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PAPER

Imaging of intracellular acidic compartments with a sensitive rhodamine based fluorogenic pH sensor

Zhu Li,[ab] Shuqi Wu,[a] Jiahuai Han[b] and Shoufa Han*[a]

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The parameters of intracellular acidic compartments are altered in apoptosis, autophagy, cancer metastasis, *etc*. Low background and selective staining of lysosomes and autophagosomes was achieved with *N*-(rhodamine 6G)-lactam-ethylenediamine (R6G-EDA) which fluoresces in acidic vesicles *via* pH mediated ring opening of the intramolecular lactam. Long retention time of R6G-EDA in lysosomes and its exceptional stability against photo-bleaching allow full time tracking of lysosome morphology changes in tumor necrosis factor-α (TNF-α) triggered cell death, suggesting its utility for acidic vesicle studies in cell biology.

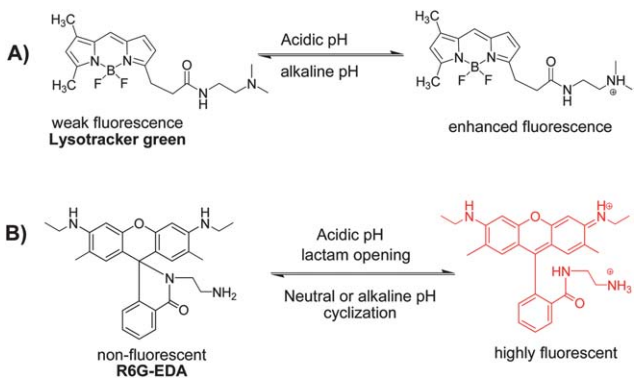
Introduction

Proton concentrations inside eukaryotic cells are not uniformly distributed. For example, the cytoplasma is slightly alkaline at about pH 7.2 whereas the luminal pH of lysosomes is in the range of 6.0–4.0. Intracellular compartments, including lysosomes, endosomes, autophagosome, *etc*., have intracompartmental acidic pH. The acidity of these vesicles is essential for a variety of biological functions ranging from optimal hydrolase activity, autophagy, to apoptosis, *etc*. The organelle specific intracellular pH topology is elaborately controlled and yet dynamically regulated to maintain various levels of pH gradients.

Changes of the parameters of acidic cellular compartments (constituted mostly by lysosomes) have been associated with cell death, cancer metastasis, cell maturation, *etc*.[1–3] The morphology of lysosomes in cancer cells is significantly altered relative to normal cells.[4] Enlargement of total volume of intracellular acidic compartments is reported to be a common feature for cells undergoing apoptotic death.[5] Dyes that could be used for noninvasive imaging of lysosomes are valuable for biomedical applications, including probing the roles of lysosomal parameters in cancer metastasis, *in vivo* cancer diagnosis and evaluation of the effects of lysosome-targeted cancer therapy.[6–10]

Many lysosome specific optical probes often consist of a luminescent core and a side chain of weak base which render the probe acidotropic and thus accumulate in acidic vesicles upon protonation. The weak base side chain, mostly the amine-containing functionality, is judiciously installed in the vicinity of the luminescent core to quench/decrease the inherent fluorescence *via* intramolecular photo induced electron transfer (PET) (Scheme 1). Upon protonation of the side chain, the PET is inhibited, leading to pH dependant recovery of fluorescence emission intensity. Due to the inadequate fluorescence quenching of the PET mechanism, such probes often show nonspecific high background fluorescence signal inside cells. Another limitation of many lysosomal markers is that they are prone to photo-bleaching, excluding long term tracking of lysosome morphology with fluorescence microscope under strong excitation light.

Rhodamine dyes have attracted considerable interest in biological studies due to their distinguished properties of high fluorescent quantum yields, bioorthogonal fluorescence spectra, and exceptional stability against photo-bleaching. Herein we report the low background fluorescent staining of lysosomes and autophagosomes in live cells with a non-fluorescent rhodamine



Scheme 1 Different pH sensing mechanisms of lysotracker green and R6G-EDA. (A) protonation of lysotracker green inhibited the PET, leading to increased fluorescence; (B) pH mediated ring opening of the intramolecular spirolactam of R6G-EDA, yielding a highly fluorescent species.

[a]*Department of Chemical Biology, College of Chemistry and Chemical Engineering, and the Key Laboratory for Chemical Biology of Fujian Province, Xiamen University, Xiamen, China. E-mail: shoufa@xmu.edu.cn*
[b]*The Key Laboratory of the Ministry of Education for Cell Biology and Tumor Cell Engineering, School of Life Science, Xiamen University, Xiamen, China*

lactam that can be activated at acidic pH to give a highly fluorescent and photo-stable rhodamine derivative.

Results and discussion

pH mediated "turn-on" fluorescence of R6G-EDA

Unmodified rhodamines remain highly fluorescent over a wide range of pH (9.0–4.0). Thus they are generally unsuitable to stain intracellular acidic compartments due to high background fluorescence signals, albeit the fact that rhodamine B stained the acidic organelles of denervated skeletal muscle cells but failed for other mammalian cell lines.[11] It would be advantageous to mark lysosomes with probes that are intrinsically non-fluorescent but could reversibly rearrange into highly fluorescent species *in situ* in acidic organelles.

