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An Amphiphilic Squarylium Indocyanine Dye for Long-term Tracking of Lysosomes

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A novel amphiphilic squarylium indocyanine (LysoCy) is reported for remarkable lysosome tracking in living cells. LysoCy performs as a promising lysosome tracker with low cytotoxicity, strong binding affinity and clear subcellular labelling. The long-term dynamics of lysosomes can be revealed by LysoCy up to 2 day's culture, while the working duration of commercial dye is no longer than 1 hour. Our work provides a good alternative tool for long-term live cell imaging.

To visualize the subcellular organelles and biological dynamics, organelle-specific markers have attracted much interest for decades.[1-8] Lysosomes, the vital organelles, are part of endomembrane system and are essential for multiple biological processes such as plasma membrane repair, cell homeostasis, energy metabolism and immune response.[9] To reveal the morphology and function of lysosomes, the application of fluorescent lysosome tracker draws increasing attention recently.[10-13]

Fluorene[14] and rhodamine[15-17] fluorophores have been used for lysosome marking and imaging. However, these dyes' absorption and emission maxima are below 600 nm which has been thought as disadvantages due to the high exciting energy and the putative autofluorescence of biomolecules. Therefore, fluorochromes whose absorption and emission maxima are in the red and near-infrared (NIR) region (wavelength range from 620 to 1000 nm), have been preferred as proper candidates for bioimaging. Among these dyes, cyanine dyes are ideal candidates owing to the high absorptivity, strong fluorescence, and acceptable photostability.[18] The general cyanine dyes consist of two nitrogen centers, one of which is positively charged and is linked by a conjugated chain of an odd number of carbon atoms to the other nitrogen. [18] Due to the use of indoline unit as the nitrogen center, the dye is called as indocyanine (ICy). ICys are popular imaging agents[19] or fluorescent probes[20] in biological applications. However, to the best of our knowledge, ICy dyes have not been reported as lysosome tracker for dynamic monitoring.

The most commercial small-molecule trackers, such as neutral red and LysoTracker DND series, preformed poorly in long-term tracking due to their high pH dependency.[21] Other macro-molecule trackers including nanoparticles[22] and quantum dots[23] can prolong the imaging period, unfortunately, with high cytotoxicity.[24-25] Therefore, there is a need to develop a fluorescent ICy dye with low cytotoxicity for long-term lysosome tracking in live cells.

In this work, a novel amphiphilic squarylium indocyanine (SICy) dye (**LysoCy,** Fig. 1), bearing two bromines and two primary amines, was designed, characterized, and.explored as a promising fluorescent marker with low cytotoxicity to track the long-term dynamics of lysosomes in live cells.

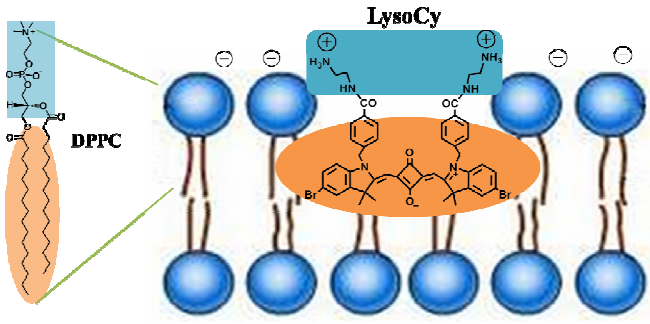
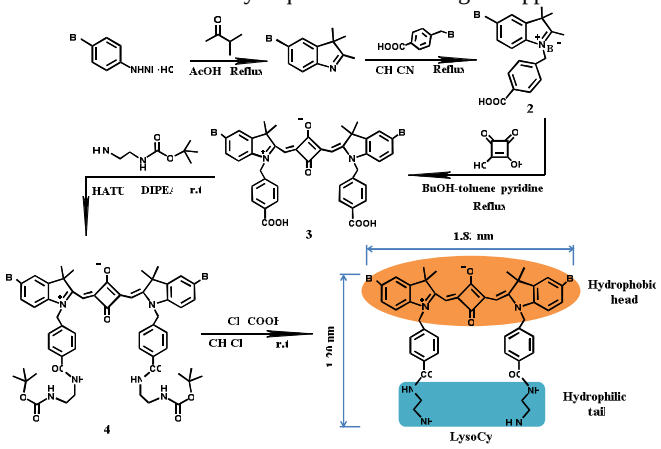


Fig. 1 Chemical structure of DPPC and the schematic diagram of **LysoCy** binding to the DPPC-mimicked lysosome membrane.

