Electronic Supplementary Material (ESI) for RSC Advances.

Supporting information for

Specific and Photostable Rhodamine-based Tracker for 3D Video Imaging Single Acidic Organelles

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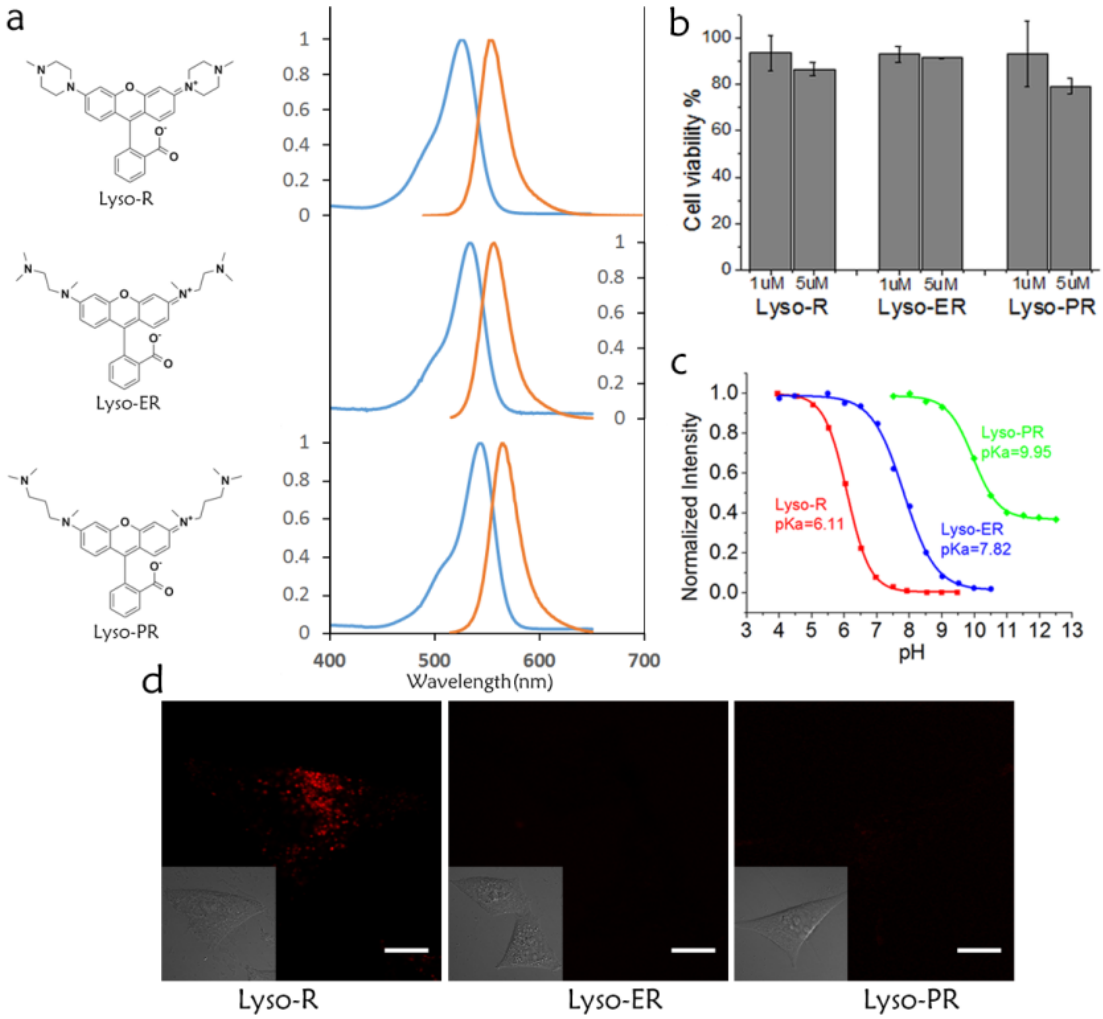
Supporting Figure S1. Properties of **Lyso-R**, **Lyso-ER** and **Lyso-PR**.

a) The normalized absorption spectrum (blue) and emission spectrum (orange) of **Lyso-R, Lyso-ER** and **Lyso-PR** at 5 μM in aqueous solution, respectively. b) 24 h cell viabilities of **Lyso-R, Lyso-ER** or **Lyso-PR** at 5 μM and 1 μM, respectively. c) pK_a plot of **Lyso-R, Lyso-ER** or **Lyso-PR** at 5 μM in PBS solution, respectively. d) Confocal images from MCF-7 cells incubated with **Lyso-R, Lyso-ER** and **Lyso-PR** for 5 minutes, respectively. Scar bar: 10 μm



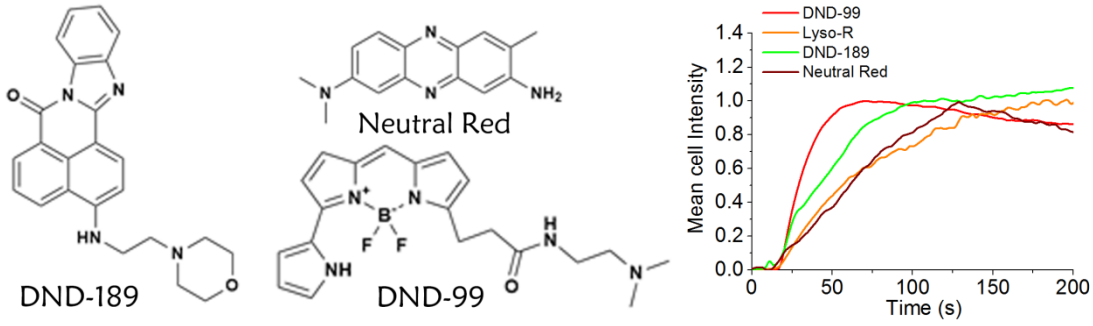
Lyso-R owns the marker potential of acidic oragnelles in vivo. First, the cytotoxicity of **Lyso-R** is relatively low as shown in Supporting Figure S1b. Cell viability is higher than 90 percent under staining concentration (1 μM) for three designing

compounds. Even at a relatively high concentration of 5 μM, cell viability is higher than 85 percent for **Lyso-R** and **Lyso-ER**. Compared with commercial dyes, the lower cytotoxicity towards cells is a favorable characteristic for application in live cells.