Rhodamine lactams, a group of non-fluorescent and colorless compounds hallmarked by intra-molecular spirolactams, are poised to form highly fluorescent rhodamine derivatives under appropriate conditions *via* ring opening of the spirolactam. Recently, rhodamine lactams containing metal ion recognition moieties have been extensively explored to sense metal ions with selectivity and sensitivity.[12] Although it has long been documented that rhodamine lactams display sharp color changes and strong fluorescence in acidic media, applications of rhodamine lactams as intracellular acidic pH sensors have been largely unexplored.[13] In this report, R6G-EDA was systematically evaluated for its efficacy to fluorescently stain lysosomes *via* proton triggered ring opening of the lactam in live cells (Scheme 1).

R6G-EDA, the non-fluorescent rhodamine lactam, was readily achieved *via* aminolysis of rhodamine 6G with ethylenediamine.[14] Analogous to commercial lysotrackers which often consist of a fluorophoric core linked to a weak base moiety, R6G-EDA also contains an amino group to allow it to preferentially accumulate in acidic organelles. To test the sensitivity of proton triggered ring opening of R6G-EDA, its pH dependant fluorescence emission spectra were recorded in buffers of various pH values (Fig. 1A). The pH titration showed that R6G-EDA, non-fluorescent at pH 7.0 or above, rearranged at acidic pH into a fluorescent rhodamine species which has fluorescence spectra similar to that of rhodamine 6G (Fig. 1B). The fluorescence intensity of R6G-EDA at pH 4 was 1500 fold brighter than that at pH 7.0, and was 100 fold brighter than that at pH 6.5, demonstrating that R6G-EDA was a sensitive acid responsive probe capable of sensing minute pH changes in the range of pH 6.0–4.0.

Selectivity of R6G-EDA for pH sensing

Many ions, like Na^+, Ca^{2+} and Mg^{2+}, are ubiquitous in mammalian cells at various concentrations. Upon appropriate stimulations, some cells could generate chemically reactive species, *e.g.*, HOCl and H_2O_2. The selectivity of R6D-EDA against representative intracellular cations and reactive chemical species was tested by fluorescence emission spectrometry. It was shown that R6G-EDA did not respond to Na^+, K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, and Co^{2+} at a concentration up to 1 mM (Fig. 2), and was unreactive with hydrogen peroxide and hyperchlorite at concentrations up to 5 mM (Fig. 2). In contrast, an acid sensitive

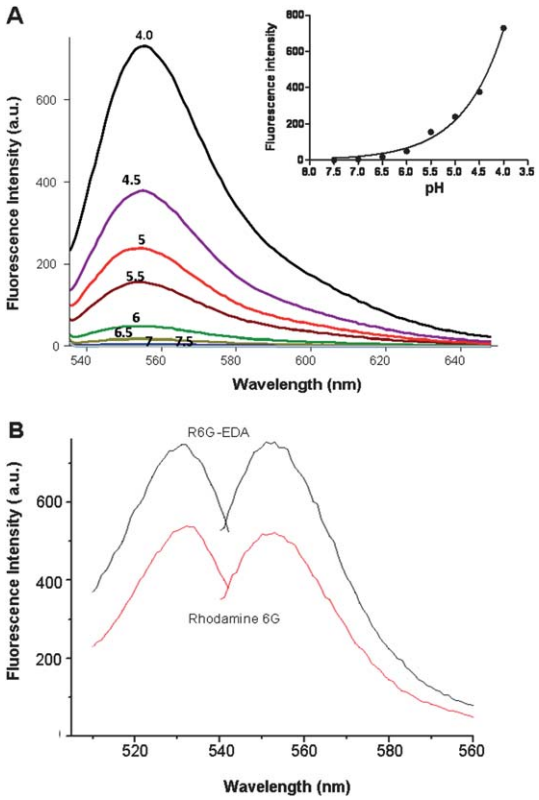


Fig. 1 Characterization of R6G-EDA by fluorometry. (A) Fluorescence emission spectra of R6G-EDA at various pH; the insert shows the pH titration curve of R6G-EDA (Ex@535 nm and Em@552 nm); (B) fluorescence spectra of R6G-EDA as compared to Rhodamine 6G in acidic buffer (pH 4).

rhodamine-hydrazide lactam was shown to be highly reactive with hyperchlorite ion.[15] The high sensitivity of R6G-EDA to proton and its high selectivity over interfering species that could be present inside cells indicate that R6G-EDA is a potential selective pH sensor for applications in live cells.

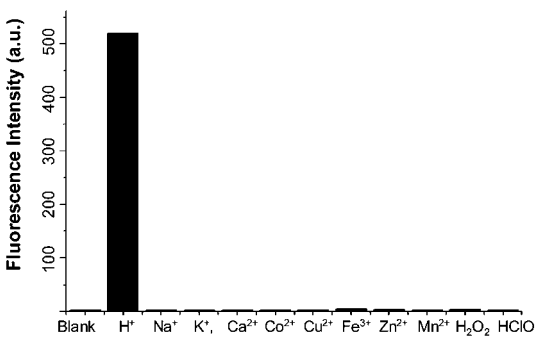


Fig. 2 Selectivity of R6G-EDA for pH over selected interferences in Dulbecco's Modified Eagle Medium (DMEM). Fluorescence emission intensity of R6G-EDA in DMEM containing H_2O_2 (5 mM), HOCl (5 mM) or various cations (1 mM) as compared to pH (0.1 mM). (Ex@533 nm, Em@553nm).

