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Thiophene-based fluorescent probes with low cytotoxicity and high photostability for lysosomes in living cells

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ABSTRACT

Background: Selective imaging of lysosomes by fluorescence microscopy using specific fluorescent probes allows the study of biological processes and it is potentially useful also for diagnosis. Lysosomes are involved in numerous physiological processes, such as bone and tissue remodeling, plasma membrane repair, and cholesterol homeostasis, along with cell death and cell signaling. Despite the great number of dyes available today on the market, the search for new fluorescent dyes easily up-taken by cells, biocompatible and bearing bright and long-lasting fluorescence is still a priority.
Methods: Two thiophene-based fluorescent dyes, **TC1** and **TC2**, were synthetized as lysosome-specific probes.
Results: The new dyes showed high selectivity for fluorescent staining and imaging of lysosomes and disclosed high photostability, low toxicity and pH insensitivity in the range 2–10.
Conclusions: The **TC** dyes exhibited high co-localization coefficients (>95%) and moderate quantum yields. They showed high biocompatibility and long-term retention, important features for biological applications.
General significance: The results of the present work disclose a new class of organic dyes with potential wide applications as specific and efficient lysosome probes in the study of various biological processes.

1. Introduction

Lysosomes (**Lyso**), which are roughly spherical bodies enclosed by a single membrane, are an important class of cellular organelles that receive and degrade macromolecules from the secretory, endocytic, autophagic, and phagocytic membrane-trafficking pathways [1]. Defects in lysosome function lead to the development of diseases with often-severe consequences for individuals. Since the discovery of lysosomes by Christian de Duve over 50 years ago [1–5], research into endocytic and lysosomal biology has allowed the development of tools to understand further the role of lysosomes in cells.

Fluorescence microscopy, which allows non-invasive imaging of organelles in live cells, is one of the most commonly used imaging technologies in cell biology. Selective imaging of **Lyso** by fluorescence microscopy using specific fluorescent probes would enable further studies in the aforementioned biological processes, and potentially also for diagnosis [6–9]. A fluorescent marker must have the capability to be internalized into the cells with long-term retention as well as long-lasting fluorescence. More importantly, the probe should be biologically inert and non-toxic. Owing to these requirements and despite the great number of dyes available today on the market, the search for new fluorescent dyes easily up-taken by cells, biocompatible and bearing bright and long-lasting fluorescence is still a priority. Several fluorescent dyes with visible wavelengths for **Lyso**, such as LysoTracker®, LysoSensor™, have been produced and are commercially available [10]. Nevertheless, many of them suffer from various practical limitations, such as a poor light fastness. This being so, a significant demand remains for lysosomal selective probes with high bio-and photostability, low cytotoxicity and long-wavelength emission peaks for a

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deeper tissue penetration. Fluorophores based on thiophene core have been widely used as organic dyes for efficient fluorescent cell staining [11], labeling of proteins and DNA [12]. This widespread use is due to the high photostability of the fluorophore skeleton, low cytotoxicity, high color-tunable emission, high quantum efficiency and large Stokes shifts. Noteworthy, these dyes have a robust aromatic backbone, which can undergo a variety of functionalizations, including bio-derivatization [12–15].

Herein, we report the synthesis and investigation of two biocompatible thiophene-based fluorescent probes, **TC1** and **TC2**, for specific imaging of lysosomes in living specimens (Fig. 1). **TC1** and **TC2** were designed by connecting the diphenylamine unit at the 4 and 4′-positions of the dithiophen-2-ylmethanone and ethyl 2-cyano-3,3-di(thiophen-2-yl)acrylate molecules (Scheme 1), respectively, in order to extend the π-conjugation of the final compounds and realize push–pull systems, which allow a fine control of the emission color of the dyes. The **TC** dyes exhibited excellent photostability, moderate quantum yield and large Stokes shift. Moreover, they were localized in lysosomes and showed high biocompatibility and long-term retention, important features for biological applications.

2. Materials and methods

2.1. Materials

All reactions were carried out under a nitrogen atmosphere. Solvents were freshly distilled prior to use, according to standard procedures. Commercial products were purchased from Sigma-Aldrich. LysoTracker Red DND-99 (**LTR**) and MitoTracker Red CMXRos (**MTR**) were purchased from Life Technologies. Glass bottom Petri dishes for confocal experiments were purchased from WillCo Dish®. The microwave used was a CEM Discover LabMate.

2.2. Measurements

UV–Vis absorption spectra were recorded on a Varian-Cary 500 spectrophotometer. Fluorescence spectra were recorded on a Varian Cary Eclipse spectrofluorimeter. Tris(bipyridine)ruthenium(II) chloride was used as the standard for determination of fluorescence quantum yields. Biological imaging tests were carried out with a Zeiss LSM700 (Zeiss, Germany) confocal microscope equipped with an Axio Observer Z1 (Zeiss, Germany) inverted microscope using an objective 100×, with 1.46 numerical aperture oil immersion lens for imaging. Laser beams with 405 nm, 488 nm and 542 nm excitation wavelengths were used for Hoechst, **TC1** and **TC2** dyes, and commercial trackers imaging, respectively.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer with chemical shifts reported in ppm ($CDCl_3$). LC–MS spectra were acquired with an Agilent 6300 Series Ion Trap interfaced to an Agilent 1200 HPLC adopting the following general conditions: atmospheric pressure chemical ionization, positive ions, chloroform as the eluent, flow rate 0.200 mL × min^{-1}, drying gas flow 5.0 L × min^{-1}, nebulizer pressure 60 psi, drying gas temperature 350 °C, vaporizer temperature 325 °C, and mass range 100–2200 m/z.