Secondly, the Log P value and pK_a plot of **Lyso-R** suggests better cell permeability and marker potential for acidic organelle than **Lyso-ER** and **Lyso-PR**. Generally, a hydrophobic compound exhibits better cell membrane permeability than a hydrophilic compound.[1] In addition, Log P (logarithm of octanol-water partition coefficient) value indicates the hydrophobicity or hydrophilicity of compound.[1] The log P values shown in Table 1 indicate the hydrophobicity of **Lyso-R** (log P=0.85) and the hydrophilicity of **Lyso-ER** (log P=-0.38) and **Lyso-PR** (log P=-0.58). This result is consistent with their pK_a plots, as shown in Supporting Figure S1c. The uncharged dominate non-protonated form of **Lyso-R** (pK_a=6.11) exhibits hydrophobic properties while the charged protonated form of **Lyso-ER** (pK_a=7.82) and **Lyso-PR** (pK_a=9.95) exhibit hydrophilic properties. Thus, compared with **Lyso-ER** and **Lyso-PR**, **Lyso-R** owns better cell membrane permeability. Moreover, the pK_a value of **Lyso-R** sits between DND-189 (pK_a=5.80[2]) and NR (pK_a=6.81[3]), indicating a potential acidic organelle target ability. The unquenched fluorescent of **Lyso-PR** might be contributed to the longer alkyl chain between the amino group and the fluorophore.

Cell imaging experiment decisively confirms the membrane permeability of **Lyso-R**. In this experiment, MCF-7 cells are stained with **Lyso-R** (1 μM), **Lyso-PR** (1 μM) or **Lyso-ER** (1 μM) for five minutes, respectively. As shown in Supporting Figure S1d, only the fluorescent signal of **Lyso-R** is detected in live cells, in accordance with the pK_a and log P results in previous discussion. In short, **Lyso-R** exhibits better cell membrane permeability, which expands **Lyso-R**'s usage for fast staining cases.

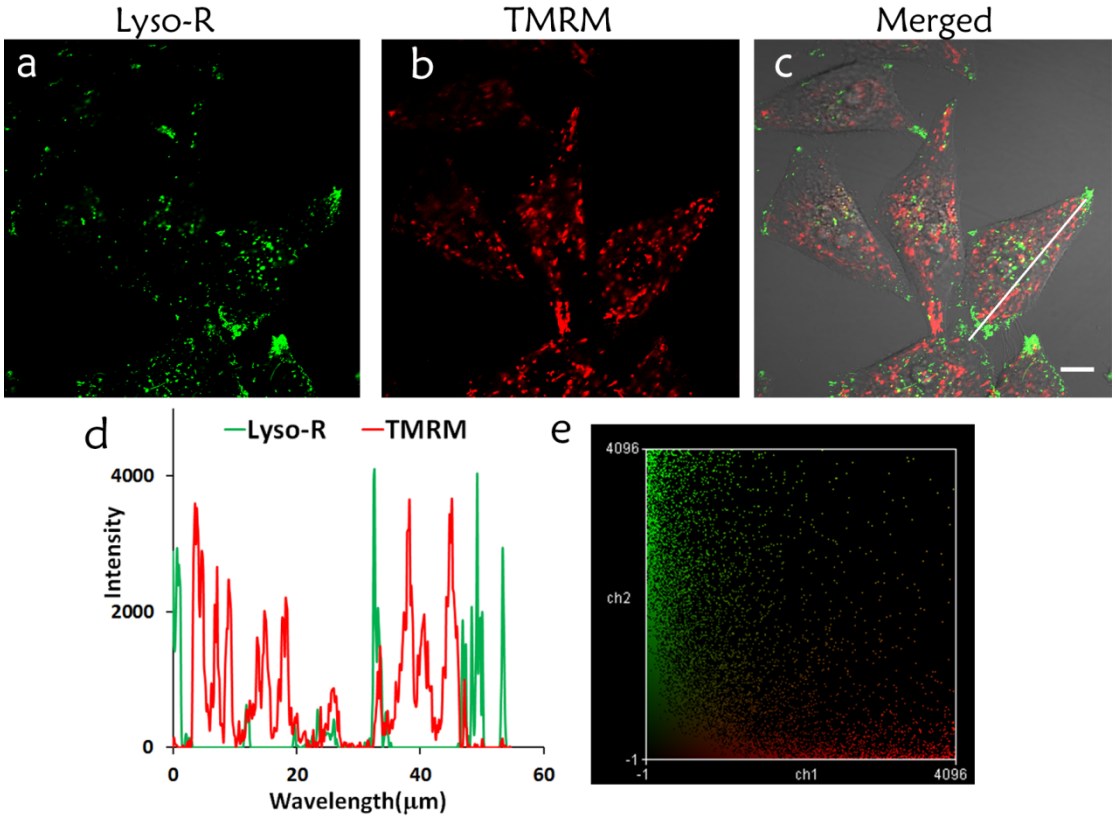
Supporting Figure S2. Stain comparison of **Lyso-R** with three commercial trackers. Left: structures of commercial trackers for acidic organelles. Right: Mean fluorescent intensity from single MCF-7 cell, shortly after the incubation of 1 μM DND-99, DND-189, NR or **Lyso-R**, respectively.



Lyso-R owns a fast staining ability for acidic organelles in cells. In order to confirm this fast procedure, the stain speed comparison of different trackers is conducted. Mean fluorescent intensity from MCF-7 cell is recorded shortly after the incubation of trackers. As shown in Supporting Figure S2, all trackers complete their staining procedures in less than three minutes. This result suggests that the stain speed of **Lyso-R** is close to commercial trackers.

Supporting Figure S3. Colocalization experiment of **Lyso-R** with TMRM in MCF-7 cell.

a) Confocal image from TMRM (0.25 μM, λ_{ex}=559 nm, λ_{em}=570 nm-600 nm). b) Confocal image from **Lyso-R** (0.25 μM, λ_{ex}=488 nm, λ_{em}=530 nm-570 nm). c) Image merging from a) and b). d) Intensity profile of ROIs across cell in c). e) Intensity correlation profile of **Lyso-R** and TMRM. Scar bar: 10 μm.