Staining of lysosomes in live cells with R6G-EDA

Demonstrated to be specific for pH sensing, R6G-EDA was evaluated for its capability to selectively stain lysosomes in live cells. Cells of mouse embryonic fibroblast (MEF), L929, and mouse leukaemic monocyte macrophage (Raw 264.7) were cultured with R6G-EDA and LysoTracker Green DND-26 from Invitrogen (referred as lysotracker green). The cells were then visualized by confocal fluorescence microscopy to probe the distribution of R6G-EDA relative to lysotracker green which is currently widely used for lysosome staining. The double staining experiments revealed that R6G-EDA stained the lysosomes in all four cell lines tested, exhibiting staining patterns that are identical to that of lysotracker green (Fig. 3). The distribution of R6G-EDA colocalized with lysotracker green, suggesting that R6G-EDA selectively stain lysosomes in live cells.

To probe if the fluorescence in cells treated with R6G-EDA resulted from pH mediated opening of the spirolactam in lysosome, L929 and MEF cells were first cultured with or without bafilomycin A1 (BFA), which is an ATP-H1 pump inhibitor and is able to dissipate the pH gradient across the lysosomal membranes. The cells were then co-stained with R6G-EDA and lysotracker green. Confocal fluorescence microscopy analysis showed that the fluorescence signals of R6G-EDA and lysotracker green were both clearly present in L929 and MEF cells in the absence of BFA in the control experiments (Fig. 4). In cells pretreated with BFA, the fluorescence of lysotracker green was greatly diminished. The remaining trace amount of fluorescence could be identified in MEF cells, which is consistent with the manufacturer's guide that the lysosomal fluorescence constituted only a portion of total cellular fluorescence (Fig. 4). In L929 and MEF cells pretreated with BFA, the fluorescence of R6G-EDA disappeared. It has been reported that some cations, *e.g.*, Fe^{3+}, could promote the ring opening of the intramolecular lactam of *N*-(rhodamine B)-lactam-ethylenediamine and lead to the formation of fluorescent species.[23] This experiment showed that R6G-EDA was a pH dependant fluorogenic lysosome marker, excluding the possibility of endogenous metal ions (*e.g.*, Fe^{3+}) mediated fluorescence of R6G-EDA inside cells.

Staining of autophagosomes with R6G-EDA

Autophagosomes are routinely visualized or quantitated with GFP-LC3, a fusion of green fluorescent protein with LC3B protein which is a marker of autophagosomes in mammalian cells.[16] Although the amount of GFP-LC3 per cell is usually an accurate measure of autophagosome number, this assay is not free of concerns.[16] Over-expressed GFP-LC3 easily forms aggregates which are often indistinguishable from true autophagosomes by fluorescence microscopy. Visualization of R6G-EDA and LC3-GFP in L929 cells undergoing autophagy was performed to probe the correlation of their spatial distributions. The confocal fluorescence microscopic images showed that the GFP-LC3 recruiting autophagosomes were greatly induced upon treatment with tumor necrosis factor-α (TNF-α), consistent with the roles of TNF-α in autophagy.[17–19] Staining of L929 cells with R6G-EDA revealed an enlarged volume of intracellular acidic compartments which colocalized with GFP-LC3 recruiting autophagosomes (Fig. 5), suggesting R6G-EDA could be used to

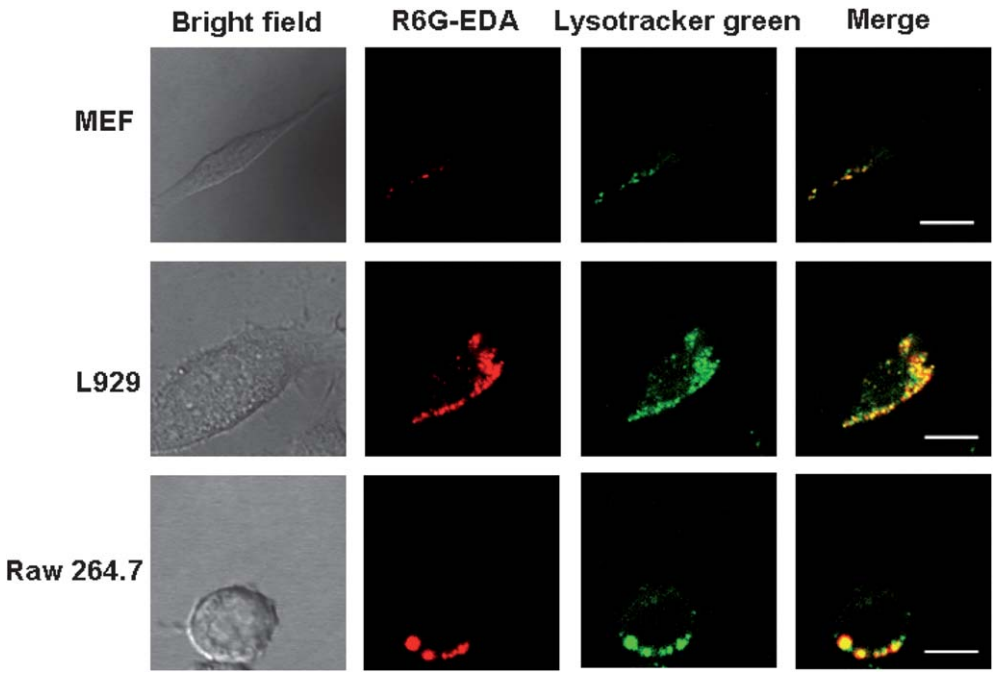


Fig. 3 Intracellular distribution of R6G-EDA as compared to lysotracker green. MEF, L929 and Raw 264.7 cells were respectively co-cultured with R6G-EDA and lysotracker green, washed with HBBS buffer, and then analyzed with confocal fluorescence microscopy to pinpoint the locations of R6G-EDA and lysotracker green inside cells. Merging of the fluorescence of R6G-EDA (shown in red) and lysotracker green (in green) was shown in yellow. Bars, 10 μm.