The SICy dye **LysoCy** was designed and synthesized in a straightforward way as shown in Scheme 1. 2,3,3-Trimethyl-5-bromo-3h-indole (**1**) was synthesized by Fisher ring reaction between 3-methyl-2-butanone and 4-bromophenylhydrazine hydrochloride in acetic acid. The further quaternary ammoniation with 4-bromomethylbenzoic acid yielded the quarternary salt **2**. The intermediate dye **3** was synthesized by the condensation of **2** and 3,4-dihydroxycyclobut-3-ene-1,2-dione (squaric acid) in toluene/n-butanol/pyridine (v/v/v = 1:1:1) solvent mixture. The rigid

squarylium ring in the conjugated center enhances the photostability of the dye.[26-27] To quantitatively confirm the improved photostability of LysoCy, the time-dependent emission of aqueous LysoCy were measured under irradiation (Supporting Information, Fig. S1). SICy5 and ICy5 were used as controls. As a result, the presence of rigid squarylium ring notably enhances the photostability of the dye. The two carboxylic acid groups in compound **3** reacted with *tert*-butyl N-(2-aminoethyl) carbamate to afford **4**. The removal of *tert*-butoxycarbonyl groups in compound **4** gave rise to the desired final product **LysoCy** dye (Scheme 1). Details of the synthetic process and material characterizations are given in the Experimental and Supporting Information (Fig. S2-S12). **LysoCy** has two bromine groups and two primary amines, which contribute to the hydrophobic property and the water solubility, respectively. The water solubility of **LysoCy** is approximate 1 mg/mL at room temperature, which fulfils the water-solubility requirement of biological applications.



Scheme 1. Synthesis of amphiphilic **LysoCy** dye.

The fluorescence quantum yield ($Ø_f$) was measured by Edinburgh Instruments' FLS 980 spectrofluorimeter at room temperature. The $Ø_f$ of **LysoCy** is 7±0.2% in water. **LysoCy** is blue under natural light and is red under UV light (Fig. 2A). Fig. 2B shows that **LysoCy** has a maximum absorption at 635 nm and an emission peak at 643 nm. A dye with absorption and emission peaks above 600 nm is ideal for bioimaging due to the advantage of avoiding of cellular autofluorescence.[28] The concentration-dependent absorption and fluorescence spectra are shown in Fig. 2C and 2D. When the concentration of aqueous **LysoCy** was higher than 10 μM, the fluorescence peaks appeared a bathochromic shift and a sign of attenuation, while the absorption peaks gradually increased without any shift. The reason can be explained that the **LysoCy** molecules tend to form dimers above the concentration of 10 μM.

Lysosomes has a single-lipid bilayer membrane, the thickness of its hydrophilic part is less than 3.5 nm,[29] while **LysoCy** is 1.83 nm long and 1.2 nm wide upon energy minimization by using Gaussian Program (Scheme 1 and Fig. S13). The phospholipid bilayer provides the incorporation space for **LysoCy** molecule. Due to the acidic environment (around pH 5) in lysosome, the primary amine groups of **LysoCy** can be fully protonated as positive charges[30-33] to electrostatically interact with the negatively charged membrane components of lysosomes. The mainly conjugated chromophore in **LysoCy** is hydrophobic that may interact with lipid tail of membranes. The bromine substitutions can increase the hydrophobic performance of **LysoCy**. Therefore, we assume that **LysoCy** dye can bind to the lysosome membrane via both electrostatic and hydrophobic interactions (Fig. 1).

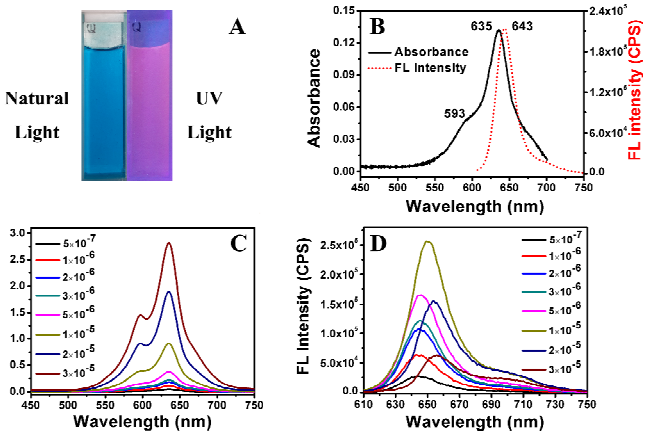


Fig. 2 (A) Colours of aqueous **LysoCy** under natural and UV light. (B) Normalized absorption and fluorescence spectra of **LysoCy** in water (2 μM). Concentration-dependent (C) absorbance and (D) fluorescence spectra of **LysoCy** in water (Excited at 593 nm, slit 5 nm).