2.3. Synthetic procedures

2.3.1. Synthesis of compound *2*

To a stirred suspension of 5-bromothiophene-2-carboxylic acid **1** (5 g, 24.15 mmol) in CH_2Cl_2 (50 mL) oxalyl chloride (3.24 mL, 120 mmol) and N, N-dimethylformamide (10 μl) at 0 °C were added, and then the mixture was warmed at room temperature. After being stirred at the same temperature for 2 h, the solvent was evaporated under a reduced pressure to give the titled compound 5-bromothiophene-2-carbonyl chloride **2** (5.42 g), as a pale yellow solid, which was used in the subsequent step without further purification.

2.3.2. Synthesis of compound *3*

Under a N_2 atmosphere, a solution of **2** (5.42 g, 24 mmol) in anhydrous CH_2Cl_2 (35 mL) was added dropwise over a period of 5 min through a dropping funnel to a 250 mL three-necked round bottom flask containing a suspension of $AlCl_3$ (8.00 g, 60.37 mmol) in anhydrous CH_2Cl_2 (30 mL) under stirring. Then, a solution of 2-bromothiophene (4.00 g, 24.5 mmol) in 20 mL of anhydrous CH_2Cl_2 was added and the resulting mixture was stirred overnight at room temperature. The reaction mixture was poured into a mixture of ice (100 mL) and concentrated HCl (20 mL), then extracted with CH_2Cl_2. The organic phase was washed with brine, dried over anhydrous sodium sulfate and concentrated to dryness. Purification by column chromatography on silica gel (eluent: hexane:CH_2Cl_2, 8:2 v/v) yielded **3** as a yellow solid (50% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.59 (d, 2H, J = 4.1 Hz); 7.51 (d, 2H, J = 4.1 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 175.98, 143.37, 133.03, 131.02, 122.58.

2.3.3. Synthesis of *TC1*

Bis(5-bromothiophene-2-yl)methanone **3** (0.40 g, 1.13 mmol), diphenylamine (0.46 g, 2.70 mmol) and sodium tert-butoxide (0.29 g, 3.00 mmol) were added to a suspension of $Pd_2(dba)_3$ (0.06 g, 0.06 mmol) and P(*t*Bu)$_3$ (0.23 mL, 0.23 mmol, 1 M in toluene) in anhydrous and degassed toluene (5 mL), previously stirred under Ar for 10 min. The resulting solution was heated under microwave irradiation at a constant temperature of 110 °C for 50 min. The solvent was removed, and the residue was dissolved in CH_2Cl_2 and filtered off on a short celite column. The solvent was removed by rotary under a reduced pressure, and the residue was purified by column chromatography on silica gel (eluent hexane:CH_2Cl_2, 1:1) to yield the dye **TC1** as an orange solid (0.42 g, 70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ; 7.57 (d, 2H, J = 4.2 Hz), 7.34 (m, 10H), 7.27 (m, 6H), 7.21 (m, 4H), 6.34 (d, 2H, J = 4.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.62, 160.60, 146.47, 132.76, 131.15, 129.43, 125.02, 124.66, 114.16. MS (APCI): calcd. for $C_{33}H_{24}N_2OS_2$ 528.13; found: m/z = 529,1 $[M + H]^+$.

2.3.4. Synthesis of *TC2*

$TiCl_4$ (0.56 mL, 5.13 mmol) was slowly added into anhydrous $CHCl_3$ (9 mL) under ice bath and Ar atmosphere. After stirring for 10 min, **TC1** (0.1 g, 0.19 mmol) and ethyl 2-cyanoacetate (0.13 g, 1.17 mmol) were added to the reaction mixture. After 10 min pyridine (0.75 mL) was added into the mixture and heated to reflux under Ar for 10 h. Then, the mixture was quenched with water and then extracted with CH_2Cl_2.

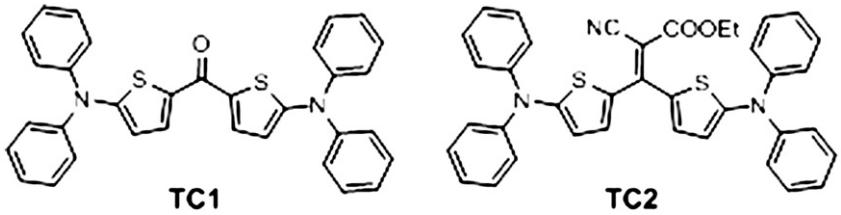
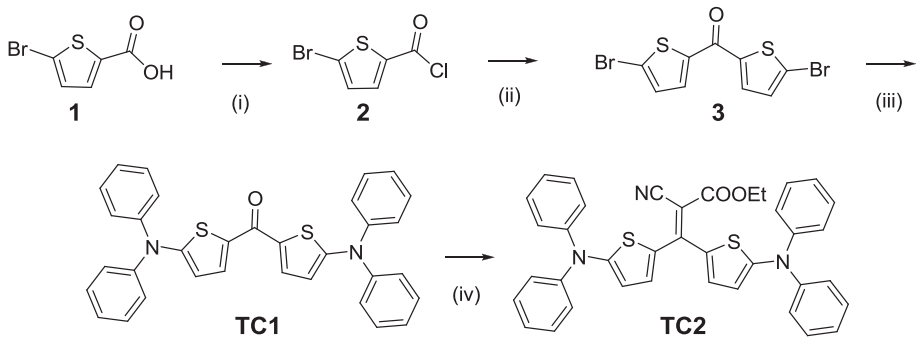


Fig. 1. Structure of **TC1** and **TC2** dyes.