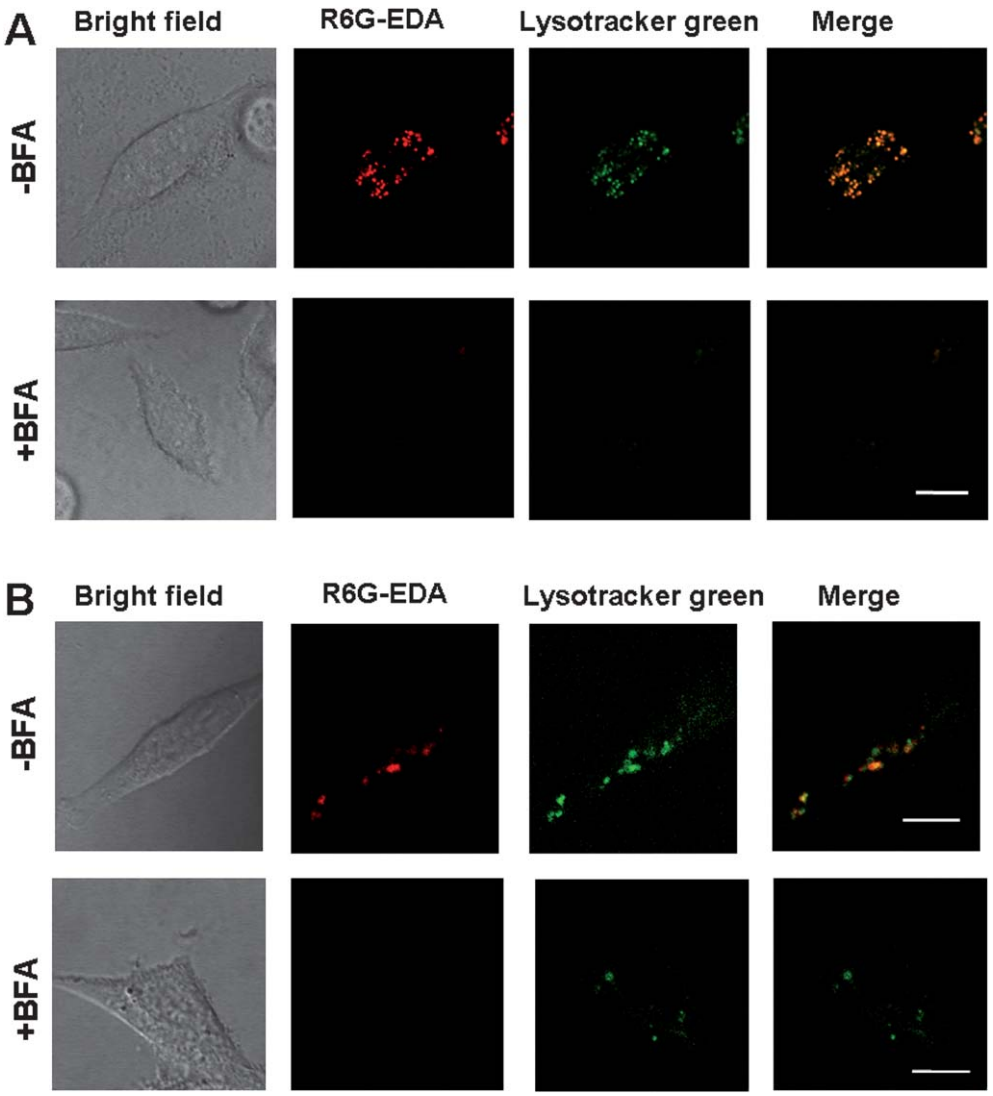


Fig. 4 pH dependant staining of L929 (**A**) and MEF (**B**) cells with R6G-EDA. Cells were incubated for 4 h at 37 °C in the absence or presence of 50 nM bafilomycin A1, and then stained with R6G-EDA (10 μg ml^{-1}) and lysotracker green (1 μM) for 30 min before characterization. Merging of the fluorescence of R6G-EDA (shown in red) and lysotracker green (in green) is shown in yellow (bars, 10 μm).

stain autophagosomes in apoptotic cells. Given the facts that proteins usually aggregate in cytoplasma, and R6G-EDA is non-fluorescent at cytoplasmatic pH, R6G-EDA might be used as the reference dye to discriminate GFP-LC3 aggregates from autophagosomes.

Properties of R6G-EDA in live cells

Non-invasive imaging of lysosomes with optical probes are valuable for *in vivo* diagnosis of viable cancer cells[10] and *in vitro* evaluation of the effects of lysosome-targeted cancer therapy.[11,20] It is advantageous that the probes show low background signals in extra-cellular settings and could selectively illuminate in lysosomes of viable cancer cells. Many other features are also beneficial for the aforementioned applications, *e.g.*, long retention time in lysosomes, low cytotoxicity, and high stability against photo-bleaching.

Shown to be able to selectively stain lysosome in live cells, R6G-EDA was further evaluated for the retention time in cells. L929 cells prestained with either R6G-EDA or lysotracker green were respectively seeded and incubated in probe-free culture medium for 24 h. Cells were analyzed with fluorescence microscopy to quantitate the amount of probes left inside cells. The levels of R6G-EDA in cells remain unaffected before and after incubation. In contrast, no lysotracker green was identified in the control cells (Fig. 6).