To test above hypothesis, we further investigated the interaction between **LysoCy** and lysosome's membrane component. DPPC micelle was chosen as a membrane model for analysis.[34] DPPC, consisting of a plasma hydrophilic head and a hydrophobic tail, is one of the components of bio-membranes and has a critical micelle concentration (CMC) of 0.46 nM in buffer solution. Here, the DPPC lipid suspension was prepared under ultrasonic at a high concentration of 10 mM. The interaction between **LysoCy** and DPPC was first studied by spectral analysis. Aqueous **LysoCy** with concentrations of 2 μM were prepared as a blank. Both the absorption and fluorescence intensity of **LysoCy** decreased remarkably after the addition of DPPC solution (Fig. 3A and 3B). These optical changes can be attributed to the aggregation of DPPC micelles-bound **LysoCy**.

Isothermal titration calorimetry (ITC) was further carried out to quantify the interaction between **LysoCy** and DPPC.[26] Under neutral condition (pH 7), the measured affinity constant (K_A) of **LysoCy**/DPPC was 2.15×10^5 M^{-1}. Both positive enthalpy change (ΔH) and entropy change (ΔS) values indicate that the interaction between **LysoCy** and DPPC was mainly driven by a hydrophobic effect (Fig. 4).[35-36] Next, to further mimic the lysosome environment, a similar titration was performed under acidic environment (pH 5). The ΔH under weak acidic condition was below zero, which is the typical sign of an electrostatic interaction.[36] The K_A (1.39×10^6 M^{-1}) was greater compared with that value under neutral condition. These data suggest that the full protonation of primary amines in **LysoCy** under acidic environment can enhance the electrostatic interaction between **LysoCy** and DPPC. Above interactions provide a good potential for **LysoCy** to specifically label the lysosome during live cell imaging.

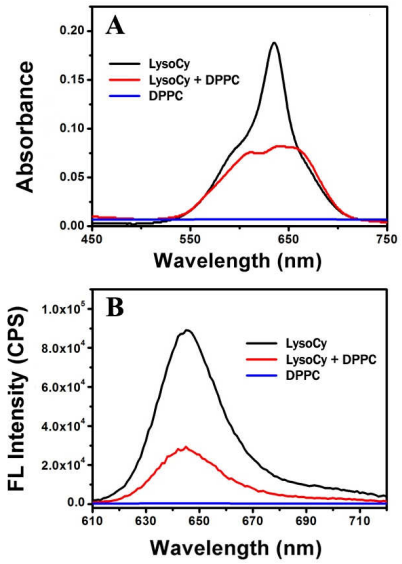


Fig. 3 Absorption (A) and fluorescence spectra (B) of **LysoCy**/DPPC in water. Both of **LysoCy** and DPPC were used at 0.1 mM.

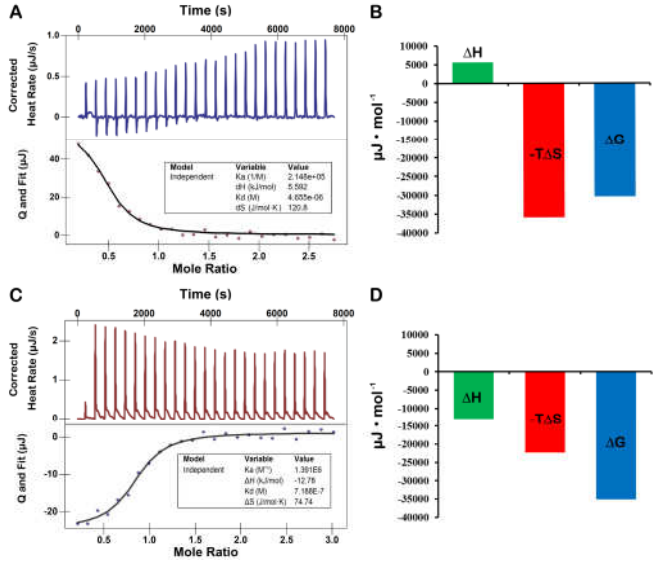


Fig. 4 ITC titration of **LysoCy** and DPPC interaction at pH 7 (A) and pH 5 (C). Thermodynamic parameter of **LysoCy** and DPPC interaction at pH 7 (B) and pH 5 (D).