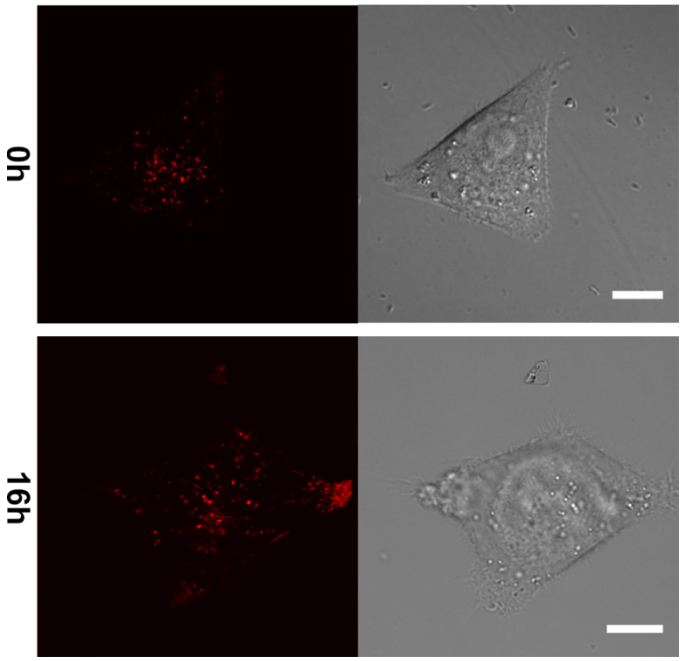


Lyso-R exhibits a segregated stain from TMRM in Supporting Figure S3. The merged image exhibits low overlapping yellow color. Intensity correlation analysis[4] supports unrelated stain from two dyes as the negative hour glass scatterplots shown in Supporting Figure S3e. In addition, the Mander's coefficient of **Lyso-R** with TMRM is barely 0.15. The unrelated intensity profiles from the line across the interest regions in cells in Supporting Figure S4d further support the separated stain. Thus, the stain of **Lyso-R** might not be mitochondria in live MCF-7 cells. **Lyso-R** is different from

TMRM with a relatively lower pKa value of 6.0 while pK_a of TMRM is at near 7.0. This slightly pK_a value difference causes **Lyso-R** exists in its ring-closed form while ring-opened cations of TMRM are accumulated by mitochondria. **Lyso-R** also owns a weak base group, piperizine group, which would further increase the accumulation of **Lyso-R** in acidic organelles. Slightly lower pK_a and the additional amine group makes **Lyso-R** different from TMRM with a specificity stain of acidic organelles other than mitochondrial.

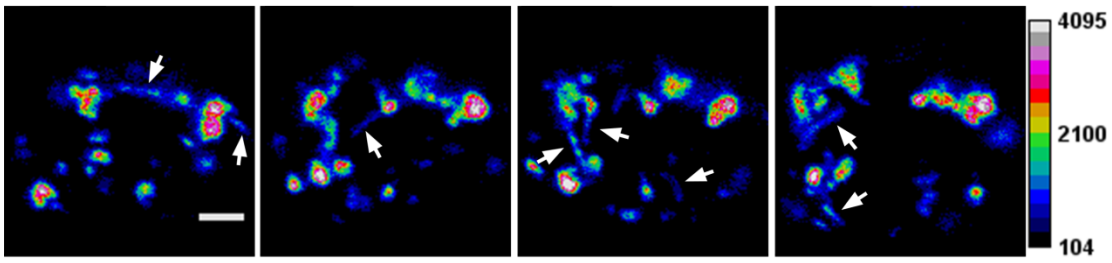
Supporting Figure S4. Stain comparison from MCF-7 cells incubated with **Lyso-R** at 0 h and 16 h.

MCF-7 cells are stained with **Lyso-R** (1 μm) and taking confocal image at 0h and 16h, respectively. Scar bar: 10 μm.



As shown in Supporting Figure S4, there is no significant difference between long time and short time stain from **Lyso-R** in MCF-7 cells. This result suggests that the stain of **Lyso-R** is stable in live cells.

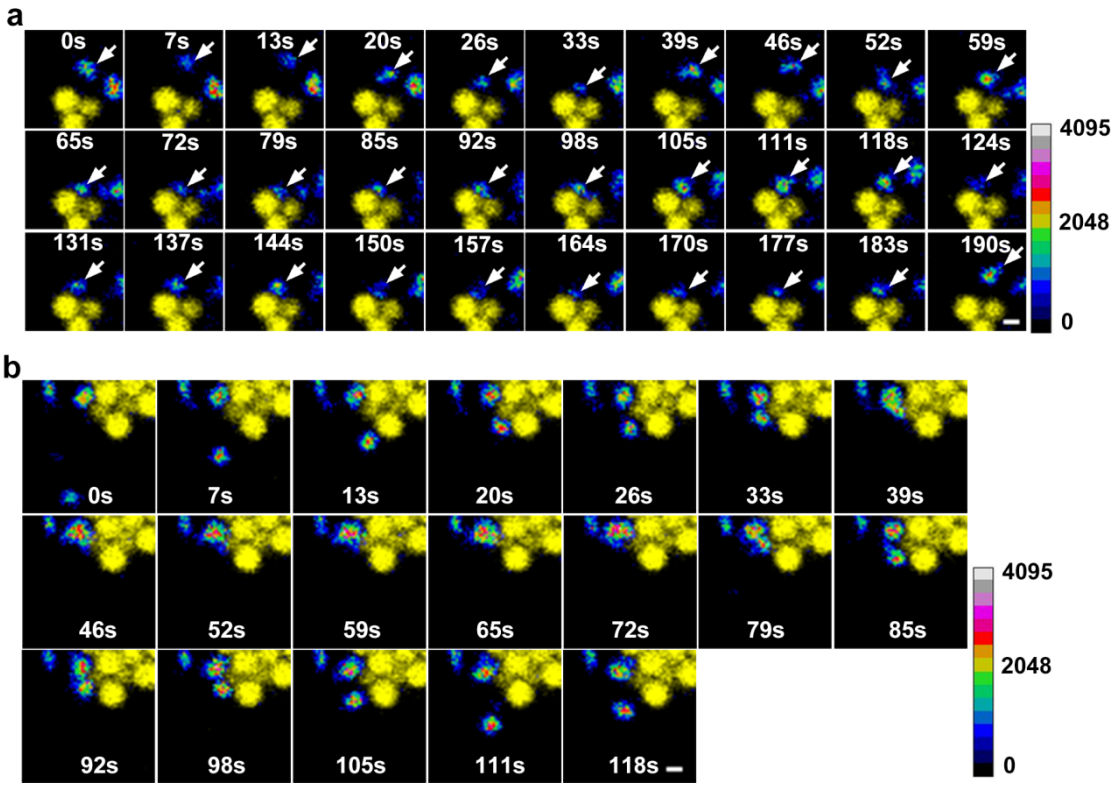
Supporting Figure S5. Tubular acidic organelles in macrophages incubated with **Lyso-R**. Scar bar: 10 μm.