The cytotoxicity of R6G-EDA was evaluated on MEF, L929, Raw264.7 and SF9 cells by trypan blue exclusion test. No detrimental effects of R6G-EDA (10 μg mL^{-1}) on the viability of MEF, L929, Raw264.7 and SF9 cells were observed before and after incubation at the concentration used for lysosome staining (Fig. 7).

Photo-bleaching is a common problem for many organic dyes, often compromising the temporal monitoring of dynamic events inside cells by virtue of the reporting dyes. L929 cells

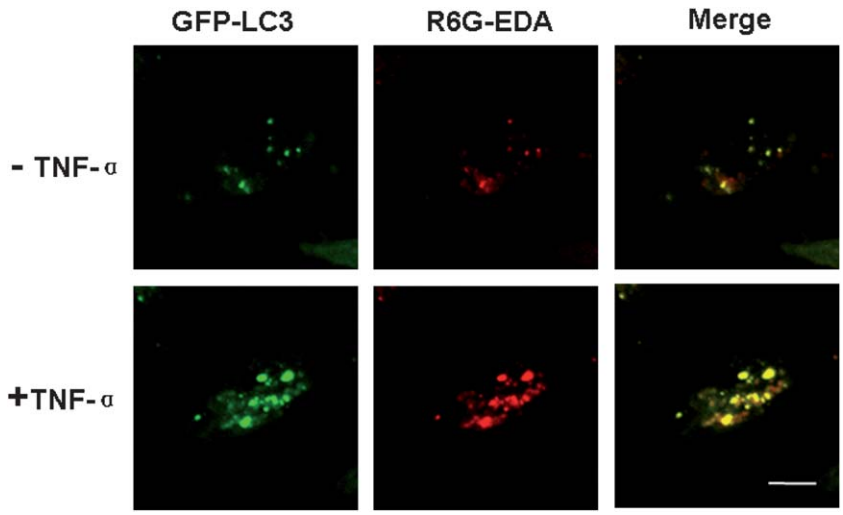


Fig. 5 Staining of autophagosomes with R6G-EDA in L929 cells. L929 cells expressing GFP-LC3 were cultured with R6G-EDA in the absence or presence of TNF-α for 6 h to induce autophagy vesicles. Both autophagic and control cells were imaged by confocal fluorescence microscopy to probe the distribution of R6G-EDA and GFP-LC3. Merging of the fluorescence of R6G-EDA (shown in red) and lysotracker green (in green) is shown in yellow (bars, 10 μm).

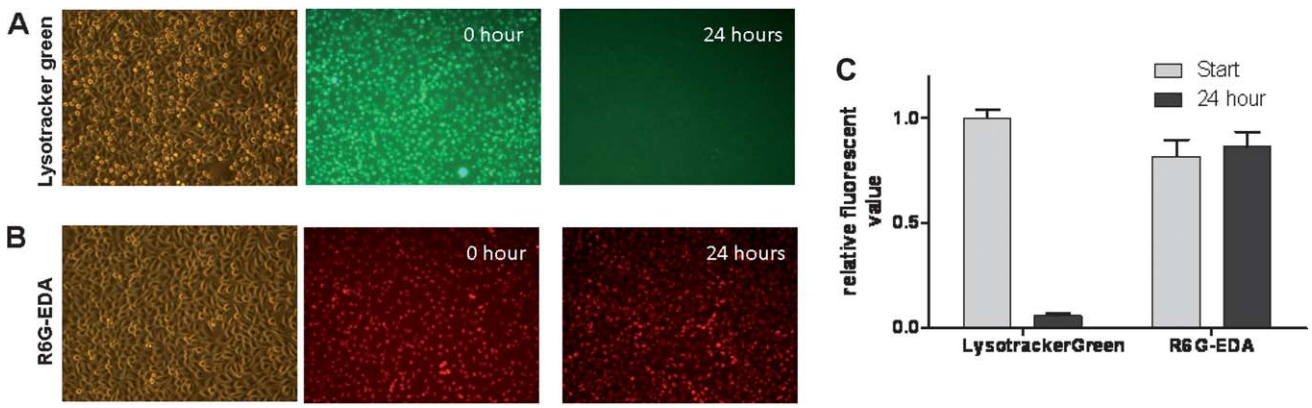


Fig. 6 Retention rates of R6G-EDA in L929 cells as compared to time dependant reduction of Lysotracker green. Cells prestained with Lysotracker green (A) or R6G-EDA (B) were cultured in fresh medium for 24 h, and then visualized by fluorescence microscopy before and after incubation; (C) Retention rates of R6G-EDA and Lysotracker green in L929 cells before and after incubation.

prestained with R6G-EDA or lysotracker green were respectively exposed to constant laser illumination, the intracellular fluorescence intensity was monitored as a function of time by fluorescence microscopy. It was shown that the fluorescence of lysotracker green quickly decayed in 5 min while R6G-EDA exhibited constant fluorescence emission, suggesting the superior photostability of R6G-EDA over lysotracker green (Fig. 8).