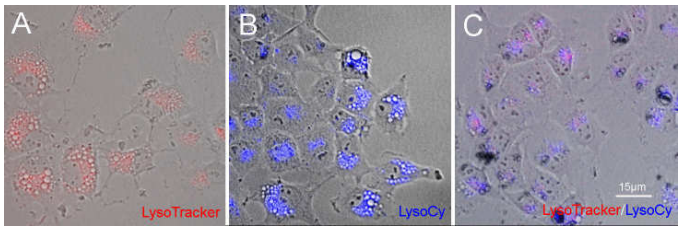


Fig. 5 Fluorescent images of live cells stained with LysoTracker alone (A, red: pseudocolour detected at emission wavelength of 570), **LysoCy** alone (B, blue: pseudocolour detected at emission wavelength of 664) and both of them (C, purple: the overlay of red and blue).

To assay the labelling effects of **LysoCy** on live cells, we chose a commercial acidotropic probe LysoTracker, which efficiently labelled the lysosomes, as a control (Fig. 5A). When live cells were incubated with **LysoCy** at extremely low concentration of 0.1 mM, the lysosomes were clearly labelled (Fig. 5B). To confirm the labelling specificity, a double labelling with both commercial LysoTracker and **LysoCy** was performed and showed well overlapped pattern (Fig. 5C), suggesting well specificity of **LysoCy** for lysosme labelling. Obviously, the fluorescence intensity of **LysoCy** is stronger than that of LysoTracker, indicating that **LysoCy** is more effective than LysoTracker to detect and track lysosomes. Base on above ITC analysis under neutral and acidic conditions, the two weak bases of **LysoCy** are only partially protonated at neutral pH, which makes the dye permeable to cellular membrane. Subsequently the two weak bases of **LysoCy** are further protonated at acidic pH, which leads to the retention and accumulation of **LysoCy** on lysosome membrane. Note that, both commercial LysoTracker and our **LysoCy** can not distinguish the endosome, early or late lysosome.

Long-term tracking of lysosome dynamics was also studied. We performed a real-time monitoring up to two days by incubation of cells with **LysoCy**. The fluorescence of **LysoCy** in lysosomes gradually increased along with the incubation time (Fig. 6A-D'), revealing ideal lysosome dynamics. In contrast, the fluorescence of LysoTracker decreased along with the incubation time (Fig. 6E-H'). The quantified fluorescence intensities of **LysoCy** and LysoTracker labelled cells at a series of time points are shown in Fig. 7. Consistently, the **LysoCy** fluorescence intensities gradually increased along with the continuous cell uptake of the dye, while LysoTracker labelling exhibited declined intensities. Obviously, **LysoCy** has a great potential to be applied as a long-term lysosome tracker with stable labelling and easy manipulation.

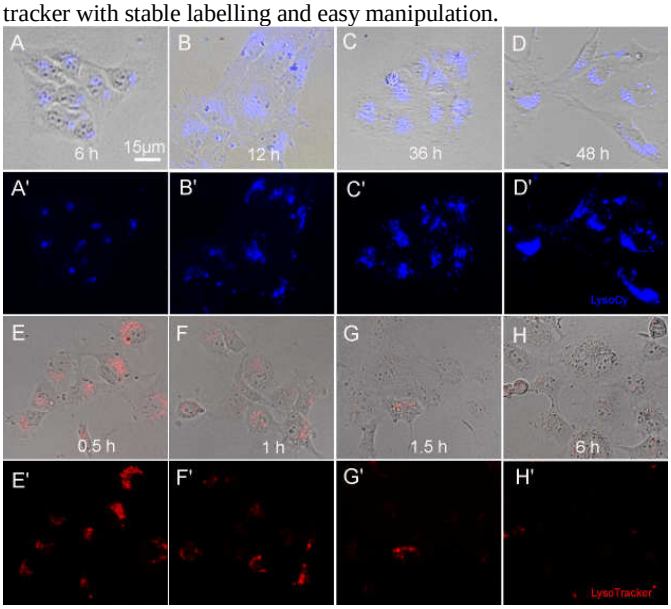


Fig. 6 Lysosome dynamics labelled with **LysoCy** (A-D', blue: pseudocolour detected at emission wavelength of 664) and LysoTracker (E-H', red: pseudocolour detected at emission wavelength of 570).