Supporting Figure S6. Behavior of acidic organelles during lipid hydrolase in MCF-7 cell.

a) "Kiss and run" event between single acidic organelle and lip droplet. b) "Fusion and fission" event. Scar bar: 0.5 μm.

The fluorescence of **Lyso-R** (1.0 μM) is exhibited in a ratio color from cold to warm. The stain of lipid from commercial lipid tracker BODIPY 493/503 (250 nM) is exhibited in yellow color.



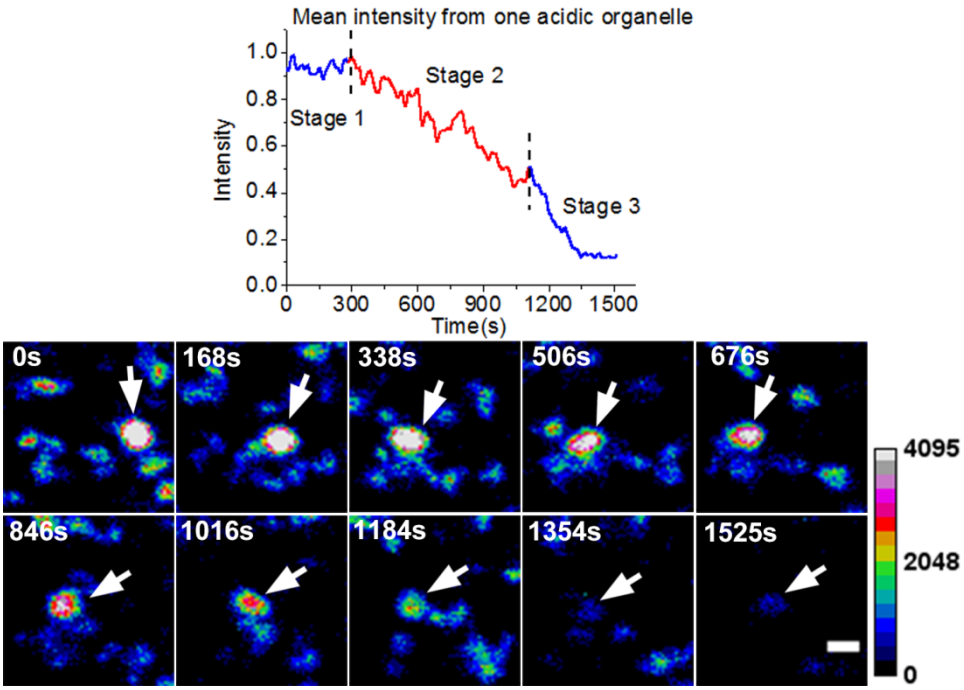
Lipid hydrolysis is crucial to maintain cell energy homeostasis. Dysfunction of lipid droplets hydrolysis causes many serious diseases such as obesity syndrome. However, lipid hydrolysis is not fully understood yet. Recently, researchers[5] have pointed out that partial lipid droplets hydrolysis is regulated by autophagy. Thus, it is significant to understand the role of acidic organelles during lipid hydrolysis.

The behavior of acidic organelles during lipid hydrolysis is video imaged in MCF-7

cells stained with **Lyso-R** and commercial lipid tracker as shown in Supporting Figure S6 and Supporting Movie 4. Supporting Figure S6a (also with Supporting Movie 4a) presents a "kiss and run" event between single organelle and lipid droplets in live cell. These images exhibit the transformation of globular single acidic organelle to a special concaved shape for better enfolding the lipid droplets. This result also indicates microautophagy might happen during the lipid droplets hydrolase. Another phenomenon (Supporting Figure S6b and Supporting Movie 4b) is a fusion event between the "transport" organelle and the "deplete" organelle close to the lipid droplet. In the first thirty seconds, the "transport" organelle moves to the "deplete" one closer to the lipid droplets. After short fusion event between two organelles, the two fuses and splits away.

Supporting Figure S7. The pH increase single acidic organelle induced by chloroquine in MCF-7 cell.

MCF-7 cells stained with **Lyso-R** (1 μM) are incubated with chloroquine (1 μM) at 0 s. The upper plot exhibits the mean fluorescent intensity of the arrowed organelle in the below image sequence. Scar bar: 1 μm.



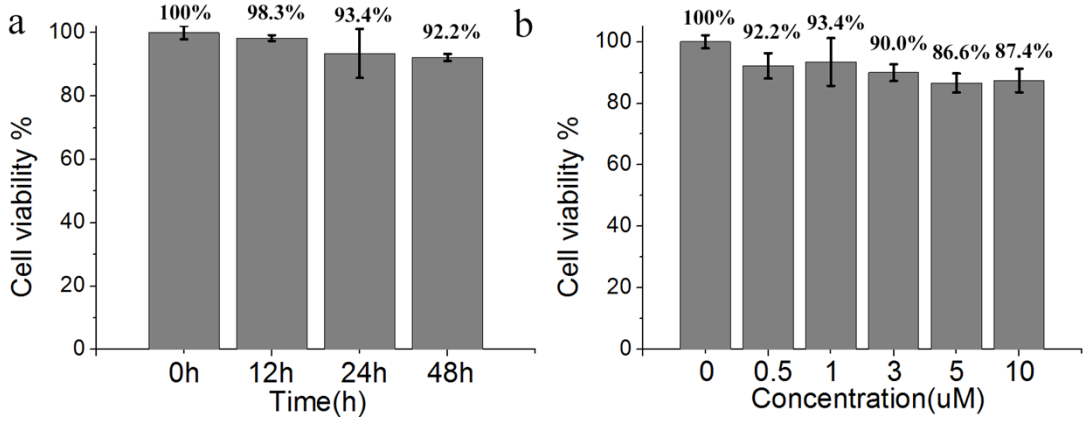
The pH of acidic organelles, especially lysosomes, is important indicator for evaluating the pharmacology of cancer drugs like chloroquine.[6] However, the pH increment of single acidic organelle during chloroquine inhibition is not exhibited yet. As **Lyso-R** exhibits its independent fluorescent pK_a plot from the existence of BSA or lecithin, **Lyso-R** is a potential pH sensor for acidic organelles. Thus, Supporting Figure S7 and Supporting Movie 5 exhibit the attempt to evaluate the pH of single acidic organelle in MCF-7 cell induced by chloroquine and stained with **Lyso-R**.

