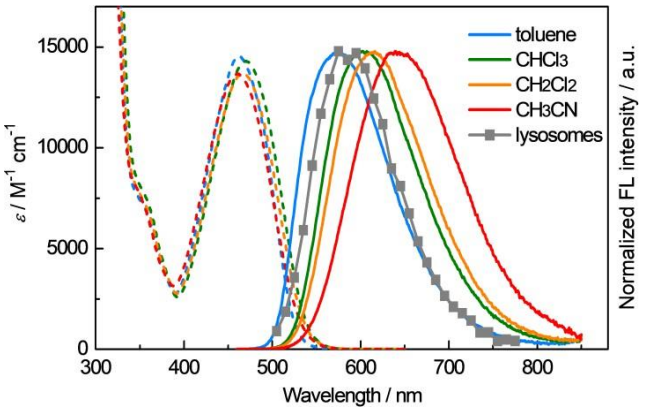

Figure 2. Absorption (dashed line) and fluorescence (solid line) spectra of LysoPB Yellow in various organic solvents. The emission spectrum of LysoPB Yellow in the lysosomes of living HeLa cells is also shown in gray.

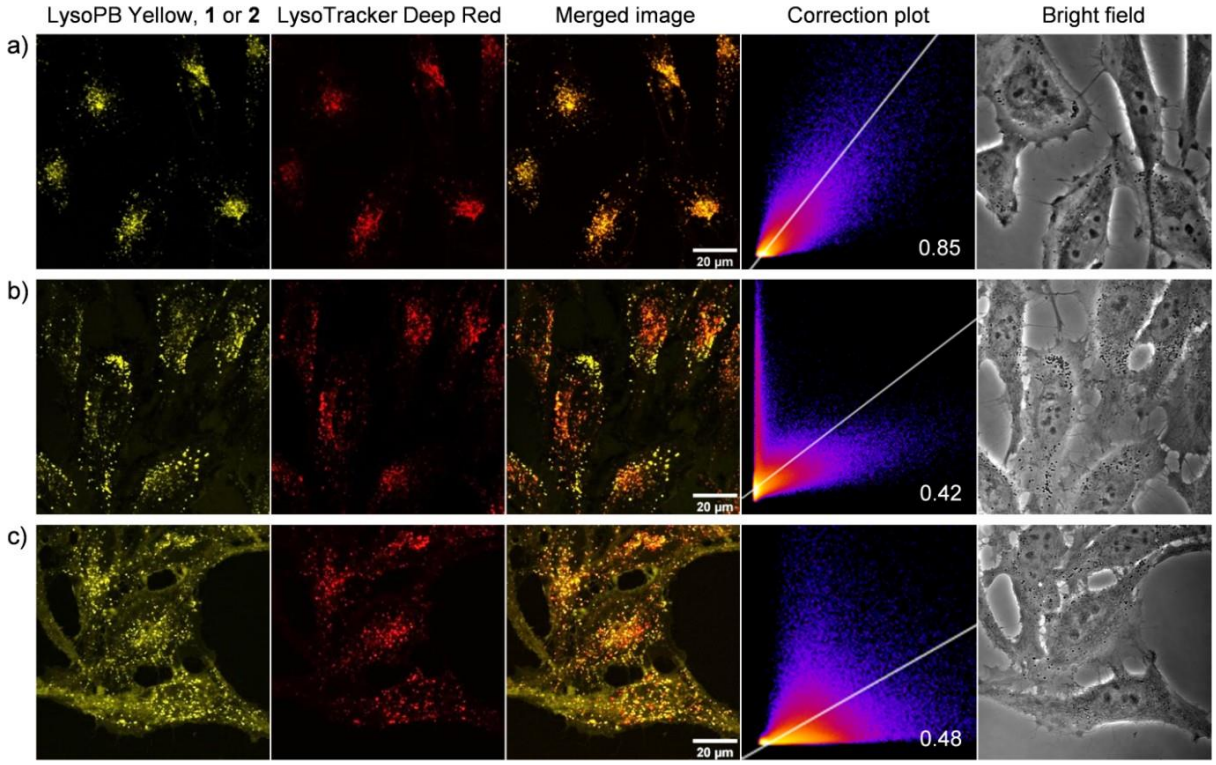

Figure 3. Co-localization imaging of live HeLa cells stained with LysoTracker Deep Red and (a) LysoPB Yellow, (b) **1**, or (c) **2**. From left to right: LysoPB Yellow, **1**, or **2** channel (λ_{ex} = 473 nm; λ_{em} = 490–590 nm); LysoTracker Deep Red channel (λ_{ex} = 635 nm; λ_{em} = 660–760 nm); merged image; Pearson correlation coefficient plot of the two channels; bright field image; scale bar: 20 μm.