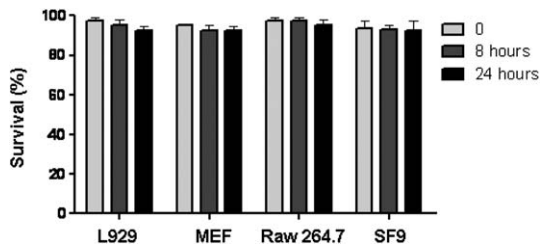


Fig. 7 Cytotoxicity of R6G-EDA on MEF, L929, Raw264.7 and SF9 cells. Cells were incubated with R6G-EDA (10 μg mL^{-1}) for 8 or 24 h. The cell number and cell viability were determined by trypan blue exclusion test.

Another advantageous feature of R6G-EDA is that imaging or quantitation of lysosomes in cells can be performed in the culturing medium without extensive washing of the cells to remove the extracellular R6G-EDA. On the contrary of R6G-EDA, which is non-fluorescent in culture medium, lysotracker green exhibits high background fluorescence in the medium (Fig. 9).

R6G-EDA, with high selectivity for pH, long retention time in lysosomes, high stability against photobleaching, low cytotoxicity and low background signals, demonstrates better performance in many aspects than lysotracker green, suggesting its novel applications for lysosome studies.

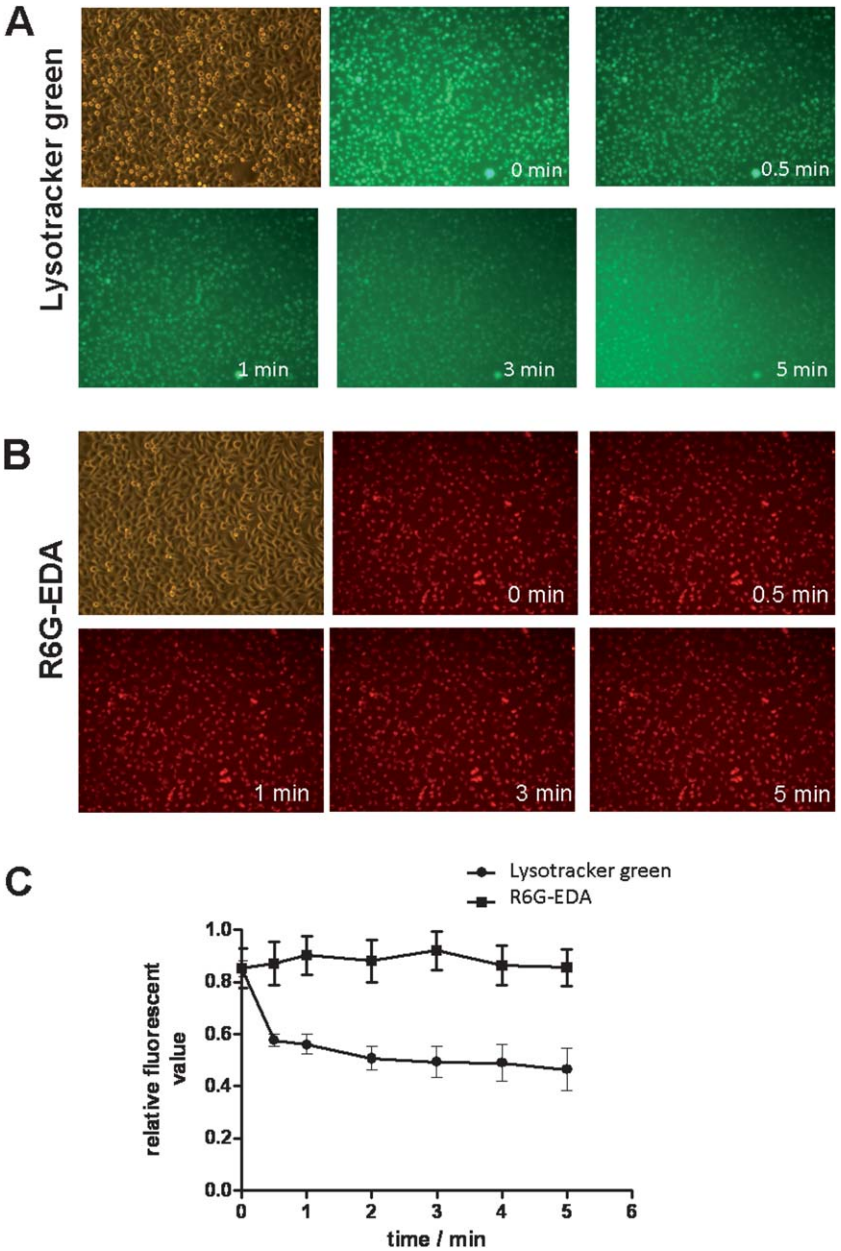


Fig. 8 Photostability of R6G-EDA or lysotracker green in cells illuminated with a laser at various time points as indicated in the images.

Visualization of temporal alteration of lysosome morphology in apoptotic cells

Lysosomes are involved in many cell signaling pathways, *e.g.* endocytosis, autophagy, and apoptosis.[4,21,22] Consequently, acidity and volumes of lysosomes could be altered in concert with these biological events. For example, enlargement of total volume of acidic compartments is reported to be a common feature for cells undergoing apoptotic death based on the observation that fluorescence of lysotracker in apoptotic cells increased compared to normal cells.[5] To date, no successive visualization of alteration of lysosome morphology in apoptotic cells have been achieved to unambiguously show the enlargement of total volume of acidic compartments.