As shown in Supporting Figure S7, the procedure could be divided into three stages. In the first stage (0 to 300 seconds), the acidic organelle maintains its acidic environment. This result suggests that the organelle resists the basicity infection from

chloroquine for the first five minutes. In second stage (300 second to 1100 second), the pH increment of the organelle might be 2.5 at a moderate increase rate, according to the fluorescent pK_a plot of **Lyso-R** in previous study. The result indicates that chloroquine might take effectiveness during this stage. In the last stage (from 1100 second to 1500 second), the pH of the organelle might increase to above 7.0 at a relatively high speed. The result supports an irreversible destruction of the pH maintaining system. This irreversible pH increase further indicates the start of cell apoptosis. In this case, **Lyso-R** exhibits potential applicability for pH evaluation of single acidic organelle in live cell.

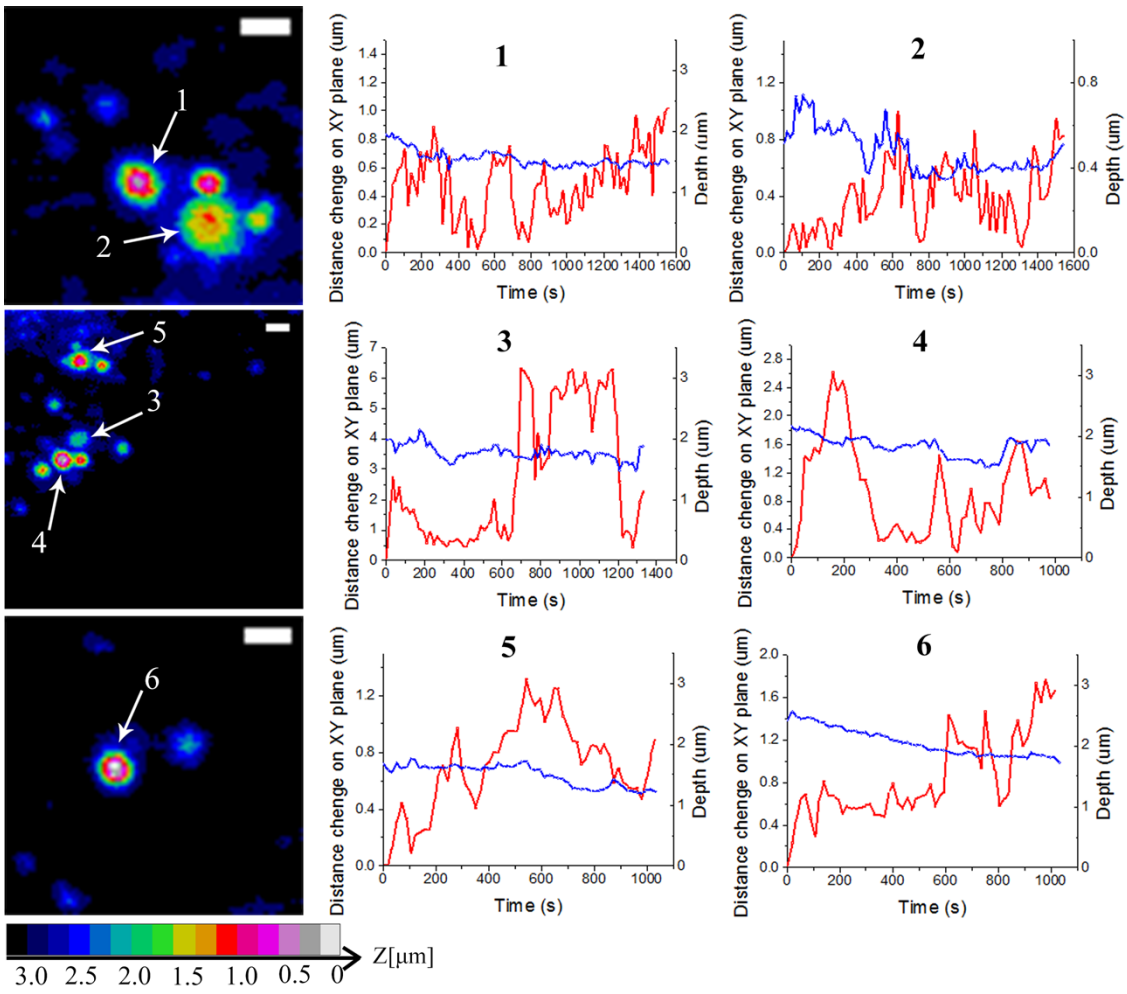
Supporting Figure S8. Viability of MCF-7 cells incubated with **Lyso-R**.

a) The cell viability of MCF-7 cells incubated with 1 μM Lyso-R for different times (0, 12, 24 and 48 hours, respectively). b) The cell viability of MCF-7 cells for 24 h at series concentrations (0, 0.5, 1, 3, 5 and 10 μM, respectively).



Supporting Figure S9. Track of single acidic organelle in **Lyso-R** incubated MCF-7 cell.

Left three confocal images exhibited six tracked acidic organelles (mapped with 1, 2, 3, 4, 5 and 6). Right six plots exhibit the depth and the distance changes on XY plane of the corresponding tracked organelles, respectively. The 3D video of six organelles are exhibited in Supporting movie 2b, 2c and 2d. Scar bar: 1 μm.



Supporting Table S1. Photo-physical property comparison of **Lyso-R** and commercial trackers.

Compounds	Absorb Peak (nm) [a]	Fluorescent Peak (nm) [a]	Quantum yields[a]	Absorption cross section[a] ($\times 10^{-19} cm^2$)
Lyso-R	526	557	0.73	2.9
Neutral Red	535	610	0.05	0.9
DND 189	443	502	0.35	1.2
DND 99	577[b]	590[b]	0.08[c]	3.0[b]

[a] All data are measured in pH=4.0 aqueous solution except for DND 99; [b] Absorption peak, fluorescent peak and absorption cross-section in methanol are adopted from The Molecular Probes Handbook[7] (session 12.3); [c] The quantum yield in pH=3.2 universal buffer is adopted from Ref[8].