Supporting Information).

LysoPB Yellow exhibits an absorption maximum (λ_{abs}) at 460–470 nm, irrespective of the solvent, with molar absorption coefficient (ε) values of ~1.4 × 10^4 M^{-1} cm^{-1} (Figure 2, Table S1 in the Supporting Information). In contrast, the emission maximum (λ_{em}) of LysoPB Yellow strongly depends on the solvent polarity due to its ICT character in the excited state (Figure S3 in the Supporting Information). As the polarity increases from toluene to CH_3CN, its λ_{em} is shifted from 573 nm to 642 nm, which is accompanied by slight decrease in fluorescence quantum yield (Φ_F) from 0.71 to 0.54.

Then, we investigated the cell-staining properties of LysoPB Yellow, **1**, and **2**. For that purpose, live HeLa cells were incubated with 100 nM of LysoPB Yellow for 2 h, followed by staining with the commercially available lysosome marker LysoTracker Deep Red. As shown in Figure 3a, the two channels displayed good co-localization with a Pearson's correlation coefficient value of 0.85, which indicates high lysosome selectivity of LysoPB Yellow. In sharp contrast, both **1** and **2** exhibited nonspecific labeling of other cellular compartments to some extent. Namely, morpholine analogue **1** stained not only lysosomes but also granular structures (Figure 3b), while phosphole sulfide analogue **2** stained both lysosomes and cellular plasma membranes (Figure 3c). Two conclusions could be drawn from these results. 1) The dimethylamino group in the side chain in LysoPB Yellow provides higher lysosome selectivity than the morpholine group in **1**. This likely arises from the inherent difference in the basicity of the two amine moieties; the pK_a values of the conjugate acids of Me_3N and *N*-methylmorpholine are 9.80 and 7.38, respectively. 2) The phosphole oxide skeleton more effectively suppresses nonspecific interactions than the phosphole sulfide skeleton, probably due to its higher hydrophilicity. The enhancement of fluorescence of LysoPB Yellow in lysosomes is due to neither the aggregation of the probe in lysosomes[20] nor the change of the electronic structure induced by protonation of the amino groups (Figures S4 and S5 in the Supporting Information).[21] Importantly, the high lysosome selectivity observed for LysoPB Yellow is general; even in other cell lines, including HepG2 and 3T3-L1, LysoPB Yellow selectively stains lysosomes, which indicates promising potential in this application (Figure S6 in the Supporting Information).

Subsequently, we evaluated the working concentration range of LysoPB Yellow for lysosomal staining. LysoPB Yellow stains lysosomes selectively over a wide concentration range (10 nM – 1 μM) without requiring cell washing. In contrast, LysoTracker Deep Red results in non-specific staining at concentrations as high as 200 nM (Figure S7 in the Supporting Information). The staining ability of LysoPB Yellow is sensitive to the lysosomal pH. When the cells were treated with GPN (Gly-Phe beta-naphthylamide), which increases the pH value oflysosomes,[22] the fluorescence signals of the lysosomes significantly decreased within 10 min, which is consistent with the behavior of LysoTracker Red (Figure S8 in the Supporting Information).[22] This result indicates that the probes located on the lysosomes diffuse into the cytosol at higher pH. One