Scheme 1. Synthetic route for **TC1** and **TC2** dyes. Reagents and conditions: (i) oxalyl chloride, CH_2Cl_2 (ii) $AlCl_3$, CH_2Cl_2, 2-bromo-thiophene (iii) diphenylamine, $Pd_2(dba)_3$, $P(tBu)_3$, NaO*t*Bu, Toluene, MW, 110 °C, 50 min (iv) $TiCl_4$, pyridine, ethyl 2-cyanoacetate, $CHCl_3$ anhydrous.

The organic phase was washed with brine and then dried over anhydrous sodium sulfate. The solvent was removed by rotary evaporation under a reduced pressure and the crude residue was purified by column chromatography on silica gel (eluent: hexane:CH_2Cl_2, 3:7), to give the product **TC2** as a dark-orange powder (70% yield). ^{1}H NMR (400 MHz, $CDCl_3$); 7.34 (m, 8H), 7.25 (m, 10 Hz), 7.18 (m, 4H), 6.43 (d, 2H, $J = 4.3$ Hz), 4.17 (quart, J = 7.1, 2H), 1.23 (t, J = 7.1, 3H); ^{13}C NMR (100 MHz, $CDCl_3$); 164.31, 160.38, 152.18, 146.08, 136.71, 134.70, 128.45, 127.66, 124.66, 114.59, 88.99, 60.95, 14.00. MS (APCI): calcd. for $C_{38}H_{29}N_3O_2S_2$ 623,17, found: m/z = 624,4 $[M + H]^+$.

2.4. Determination of quantum yield

The fluorescence quantum yield was determined using tris(2,2′-bipyridyl)dichlororuthenium(II) hexahydrate [Ru(bpy)$_3$] as the standard with a known Φ value of 0.028 in H_2O. The quantum yield was calculated according to the following Eq. (1):

$$\Phi_S/\Phi_R = (A_S/A_R) \times (Abs_R/Abs_S)x\left(\eta^2{}_S/\eta^2{}_R\right), \quad (1)$$

where Φ_S and Φ_R are the fluorescence quantum yields of sample and standard; A_S and A_R are the emission areas of sample and standard; Abs_S and Abs_R are the corresponding absorbance of the sample and the standard solution at the wavelength of excitation; η_S and η_R are the refractive indices of the sample and the standard, respectively.

2.5. Cell culture and staining

MCF-7 (human breast adenocarcinoma cell line), Hela (cervical cancer cells), HLF (human hepatoma cell line) and 3T3 (fibroblast cell line) cell lines were maintained in DMEM medium supplemented with FBS (10%), penicillin (100 U/mL culture medium), streptomycin (100 μg/mL culture medium), and glutamine (5%). Cells were grown in a humidified incubator at 37 °C, 5% CO_2, and 95% relative humidity. Cell lines were serum-starved for 24 h before any test.

Cancer cell line (5×10^4) was seeded onto 35 mm glass bottom Petri dish and incubated in complete media for 24 h. The cells were incubated with the fluorescent probes (**TC1** and **TC2**) at a concentration of 2.0 μM for 60 min in dark in a humidified incubator at 37 °C, 5% CO_2, and 95% relative humidity.

For the co-localization experiments, living cells were first incubated with **TC1** or **TC2** for 60 min, then stained with 0.1 μM **MTR** for 30 min or 0.075 μM **LTR** for 60 min. All samples were also stained with Hoechst 33342 (0.2 μM) for 5 min to check the nuclei morphology. The concentration and labeling conditions of each tracker were suggested by manufacturer. For each experiment, the cells were washed every time to remove the unbound marker before staining with another marker. After rinsing with PBS twice, cells were imaged under confocal microscopy immediately adding complete media without red phenol.

2.6. Cytotoxicity determination by MTT method

All cell lines were used in the general cytotoxicity test. The MTT method was used for measuring the activity of living cells via mitochondrial dehydrogenase activity. The key component is 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide or **MTT**. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple **MTT** formazan crystals which are insoluble in aqueous solutions. 20 μL of dyes was diluted within the complete culture medium. The MTT method is more effective when cultures are prepared in multiwell plates. Cells (10^4 cells/mL) were added to 12-well culture plates at 1000 μL/well, serum-starved for 24 h, and incubated at 37 °C in 5% CO_2, 95% relative humidity for 24–48–72–96–120 h with the dye suspension. The complete culture medium was used as the reference. After an appropriate incubation period, cultures were removed from incubator and a **MTT** solution, in an amount equal to 10% of the culture volume, was aseptically added. Then, cultures were returned to the incubator and incubated for 3 h, again. After the incubation period, cultures were removed from the incubator and the resulting **MTT** formazan crystals were dissolved in 1000 μL/well of DMSO. The plates were ready within 15 min after adding DMSO solution. After the incubation time, pipetting up and down was required to completely dissolve the **MTT** formazan crystals. Absorbance at a wavelength of 570 nm was measured using the ELISA plate reader. Results were expressed as a mean ± S.D. of three separate trials.

2.7. Fluorescence imaging and photostability

Laser scanning confocal microscopy was performed on a Zeiss LSM700 (Zeiss, Germany) confocal microscope equipped with an Axio Observer Z1 (Zeiss, Germany) inverted microscope using an objective 100×, with 1.46 numerical aperture oil immersion lens for imaging. Laser beams with 405, 488 and 542 nm excitation wavelengths were used for Hoechst, **TC1** and **TC2** dyes, and commercial tracker imaging, respectively.