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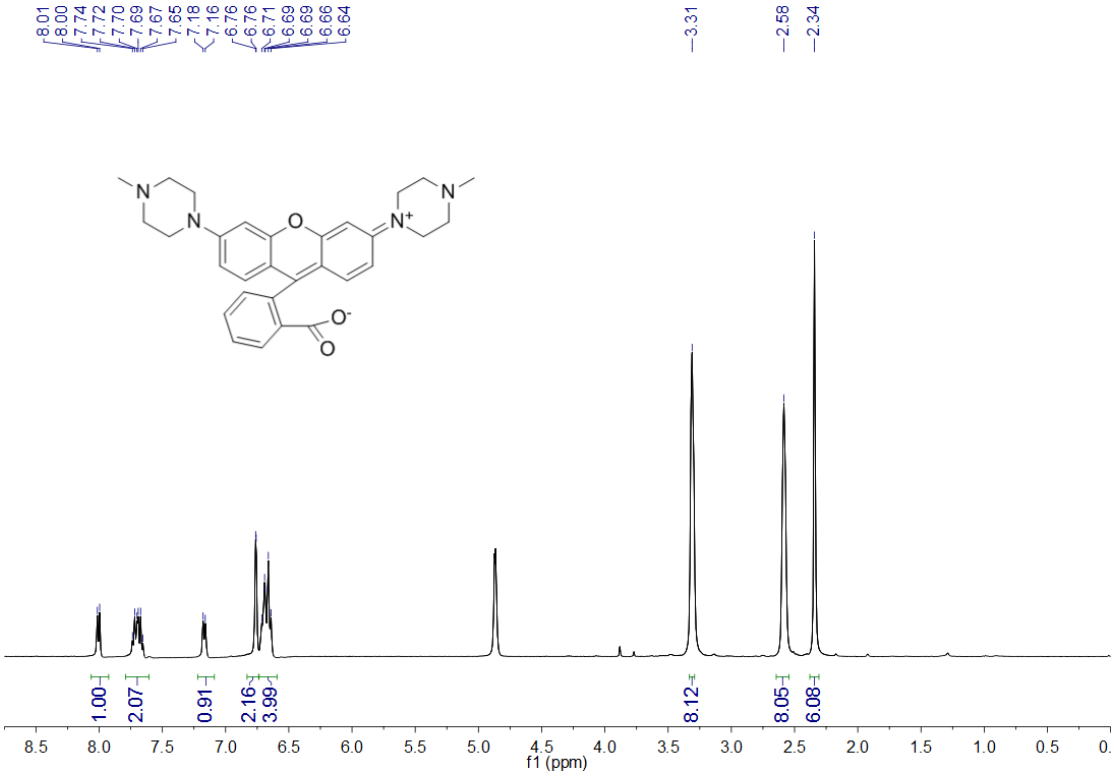
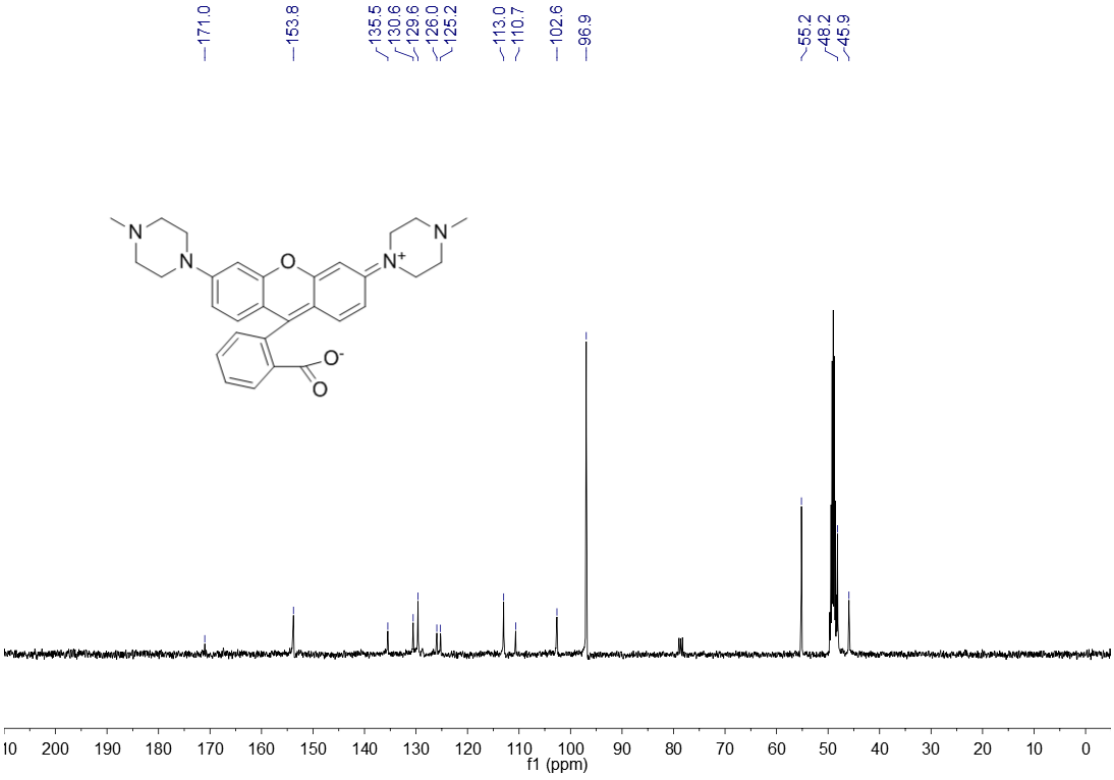
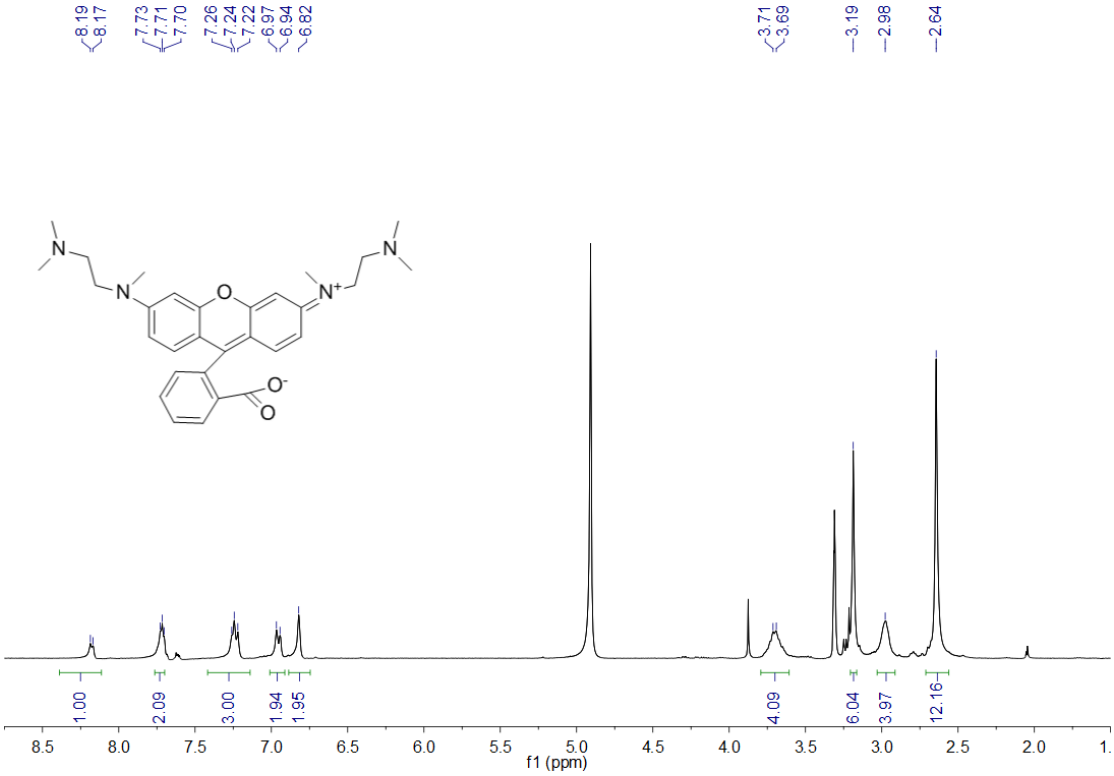
Supporting Figure S9. ^{1}H NMR spectra of **Lyso-R**.