As good biocompatibility is a significant requirement for the live imaging.[37-38] We assayed the cytotoxicity of **LysoCy**, and compared with the commercial LysoTracker. **LysoCy** shows low cytotoxicity comparable to the control (Fig. S14). The cell viability of **LysoCy**

was above 80% at all the tested concentration, suggesting the good biocompatibility of **LysoCy**.

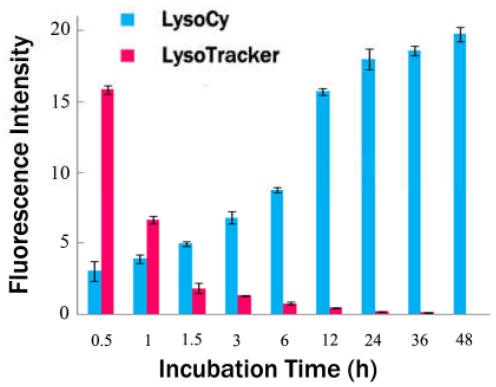


Fig. 7 Quantified fluorescence intensities of **LysoCy** and LysoTracker at series of time points.

In summary, a novel amphiphilic lysosome tracker **LysoCy** bearing two bromins and primary amines has been synthesized. The dye has absorption and emission in red region, thus avoiding the autofluorescence in bioimaging. The amine groups ensured water solubility and provided an electrostatic binding to the lysosome membrane due to the protonation in acidic aqueous environment. On the other hand, the indole part can insert and interact to the membrane of lysosomes due to a hydrophobic driven effect. In the live cell labelling, **LysoCy** showed remarkable lysosomes tracking ability with low cytotoxicity. The long-term dynamics of lysosomes can be revealed by **LysoCy** up to 2 day's culture. Therefore, a novel classic of SICy dye was explored as a long-term lysosome tracker for real time monitoring of the cellular dynamics.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Synthesis procedures and material characterizations. See DOI:10.1039/b000000x/

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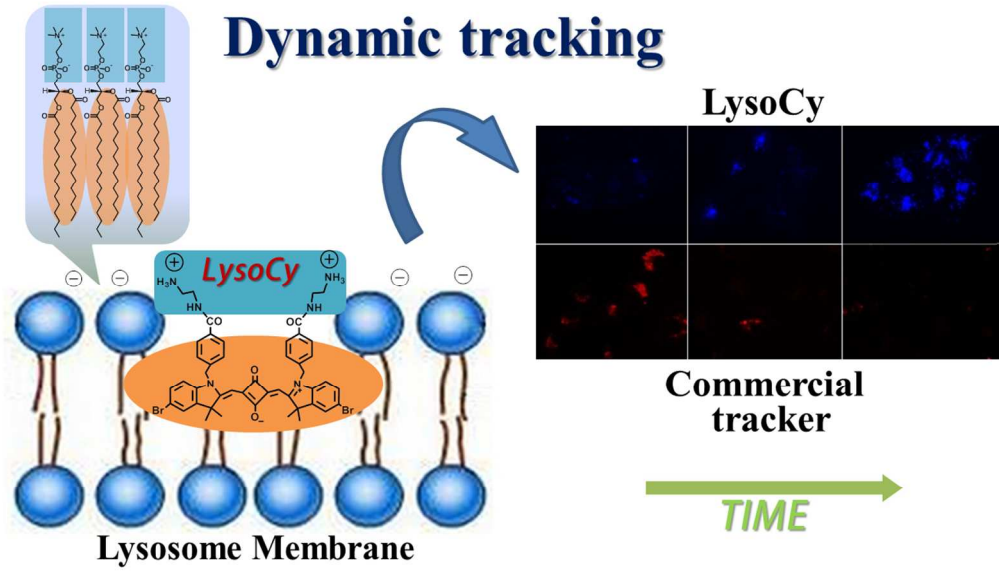

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