In order to quantitatively investigate the photostability of the dyes, MCF-7 cells stained with **TC** dyes, or **LTR**, were exposed to constant laser beam. The fluorescence intensity of intracellular probes in fluorescence microscopy was normalized and plotted as a function of time.

2.8. Flow cytometry analysis

For uptake measurements, MCF7 cells were seeded on 24 well culture plates two days before the experiment, in order to reach half confluence. Cells were incubated at 37 °C with 2 μg × mL^{-1} of **TC1** or **TC2** for the indicated periods of time. Incorporation was analyzed with a Canto II flow cytometer (BD Biosciences) using a 405 nm laser and a 488 nm emission filter. At least 10,000 cells per condition were recorded

and the mean fluorescence intensity was calculated among live cells using an FSC/SSC gate to exclude dead cells. Results were analyzed using FlowJo software (Tree star).

3. Results and discussion

3.1. Synthesis of the materials

The new thiophene-based fluorescent probes **TC1** and **TC2** (Fig. **1**) were prepared following the synthetic route reported in Scheme 1. Compound **3** was synthesized by Friedel–Crafts acylation of 5-bromothiophene-2-carbonyl chloride with 2-bromothiophene, in the presence of anhydrous aluminum chloride as the catalyst and in dichloromethane. **TC1** was synthetized by a C–N cross-coupling reaction between the compound **3** with 2.2 equivalents of diphenylamine, in the presence of $Pd_2(dba)_3$ and $P(tBu)_3$ [16], under microwave irradiation at a constant temperature of 110 °C for 50 min. **TC2** was prepared via a Knoevenagel condensation of **TC1** with ethyl 2-cyanoacetate, in the presence of $TiCl_4$ [17].

3.2. Spectroscopic properties of TC1 and TC2 probes

The absorption and emission spectra of **TC1** and **TC2** in CH_2Cl_2 are reported in Fig. 2, while in DMSO, H_2O and DMSO/H_2O in different ratios are reported in the supporting data (Fig S1–S4). In Table S4 the spectroscopic properties are summarized. **TC1** and **TC2** showed absorption maxima at 436 and 496 nm with fluorescence maxima at 528 and 536 nm, respectively. The emission quantum yield for **TC1** and **TC2** was found 0.051 and 0.007, respectively. By comparing the absorption and emission spectra, large Stokes shifts of 92 and 40 nm were observed for **TC1** and **TC2**, respectively. In hydrophilic environments, like H_2O, the fluorescence intensity is quenched and the extinction coefficients of the two dyes result lower than those in dichloromethane. These results are in accordance with the general observations for organic dyes, that fluorescence is strong in organic solvents and many often quenched in aqueous media. It can be assumed, that **TC1** and **TC2**, with low background signals, are suitable for staining of hydrophobic biomembranes [18–23]. In addition, these dyes were found to be pH-insensitive, maintaining the unchanged absorption and emission intensities in the range 2.0–10 (Fig. S5). This property is a key requisite for cell environment imaging.

3.3. Uptake kinetics and fluorescence bioimaging

TC1 and **TC2** were incubated with MCF-7 in complete culture medium and the uptake was studied both by fluorescence microscopy and by flow cytometry. Upon one-photon excitation, the fluorescence of the dyes clearly indicates that the chromophores efficiently stain the cells and is mainly localized in the cytoplasm (Figs. 4 and 5).

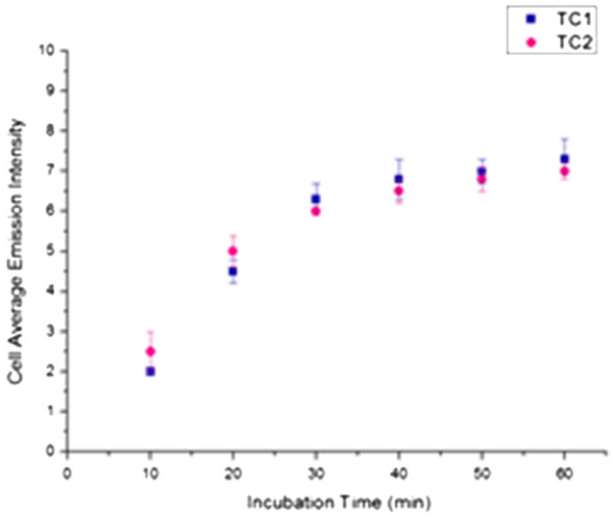


Fig. 3. Internalization kinetics of **TC1** and **TC2** in MCF7 cells measured by flow cytometry.

To assess the kinetics of the internalization process, the increase of the fluorescence signal inside the cells with time was measured by flow cytometry. The results indicated a rapid loading into the cells: after 30 min of incubation the dyes stained the cells (Fig. 3), reaching a plateau of intensity after 1 h of incubation, so we decided to fix this time for cell staining.

Furthermore, the long time of retention of the intracellular **TC1** and **TC2**, an important feature for a marker, especially for a tracer, was evaluated. Confocal microscopy results of living MCF 7 cells showed that the strong fluorescence in the cytoplasm and the total fluorescence intensity in cells remained almost unchanged for at least 120 h after removing the dyes from culture media. In particular, no fluorescence was observed in extracellular regions, indicating that **TC1** and **TC2** loaded within living cells did not leaked.