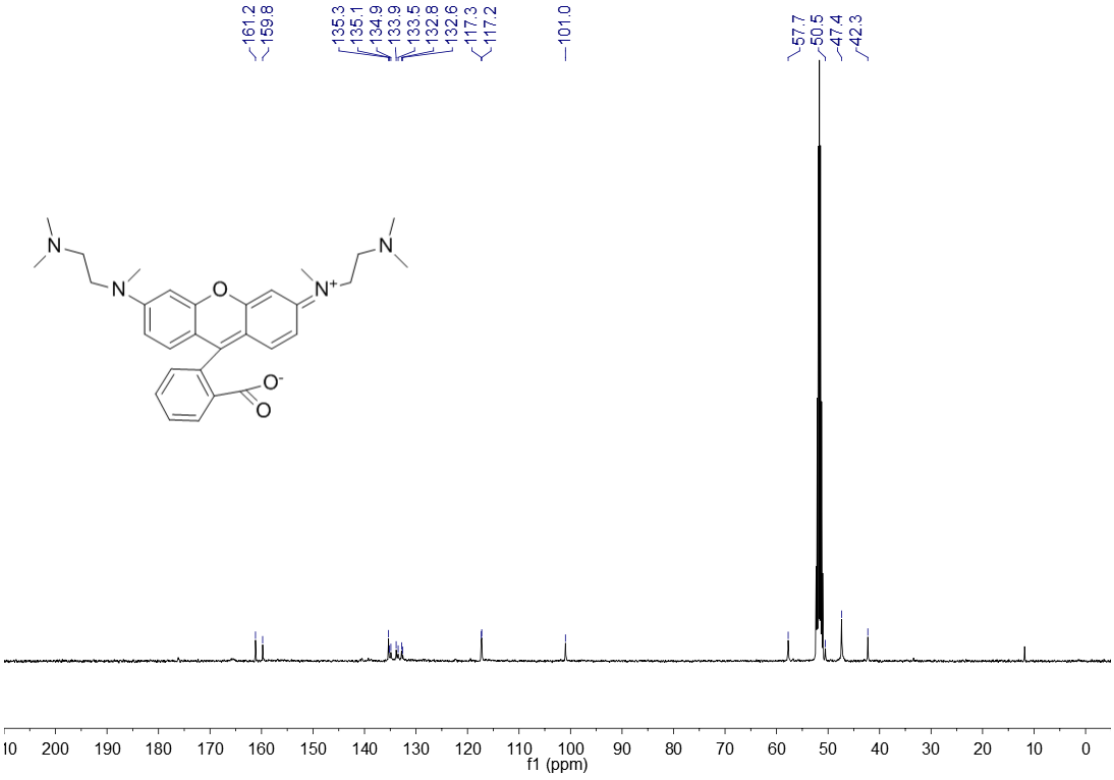
Supporting Figure S10. ^{13}C NMR spectra of **Lyso-R**.



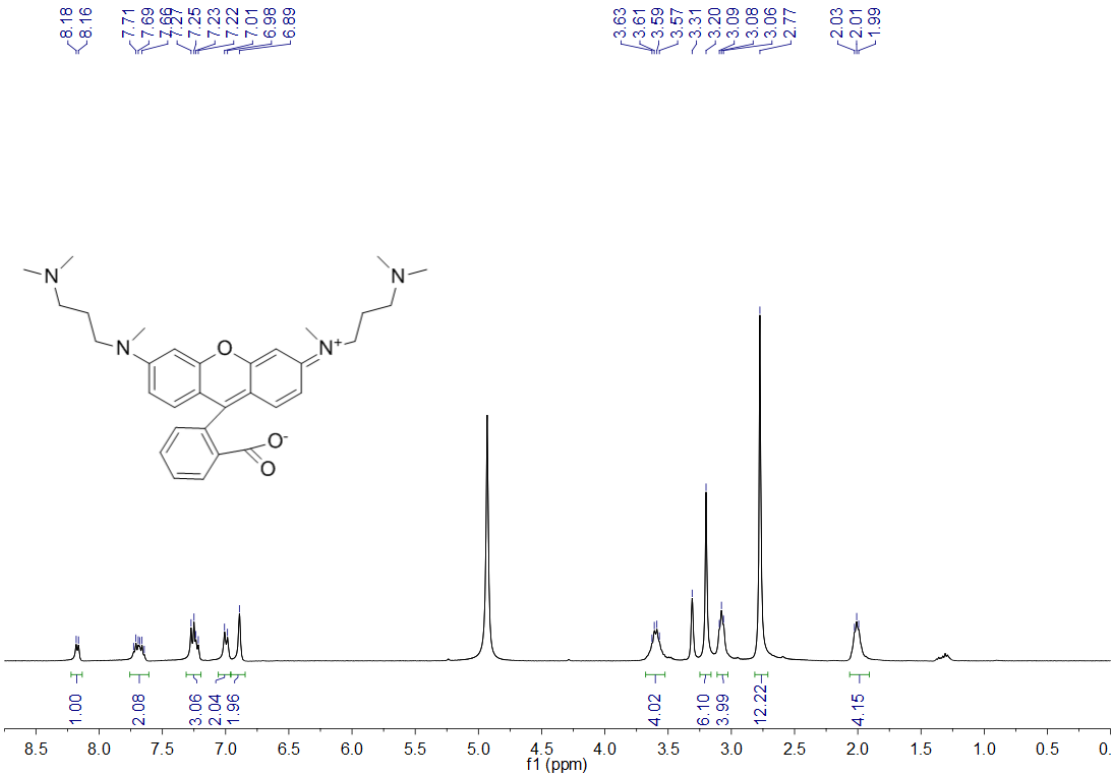
Supporting Figure S11. ^{1}H NMR spectra of **Lyso-ER**