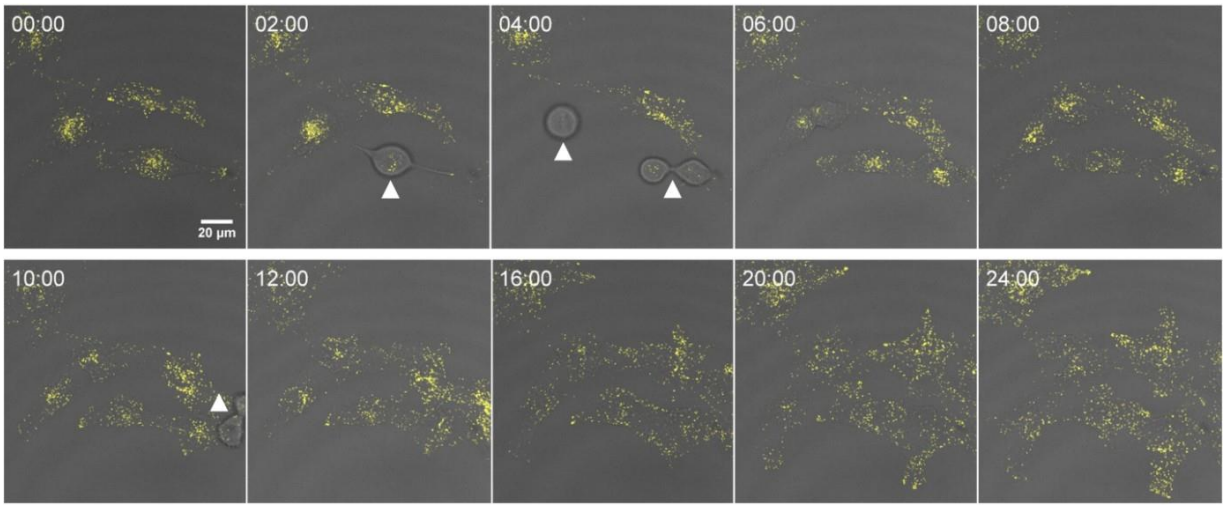

Figure 4. Time-lapse confocal imaging (λ_{ex} = 488 nm, λ_{em} = 500–700 nm) of living HeLa cells stained with LysoPB Yellow. The elapsed time since the beginning of acquisition is indicated. Cell division is marked with white triangles. Scale bar: 20 μm.

of the most advantageous characteristics of LysoPB Yellow compared to LysoTracker Deep Red is its long retention time in the lysosomes. The fluorescence signal intensity of lysosomes stained with LysoTracker Deep Red significantly decreases after washing the cells for 3 h (Figure S9 in the Supporting Information). In stark contrast, the fluorescence signal intensity of LysoPB Yellow remains almost unchanged, even after 24 h of washing (*vide infra*). Moreover, the MTT assay indicated that LysoPB Yellow was not cytotoxic at working concentrations of up to 2 μM (Figure S10 in the Supporting Information). All these results demonstrate the outstanding utility of LysoPB Yellow for lysosomal labeling.

The ICT character of LysoPB Yellow in the excited state makes its emission maximum (λ_{em}) strongly dependent on the environmental polarity. This solvatochromic character of its fluorescence allowed us to obtain in-depth information regarding the microenvironment of the probe in the cells.[23] Namely, we measured the emission spectrum of LysoPB Yellow in lysosomes of living HeLa cells by conducting a lambda scan. The emission spectra showed an emission maximum wavelength of around 585 nm, which is similar to that measured in toluene (Figure 2). This result implies that the probe was located in a very hydrophobic microenvironment.

The fluorescence lifetime (τ) of LysoPB Yellow in lysosomes was also determined using pico-second-pulsed laser excitation and time-gated detection (Figure S11 in the Supporting Information).[24] The same region of LysoPB Yellow-stained HeLa cells was repeatedly imaged with a varying gate delay time (i.e., the delay between the excitation pulse and the start of fluorescence detection, t_g) while keeping the gate width constant (Δt = 3.5 ns). The fluorescence intensity of the images as a function of the t_g value were fitted with an exponential curve, from which the fluorescence lifetime of LysoPB Yellow on the lysosomal membrane was determined to be 6.3 ± 0.2 ns (n = 3). This τ value is higher than that of representative yellow-emissive dyes such as Rhodamine B (1.7 ns) and Cyanine-3B (2.8 ns). The high τ value of LysoPB Yellow should be beneficial for time-gated detection, as it minimizes short-lived background signals in the fluorescence imaging.

It should be noted here that the τ value of LysoPB Yellow in the lysosome is meaningfully shorter than that of MitoPB Yellow in the mitochondria (7.5 ± 0.2 ns, n = 6), despite the fact that both probes employ the same fluorescence skeleton. This result stands in sharp contrast to that previously reported for the imaging of organelle membranes, in which dyes in lysosomes tend to show higher τ values than those in the mitochondria, which was attributed to differences in membrane organization.[25] MitoPB Yellow can covalently bind to inner mitochondrial membrane proteins.[19] The smaller τ value, together with the longer λ_{em} of LysoPB Yellow (585 nm) than MitoPB Yellow (568 nm), suggests that the microenvironment of the lysosomal membrane is relatively hydrophilic as compared to that of the mitochondrial proteins. These results suggest the following mechanism for the staining of lysosomes with LysoPB Yellow. The dimethylamino group of LysoPB Yellow induces the localization of the probe inside the lysosomes. However, due to the hydrophobic character of its fluorophore skeleton, LysoPB Yellow accumulates in the lysosomal lipid bilayer, resulting in the emission of intense yellow fluorescence. Even if the probe is still present in the hydrophilic lysosomal matrix, only faint emission should be observed, due to the decreased fluorescence quantum yields in such a polar medium, reflecting the ICT character of the fluorophore skeleton.