Confocal images (Figs. 4 and 5) show the ability of both dyes **TC1** and **TC2** to persistently staining the cells. Moreover, the dyes fluorescence can be also found in the daughter cell populations.

In particular, we studied the long term retention and the persisting staining of fluorescence by confocal microscopy. As shown in Fig. 4, the **TC1** fluorescence persisted up to four days (Fig. 4f). We cannot study the process for a long time because the cells are living and reach the confluence after the same days in culture. The same behavior was observed with **TC2** (Fig. 5), so we can assert that the fluorescence of our probes was transmitted after various replication processes.

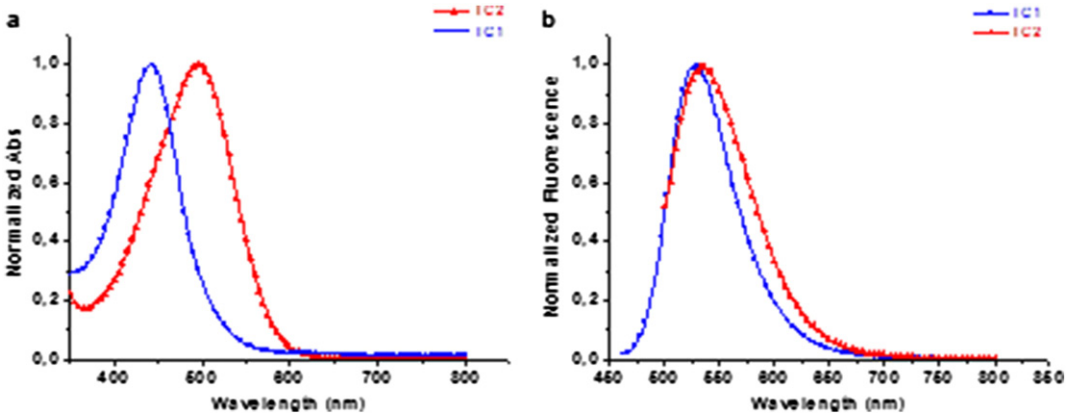


Fig. 2. Normalized absorbance (a) and emission (b) spectra in CH_2Cl_2 of **TC1** and **TC2**.

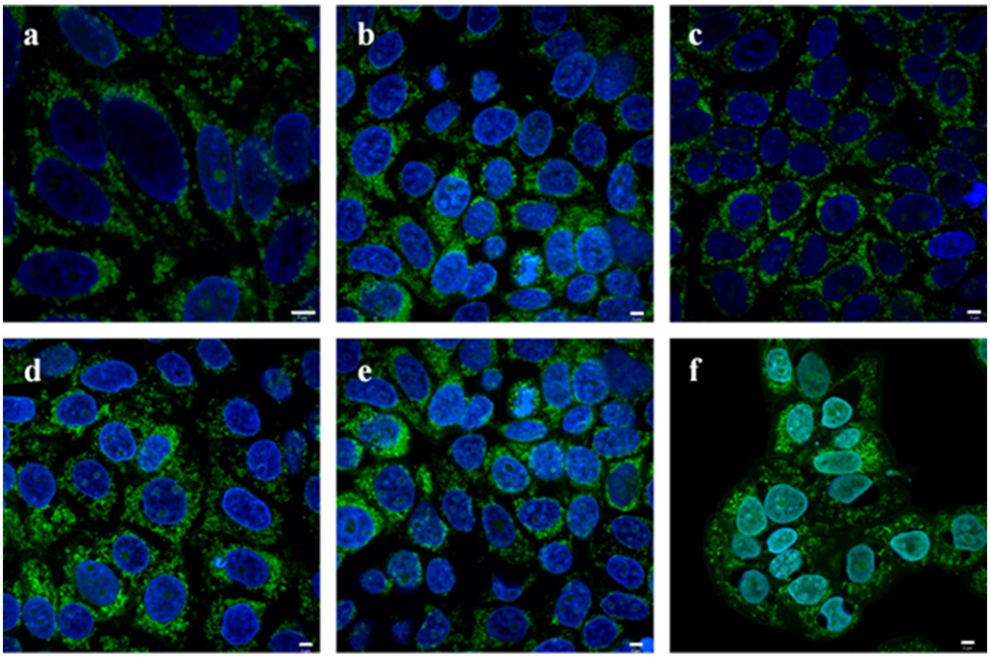


Fig. 4. Permanent staining of **TC1** in MCF7 cells measured by fluorescence microscopy (a) 30 min (b) 60 min (c) 24 h (d) 48 (e) 96 and (f) 120 h. Scale bar, 5 μm.

3.4. Specific labeling of lysosomes

To determine the subcellular distributions of **TC1** and **TC2** in living cells, the commercially available lysosome-specific staining tracker, **LTR**, and mitochondria-specific staining tracker, **MTR**, were co-incubated with **TC1** and **TC2**. To prevent the overlapping of spectra we perform confocal imaging experiments exciting before the commercial dye and then our probes. The merge of the two images was made after acquisition by software, allowing also the study of the overlapping coefficient of two fluorescent channels.