R6G-EDA, immune from photo-bleaching and exhibiting long retention time in cells, is suitable for monitoring the temporal and spatial changes of lysosome in cells undergoing apoptosis. L929 cells pre-cultured with R6G-EDA were treated with TNF-α and then imaged with confocal fluorescence microscope. The lysosome morphology was recorded every 15 min for 7 h. The images clearly showed that the total volume of lysosomes increased significantly from resting state to final stage of cell death. The rupture of the enlarged lysosomes was observed in the final stage of apoptosis (Fig. 10). The observation confirmed the previous studies that total cellular acidic compartments of L929 cells treated with TNF-α increased during apoptosis.[5] The successful full time tracking of the lysosome morphology in TNF-α treated L929 cells indicated the utility of

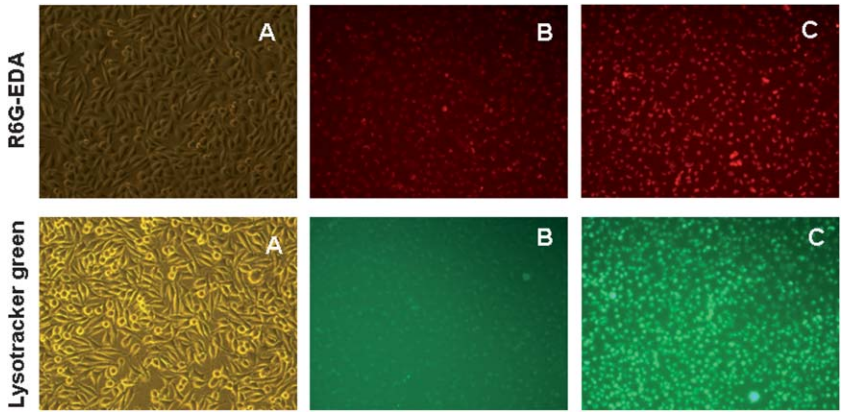


Fig. 9 Microscopic views of L929 cells prestained with lysotracker green or R6G-EDA. (A) Bright fields of cells in media containing the corresponding probes, (B) fluorescent images of the cells in media containing the corresponding probes, (C) fluorescent images of cells that were washed and incubated in fresh media.

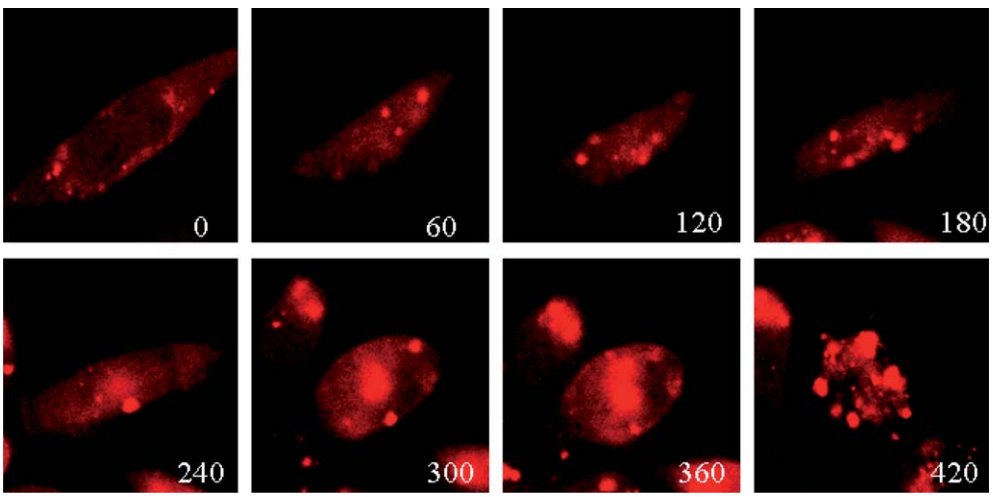


Fig. 10 Snapshots of lysosomes in L929 cells undergoing apoptotic death. Images of lysosomes of TNF-α treated L929 cells were recorded by confocal fluorescence microscopy at various time points (in minutes) as indicated.

R6G-EDA as a photo-stable pH responsive marker in cell biology.

Conclusions

The parameters of lysosomes, including number, volume, acidity, location, *etc*., are significantly altered in cancer cells relative to normal cells.[4] Optical dyes that could be used for noninvasive imaging of lysosomes are valuable for probing the roles of lysosome in cancer metastasis, *in vivo* cancer diagnosis and evaluation of lysosome-targeted cancer therapy.[6–9] In the aforementioned applications, fluorogenic probes activated in lysosomes *in situ* are advantageous due to high selectivity and low background signals which stem from reduction of staining of nonspecific intracellular organelles and extracellular background signals. In recent work, a pH activated fluorophore conjugated monoclonal antibody (Trastuzumab) was used to selectively image viable cancer cells in mice.[10]

In this report, selective imaging of lysosomes and autophagosomes were achieved with R6G-EDA which is a fluorogenic rhodamine-lactam based pH sensor. R6G-EDA fluoresces in lysosome *via* pH mediated ring opening of the intramolecular spirolactam, which is different from current lysosome probes in the sensing mechanism. Apart from being a sensitive and selective intracellular pH sensor, R6G-EDA is highly stable against photo-bleaching and exhibits long retention time in lysosomes, allowing tracking of morphological alteration of lysosomes from start to finish in the process of TNF-α triggered cell death. The advantageous features of R6G-EDA suggest its novel utility for acidic vesicles studies in cell biology.