LysoPB Yellow enables the long-term tracking of the dynamic behavior of lysosomes via time-lapse imaging with confocal microscopy. Living HeLa cells were incubated for 2 h with 100 nM of LysoPB Yellow in DMEM+ (Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS)) and 0.5% DMSO as a co-solvent. The cells were washed three times to remove free dye and then maintained in fresh DMEM+ at 37 °C and 5% CO_2 for

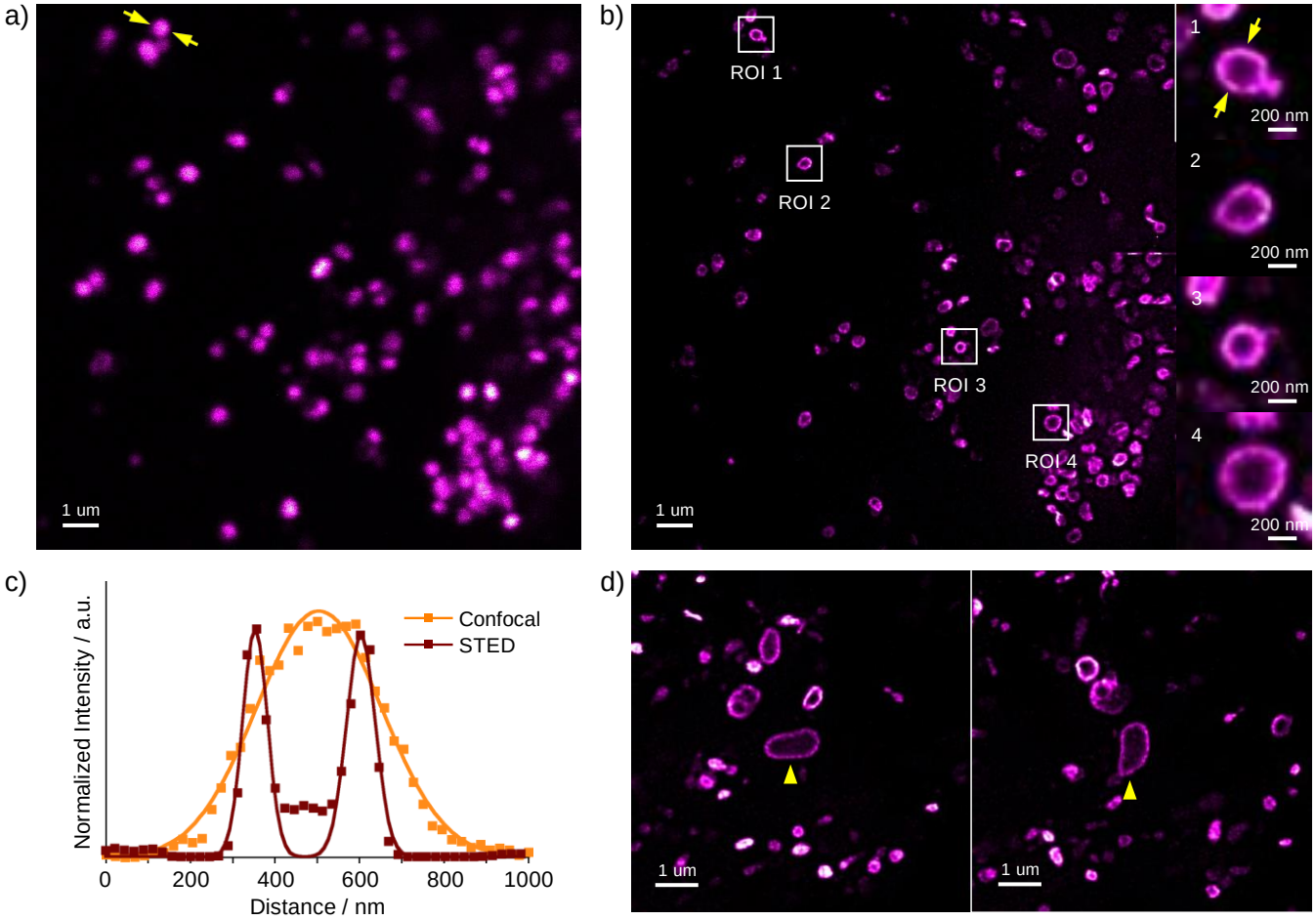

Figure 5. (a) Confocal and (b) STED (deconvoluted) images of lysosomes of living HeLa cells stained with LysoPB Yellow; regions of interest (ROI) in the STED image are shown on the right side. (c) Signal intensity profiles across the lysosomes (ROI 1 in panel b) are indicated with yellow arrows in the confocal and STED images. The data were fitted with single and double Gaussian functions for orange and brown lines, respectively. (d) STED images of unusually large lysosomes with an elliptical shape.

time-lapse confocal imaging. Images were acquired every 2 min for 24 h under excitation at 488 nm (721 frames in total; Figure 4, Movie S1 in the Supporting Information). Notably, the fluorescence intensity of the lysosomes did not decrease over the acquisition period, demonstrating the high photostability of LysoPB Yellow. The cells maintained their function, and several cell division events were observed, demonstrating the biocompatibility of LysoPB Yellow.