As reported in confocal images (Figs. 6 and 7) both **TC1** and **TC2** are localized in the cytoplasmatic region. The study of their subcellular distribution, comparing with commercial probes of subcellular organelles, lysosomes and mitochondria, led to asses that both dyes co-localize with lysosomes. In particular, as shown in confocal pictures, clearly both dyes are able to enter inside cells with no apparent surface binding on the cellular membrane. Only live cell imaging gives an accurate picture of the intracellular location of probes. Therefore, to further explore whether dyes were targeting the mitochondria or lysosomes, a co-incubation experiment was performed. This was accomplished by first incubating cells with dye **TC1** or **TC2** as described above, and then with **MTR** or **LTR**, in order to label mitochondria or lysosomes, respectively. Figs. 6 and 7 panel displays co-localization of **TC1** and **TC2** with MitoTracker Red, in the merge image the yellow areas

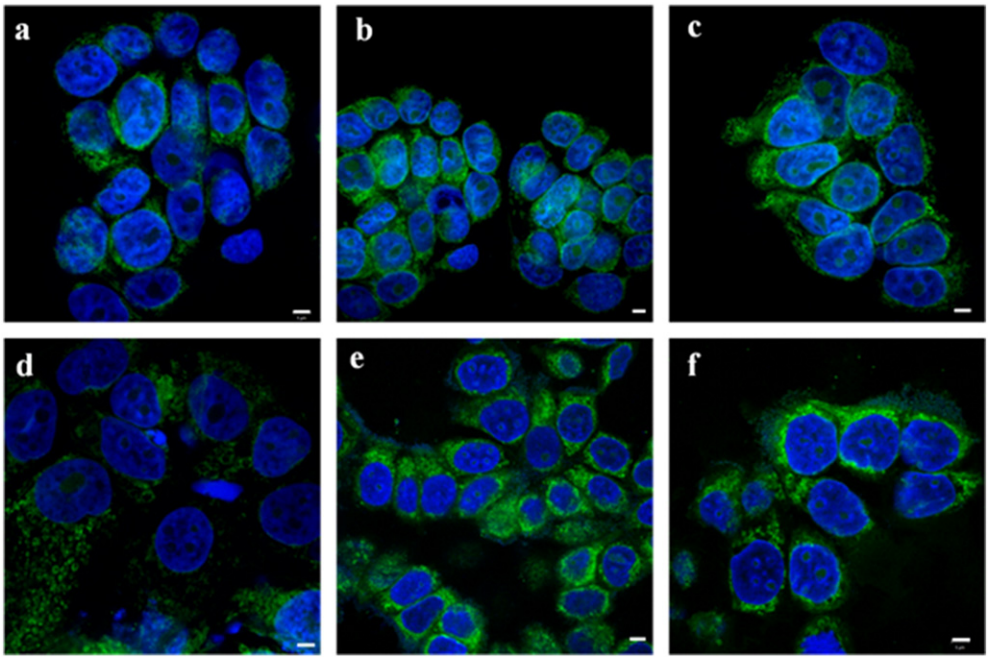


Fig. 5. Permanent staining of **TC2** in MCF7 cells measured by fluorescence microscopy (a) 30 min (b) 60 min (c) 24 h (D) 48 (e) 96 and (f) 120 h. Scale bar, 5 μm.

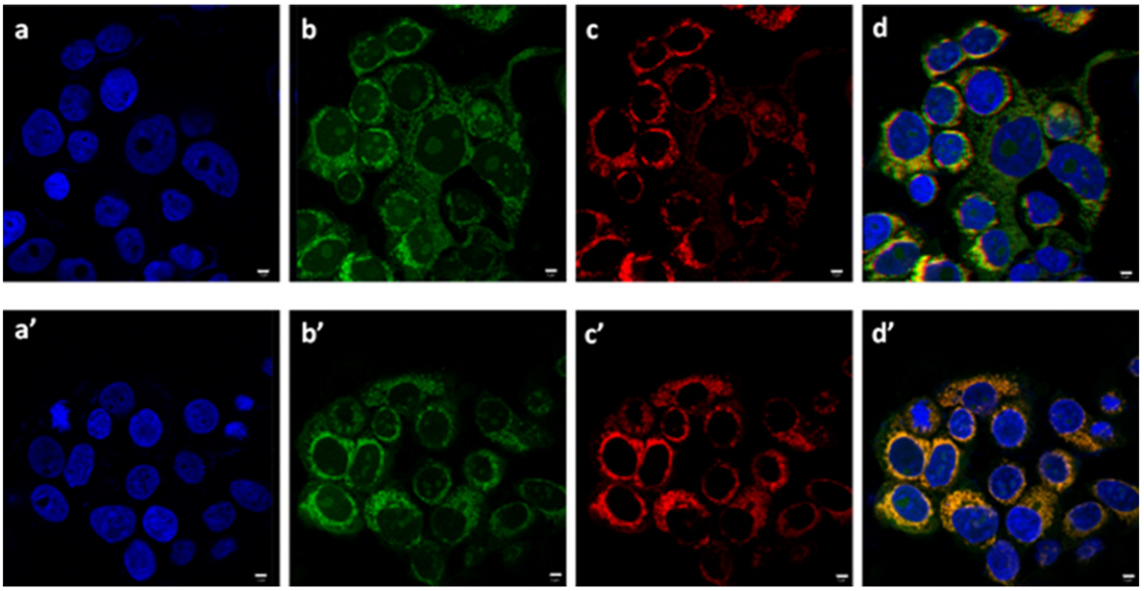


Fig. 6. TC1 colocalization study: (a–a′) nuclei; (b–b′) **TC1**; (c) commercial MitoTracker-Red; (c′) commercial LysoTracker-Red; (d–d′) merge of three previous images. Scale bar, 5 μm.

indicate the overlap regions, while red and green indicate areas where **TC1** and **TC2** (green) and the mitochondrial marker (red) are not coincident. In both case the red and green fluorescence does not overlap. In contrast, the bottom panel of Figs. 6 and 7 shows the overlapping between dyes and commercial **LTR**: it is encouraging to see that most of the green converge to yellow, indicating that the majority of **TC1** and **TC2** is in the same location of the cell as the **LTR**.