Experimental section

Materials and methods

Lysotracker green DND-26 was purchased from Invitrogen. R6G-EDA was synthesized according to a reported procedure.[14] All other reagents were obtained from Sigma-Aldrich unless otherwise specified. The fluorescence spectra of R6G-EDA and rhodamine 6G were performed on a spectrofluorimeter (Shimadzu, RF-5301, Japan) using the excitation wavelength (λex) of

525 nm. Cells were analyzed using a fluorescence microscope (Ti–S; Nikon eclipse) equipped with a 100 W mercury lamp (C–SHG1, Nikon). Confocal fluorescence microscopic images were obtained on LeicaSP2 using the following filters: λex@514nm and λem@545–580 nm for R6G-EDA; λex@488 nm and λem@500–530 nm for lysotracker green. The fluorescence of R6G-EDA in cells was shown in red in the figures while the fluorescence of Lysotracker green in cells was shown in green. Images of the fluorescence of R6G-EDA and that of Lysotracker green in cells were merged (overlapped) using Photoshop CS 3.0. Graph by GraphPad Prism5 software. Mammalian cells, L929, Raw 264.7 and MEF were obtained from American Type Culture Collection.

Staining of lysosomes in live cells

Mammalian cells, L929, Raw 264.7 and MEF were grown at 37 °C under 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM, Gibco; Invitrogen) supplemented with 10% fetal bovine serum. Insect cells, SF9, were grown at 27 °C in SF900II (Invitrogen) supplemented with 2% FBS. Cells were seeded on 35 mm glass-bottom dishes (NEST) and incubated for 24 h, followed by addition of lysotracker green (1 μM) and R6G-EDA (10 μg mL^{-1}). The cells were further incubated for 30 min. The medium was removed and replaced with fresh medium. Cells were then analyzed with confocal fluorescence microscope. For reversal staining of lysosomes, MEF cells were pre-incubated for 4 h at 37 °C in the absence or presence of 50 nM bafilomycin A1, and then incubated in media supplemented with R6G-EDA (10 μg mL^{-1}) and lysotracker green (1 μM) for 30 min. The cells were analyzed with confocal fluorescence microscope.

Comparison of the fluorescence spectra of R6G-EDA with rhodamine 6G

The fluorescence emission spectra of R6G-EDA or rhodamine 6G (1 μg mL^{-1}) in sodium-acetate-acetic acid buffer (200 mM, pH 4) were recorded using with λex of 533 nm. The fluorescence excitation spectra were scanned by monitoring the fluorescence intensity using λem of 553 nm.

pH titration of R6G-EDA

Aliquots of R6G-EDA stock solution in DMSO was added to Britton-Robinson buffers[3] of various pH values to a final concentration of 1 μg mL^{-1}. The fluorescence emission spectra were recorded as a function of pH using λex at 525 nm.

Selectivity of R6G-EDA towards interfering species

A series of solutions of R6G-EDA (1 μg mL^{-1}) in DMEM were prepared to contain one of the following cations (1) $HClO_4$ (0.1 mM), NaCl (1 mM), $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), $ZnCl_2$ (1 mM), $CuSO_2$ (1 mM), $CoCl_2$ (1 mM), KCl (1 mM); H_2O_2 (5 mM) or HOCl (5 mM). Fluorescence emission spectra of the solutions were recorded using excitation wavelength at 533 nm.

Imaging of autophagosomes in L929 cells with R6G-EDA

L929 cells stably expressing GFP-LC3 was stained with R6G-EDA using the same procedure as described for lysosome staining, and then treated with or without 10 ng mL^{-1} of TNF-α for 6 h to induce autophagy. The cells were then analyzed with confocal fluorescence microscope.

Retention time of R6G-EDA in L929 cells

L929 cells, pre-stained with lysotracker green and R6G-EDA using the same procedure as described for the staining of lysosomes, were grown on 24-well plate, and then incubated with R6G-EDA (10 μg mL^{-1}) or lysotracker green (1 μM) for 30 min. The culturing media were replaced with fresh culture medium. Cells were analyzed using fluorescence microscope.

Low background staining of lysosomes with R6G-EDA

L929 cells were seeded on 35 mm glass-bottom dishes (NEST). After 24 h, lysotracker green (1 μM) and R6G-EDA (10 μg mL^{-1}) were added to the medium. Cells were incubated for 30 min and then directly analyzed by confocol microscopy.

Photobleaching of R6G-EDA and lysotracker green

L929 cells were grown on 24-well plate, then incubated with R6G-EDA (10 μg mL^{-1}) or lysotracker green (1 μg ml^{-1}) for 30 min using the same procedure as described for staining of lysosomes. The culturing media were removed and replaced with fresh medium. Cells were exposed to laser illumination with a 100 W mercury lamp (C–SHG1, Nikon) and imaged at indicated time points (0, 0.5 min, 1 min, 3 min, 5 min) with a fluorescence microscope. The cells were further incubated for 24 h and imaged again. The images were analyzed using Image J (1.43; NIH) software.

Cytotoxicity of R6G-EDA

The cytotoxicity of R6G-EDA was evaluated on MEF, L929, Raw 264.7 and SF9 cells. The cells were cultured with medium containing R6G-EDA (10 μg mL^{-1}) for 8 or 24 h at 37 °C with 5% CO_2. The cells before and after incubation were stained with trypan blue. Cell number and cell viability were determined using the trypan blue exclusion test.

Temporal tracking of lysosome morphology of apoptotic L929 cells

L929 cells were prestained with R6G-EDA using the procedure described for lysosome staining. The cells were treated with 10 ng mL^{-1} of TNF-α to induce autophagy. The cells were then analyzed with confocal fluorescence microscope every 15 min for 7 h.

Acknowledgements

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