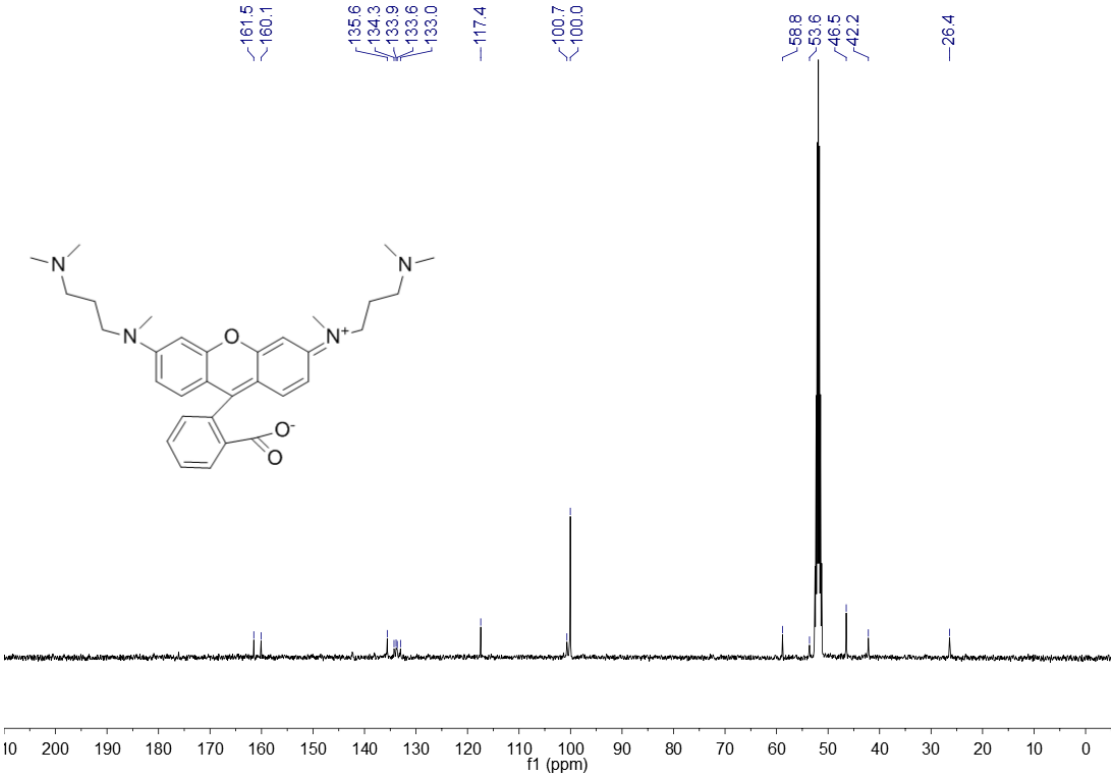
Supporting Figure S12. ^{13}C NMR spectra of **Lyso-ER**



Supporting Figure S13. ^{1}H NMR spectra of **Lyso-PR**



Supporting Figure S14. ^{13}C NMR spectra of **Lyso-PR**



Contents of Supporting movies.

Supporting movie 1: 3D reconstruction of MCF-7 cells stained with **Lyso-R** (red) and **DND-189** (green).

DND-189 exhibited diffusive and continuous fluorescence signals in broad intracellular staining regions in MCF-7 cells. **Lyso-R**, on the contrary, exhibited a punctual like specific staining in acidic organelles.

Supporting movie 2: The whole MCF-7 cell stained with **Lyso-R** (0.25 μM) in 3D tracking.

This movie exhibited 3D moving of all acidic organelles in the MCF-7 cell. A color sequence (runs cold to hot) was used as a look up table of depth.

Supporting movie 2a, 2b, 2c and 2d: 3D track of acidic organelle in **Lyso-R** (0.25 μM) stained MCF-7 cells.

Seven acidic organelles were tracked in these movies. 2a presented the organelle tracked in Figure 3. 2b, 2c and 2d exhibited the six tracked organelles in Supporting Figure S9 (Organelles 1 and 2 wer tracked in movie 2b; Organelles 3, 4 and 5 were tracked in movie 2c; Organelles 6 was tracked in movie 2d).

Supporting movie 3a: Tubular acidic organelles observed in LPS and **Lyso-R** incubated macrophages.

Different tubular formation process was observed in this movie. Macrophages were stained with **Lyso-R** and then treated with 10 μg/ml LPS for 1 h.

Supporting movie 3b: The control group of macrophages incubated with only **Lyso-R**.

This was the control macrophages treated with only **Lyso-R**. Almost no tubular organelle was observed during imaging time.

Supporting movie 4: The panorama of single cancer cell during lipid hydrolase.

Acidic organelles in MCF-7 cell were stained with **Lyso-R** (1.0 μM) in a ratio color and lipids were stained with BODIPY 493/503 (250 nM) in yellow color. In this video, three different behaviors were observed in the arrow pointing region.

Supporting movie 4a: "Kiss and run" behavior during lipid hydrolase.

The zoom in video of the "kiss and run" acidic organelle from supporting movie 4 was presented here. Acidic organelles seemed to change its shape to better surround the lipid droplets.

Supporting movie 4b: "Fusion and fission" behavior during lipid hydrolase.

The zoom in video of the "fusion and fission" from supporting movie 4 was presented here. Two single organelles were recorded in this video. One seemed like to stay near the lipid droplets; the other one seemed like to move towards the staying one; next, a fusion event happened between these two acidic organelles. In no more than fifty seconds, the two spilt up and kept their origin movement as the first one stayed near the lipid droplet and the second one moved backwards to the cytoplasm.

Supporting movie 5: pH increase of single acidic organelle induced by chloroquine.

This video presented the fluorescent change of single acidic organelle in MCF-7 cells during chloroquine treatment. As **Lyso-R** presented its independent pK_a changes upon proteins or lipids, the fluorescent of **Lyso-R** could be used to evaluate the pH changes of single acidic organelle under confocal video imaging.