Moreover, both dyes are co-localized and they do not label other parts of the cell, such as the nucleus.

Furthermore, to quantify the amount of co-localization of dyes, a computational method was used. In particular, all image pixels are displayed on the scatter diagram, in which the two channels of the image are compared with one another. Pixels of both channels having identical positions in the image can be regarded as a pair. Accordingly, each pixel pair has two intensities, one for each channel, Fig. 8. The intensities of the two channels are reported on the axes of the scatter graph. In the scatter region 3 are presented the co-localizing pixels. Results evidenced that for **TC1** and **TC2** with **MTR** co-localizing pixels are 40 and 30% of the total, respectively. It is interesting to note that co-localizing pixels with **LTR** are equal to 97 and 99%, respectively.

3.5. Cytotoxicity

MCF-7, Hela, HLF and 3T3 cells were incubated with **TC1** and **TC2** at a concentration 10 times higher than that used in staining experiments. Cell viability was studied in a time-course experiment by MTT test (Fig. 9) at 24–48–72–96 h. Our data indicated a clear cyto-compatibility of dyes over time. In Fig. 9 only the data obtained after 96 h of incubation is reported; cell viability was around 100% in all cell lines, only in 3T3 there was a decrease until 95% after 96 h. Within experimental errors no detectable difference could be discerned between **TC1** and **TC2**: both dyes are highly cyto-compatible over long time (up to 4 days).

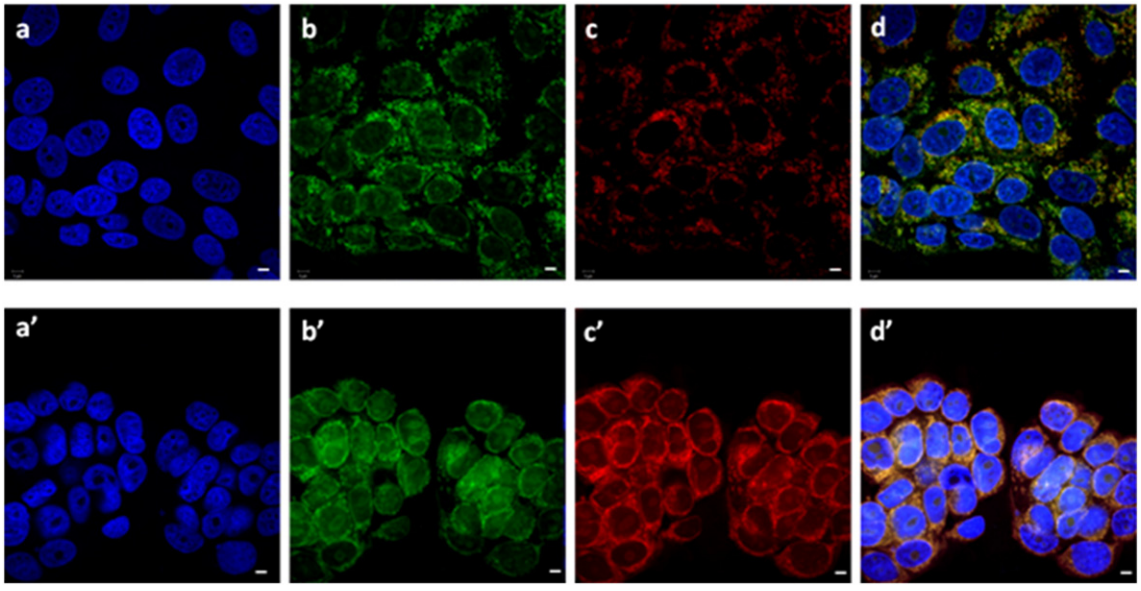


Fig. 7. TC2 colocalization study: (a–a′) nuclei; (b–b′) **TC2**; (c) commercial MitoTracker-Red; (c′) commercial LysoTracker Red; (d–d′) merge of three previous images. Scale bar, 5 μm.

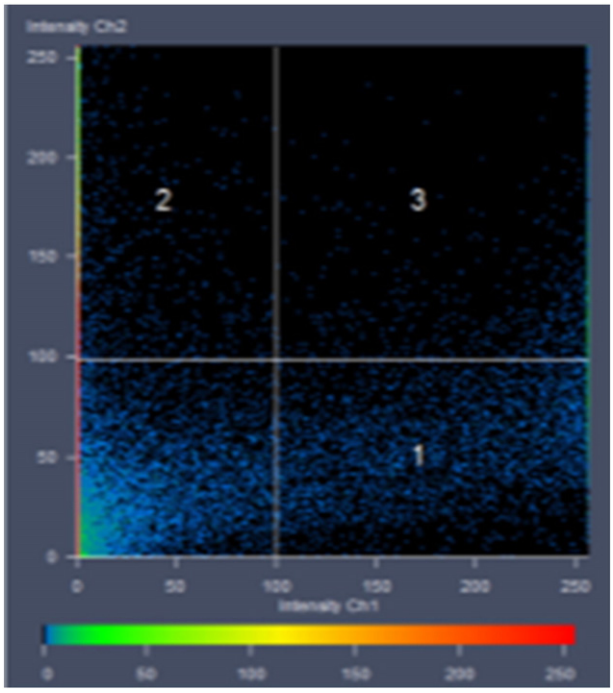


Fig. 8. Representative co-localization analysis. All image pixels are displayed on the scatter diagram, in which the two fluorescence channels of the image are compared with one another. Pixels of both channels having identical positions in the image can be regarded as a pair. Accordingly, each pixel pair has two intensities, one for each channel. The intensities of the two channels are reported on the axes of the scatter graph. In the scatter region 3 are presented the co-localizing pixels.