Over the course of long-term imaging, many new lysosomes should be generated, especially upon cell division. An important question is whether only the originally existing lysosomes or all lysosomes including the newly generated ones will be visualized. To investigate this, we performed a pulse-chase experiment using LysoPB Yellow and LysoTracker Deep Red (Figure S12 in the Supporting Information). The cells were first labeled with LysoPB Yellow and incubated for 24 h, followed by treatment with LysoTracker Deep Red. If most of the LysoPB Yellow is retained in the original lysosomes, the newly generated ones should only be stained with LysoTracker Deep Red. However, if LysoPB Yellow is released from the original lysosomes but retained in the cell, the fluorescence signals of these dyes should overlap. As shown in Figure S12, the image channels of LysoPB Yellow and LysoTracker Deep Red exhibit a high Pearson's Correlation Coefficient (0.80). This result implies an equilibrium between the accumulation of LysoPB Yellow in the lysosomes and its cytosolic release inside the cell.

The characteristic features of LysoPB Yellow, including its high photostability, long fluorescence lifetime, membrane permeability, and environment sensitivity, allowed us to utilize the probe for the super-resolution STED imaging of lysosomes in living cells. Upon depletion at 660 nm and detection with a delay time of 3 ns, the deconvoluted STED image of the lysosomes clearly showed donut-shape structures corresponding to the lysosomal membrane (Figures 5a–5b). Such structures cannot be observed using confocal microscopy. The saturation intensity of LysoPB Yellow, at which the fluorescence intensity is reduced to half of its original value, has been estimated to be 1.0 MW cm^{-2}. The low saturation intensity should be mainly contributed by the long fluorescence lifetime.[19] The intensity profiles across the lysosome exhibited a FWHM value of 70 ± 4 nm (n = 10) (Figure 5c). The relatively high FWHM value compared to the expected inherent size of the membrane may result from the fast motion of the lysosome. The high-contrast STED images imply that LysoPB Yellow is selectively

localized on the membranes and/or the fluorescence of dye molecules located in the lysosomal matrix is effectively quenched. Although most of the lysosomes observed in the STED images exhibit a circular shape with a diameter of ~200–500 nm, a few lysosomes are obviously larger than others (size up to 1 μm) with an elliptical shape (Figure 5d). These large lysosomes likely represent the fusion of an autophagosome with a lysosome, as has been previously observed by TEM.[26] Although several useful small molecule-based lysosome fluorescent markers have been reported to date, they generally accumulate in the lysosomal matrix; as a result, the lysosomes appear as filled circles in super-resolution images.[8–14] The commercially available lysosome markers LysoTracker Green and LysoTracker Red were also tested for STED super-resolution imaging. LysoTracker Green was not suit for STED imaging because of its low photostability (Figure S13 in the Supporting Information). LysoTracker Red could be used for STED imaging, but the lysosomes appeared as filled circles (Figure S14 in the Supporting Information). To the best of our knowledge, LysoPB Yellow is the first lysosomal membrane marker that enables super-resolution live imaging in untransformed cells.

In conclusion, we have developed the new lysosome fluorescent probe LysoPB Yellow, which exhibits outstanding photostability, a long fluorescence lifetime, and membrane-selective staining. Using LysoPB Yellow in STED microscopy, it was possible to clearly visualize the membrane structures of lysosomes in living cells with a resolution of up to 70 nm. Moreover, LysoPB Yellow displays excellent retention ability as well as negligible cytotoxicity, which enables the long-term time-lapse imaging of living cells. Further applications of LysoPB Yellow in time-lapse STED and SIM super-resolution imaging to reveal the lysosomal membrane dynamics during autophagic processes are currently in progress and the results will be reported in due course.

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge at http://pubs.acs.org.

Chemical synthesis and characterization data of all new compounds, photophysical data for **1** and **2**, additional fluorescence imaging data, and MTT assay results (PDF).

Time-lapse confocal imaging of living HeLa cells stained with LysoPB Yellow (avi).

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Notes

The authors declare no competing financial interests.

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