These data are very important, overall if compared with commercial trackers, which often cause a decrease in cell viability [24], also using lower concentrations with respect to those texted with the organic dyes of the present work.

3.6. Photostability in MCF-7 cell

Under the high-intensity illumination conditions used for fluorescence microscopy, the irreversible destruction of the excited fluorophore

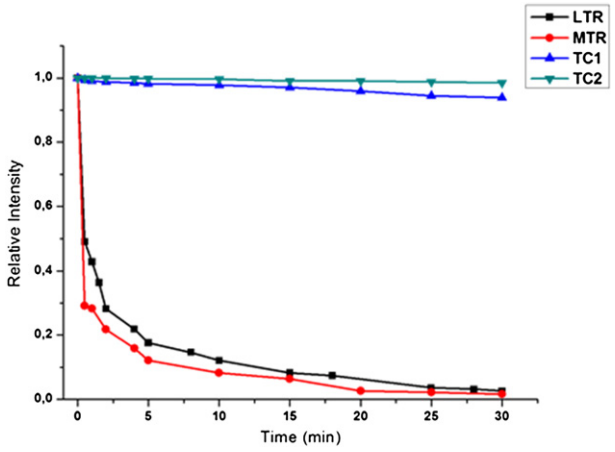


Fig. 10. The fluorescence intensity in MCF-7 cells was determined staining with **TC1** (2.0 μM), **TC2** (2.0 μM) for 60 min, **MTR** (0.1 μM) and **LTR** (0.075 μM for 60 min) by confocal microscopy. The changes of fluorescence intensity in the consecutive t-scan mode were determined for 30 min (time interval of data determination = 2 min). Excitation wavelength for our probes was 488 nm, instead for commercial trackers was 514 nm. Data were obtained from replicate experiments (n = 5).

(photobleaching) often becomes the factor limiting fluorescence detectability. In order to quantitatively investigate the photostability of **TC1** and **TC2**, MCF-7 cells stained with **TC1**, **TC2**, **MTR** or **LTR** were exposed to constant laser beam. The fluorescence intensity of intracellular **TC1** and **TC2** in fluorescence microscopy was normalized and plotted as a function of time. In Fig. 10 the dyes exhibited constant fluorescence emission during 10 scans with a total irradiation time of about 10 min. In contrast, the signal intensity of **LTR** and **MTR** quickly decreased. The slight fluctuation of the signal intensity may be due to the metabolic activity of the living cells. These results suggested a superior photostability of **TC1** and **TC2**.

4. Conclusion

In summary, we developed two thiophene-based fluorescent probes specifically targeting the lysosomes in cancer cells. The organic dyes showed high specificity for lysosomes, excellent and superior photostability compared with commercial **LTR** and **MTR**

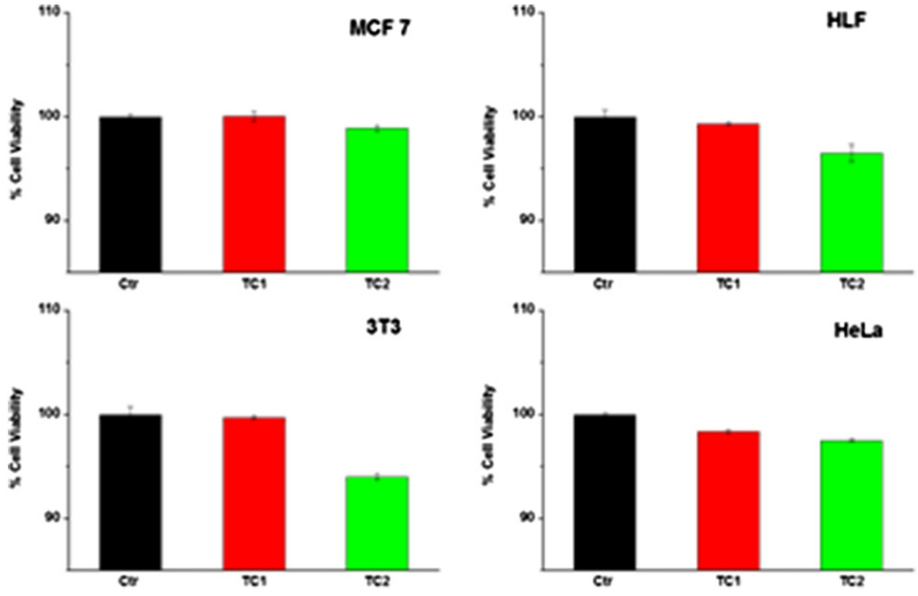


Fig. 9. MTT cytotoxicity tests for MCF-7, HLF, 3T3 and Hela cells treated with **TC1** and **TC2** compared to untreated cells (Ctr).

trackers, and low phototoxicity. In addition, **TC1** and **TC2** showed a complete insensitivity to the pH in the range 2–10, maintaining intact their fluorescence capability. The results of the present work disclose a new class of organic dyes with potential broad applications as specific and efficient **Lyso** trackers in the studies of various biological processes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.10.010.

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