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A Non-Toxic Fluorogenic Dye for Mitochondria Labeling

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Abstract

Background—Mitochondria, powerhouses of cells, are responsible for many critical cellular functions, such as cell energy metabolism, reactive oxygen species production, and apoptosis regulation. Monitoring mitochondria morphology in live cells temporally and spatially could help with understanding of the mechanisms of mitochondrial functional regulation and the pathogenesis of mitochondria-related diseases.

Methods—A novel non-cytotoxic fluorogenic compound, **AcQCy7**, was developed as a mitochondria-specific dye.

Results—AcQCy7 emitted no fluorescent signal outside of cells, but it became fluorescent after intracellular hydrolysis of the acetyl group. The hydrolyzed fluorescent product was well retained in mitochondria, enabling long-lasting fluorescence imaging of mitochondria without cell washing. A 2-day culture study using **AcQCy7** showed no sign of cytotoxicity, whereas a commonly used mitochondria-staining probe, Mitochondria Tracker Green, caused significant cell death even at a much lower concentration. Apoptosis-causing mitochondria fission was monitored clearly in real time by **AcQCy7**.

Conclusions—A simple add-and-read mitochondria specific dye **AcQCy7** has been validated in various cell models. Bright mitochondria specific fluorescent signal in treated cells lasted several days without noticeable toxicity.

General Significance—The probe **AcQCy7** has been proofed to be a non-toxic agent for long-term mitochondria imaging.

Keywords

fluorogenic dye; mitochondria; cyanine dye; cytotoxicity; apoptosis

1. Introduction

Mitochondria play important roles in cellular pathways, such as cellular energy metabolism, apoptosis regulation, cell redox signaling, as well as reactive oxygen species production [1–5]. The multiple functionality of mitochondria is highly associated with their dynamic heterogeneous structures [6]. Additionally, mitochondria morphological changes are

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indications of many cellular processes [7–12]. Monitoring mitochondria morphological changes temporally and spatially could help with understanding of the mechanisms of mitochondrial functional regulation, leading to possible therapeutics [5, 13, 14]. Mitochondria morphological changes are usually followed in living cells non-invasively using an appropriate mitochondria-selective dye by fluorescence microscopy. Because of the unique negative membrane potentials, mitochondria passively collect positively charged compounds. Based on this phenomenon, most of the mitochondria-selective fluorophores are generally cationic lipophilic dyes, including conventional fluorescent mitochondria stains, such as rhodamine 123 [15], JC-1 dye [16], and tetramethylrhodamine [11], as well as chloromethyl moiety-containing dyes, such as Mitotracker Green (MTG), Mitotracker Orange (CMTMRos), and Mitotracker Red (CMXRos) [17]. The chloromethyl moiety-containing dyes react with thiol groups associated with mitochondria, resulting in long retention of the stains; thus, they are also compatible with commonly used fixation or permeabilization protocols. However, of numerous mitochondria-selective fluorescent probes, including the ones mentioned above, very few are non-cytotoxic. Here, we report the novel usage of a recently reported compound, **AcQCy7** [18], for mitochondria-specific labeling, and importantly, **AcQCy7** is not toxic to cells. Moreover, this probe has the fluorogenic advantage in that it only becomes fluorescent once it is picked up by cells, and the fluorescent signal is retained inside of mitochondria, enabling long-term fluorescent imaging of mitochondria in live cells without cell washing.

2. Materials and methods

2.1. Reagents and cells

The probes **AcQCy7** and **QCy7** were prepared following a published procedure [18]. The key fluorogenic compound **AcQCy7** was prepared conveniently in 84% yield through just one step from two commercially available starting materials. Mito Tracker Green FM™ (MTG), Hoechst 33324, Endoplasmic Reticulum (ER) Tracker™ Blue-White, and Lyso Tracker Green were purchased from Life Technologies (Carlsbad, CA). They were dissolved in dimethylsulfoxide and stored as a stock solution (1 mM) at – 20 °C in the dark. Staurosporine was purchased from Sigma-Aldrich (St Louis, MO).

The human glioma C6 and C6/LacZ7 cell lines, the HeLa human cervical cancer cell line, and the MDA-MB-231 breast cancer cell line, obtained from the American Type Culture Collection (ATCC, Manassas, VA), were maintained in Dulbecco's modified Eagle's medium (DMEM), which was supplemented with 10% fetal bovine serum, 1% antibiotics (penicillin-streptomycin), 200 nM L-glutamine, and 100 nM NEAA at 37 °C under humidified 5% CO_2.

2.2. Fluorescence and confocal microscopy

Images of live cells were acquired using either an inverted fluorescence microscope (Olympus 81X; Tokyo, Japan) or a confocal microscope (Olympus FluoView TM FV1000) as indicated in the figure legends. The emission of **AcQCy7** was collected through the TRITC band pass filter set (excitation: 510–550 nm, emission: 573–648 nm). The fluorescence of MTG and Lyso Tracker Green was obtained through the FITC filter set (excitation: 450–490 nm, emission: 500– 550 nm), whereas Hoechst 33342 and ER Tracker Blue was taken via the DAPI filter set (excitation: 325–375 nm, emission: 435–485 nm).

2.3. Co-localization staining and fluorescence imaging

HeLa cells (5×10^3/well) were cultured in wells on a μ-Slide 8-well chamber (ibidi, Munich, Germany) in complete media for 20 h. The cells were incubated in media in the presence of **AcQCy7** (1 μM) for 40 min at 37 °C under humidified 5% CO_2. The cells were washed

using Hank's balanced salt solution (HBSS), followed by incubation with MTG (250 nM), Hoechst (200 nM), ER Tracker (500 nM), or Lyso Tracker (500 nM) in HBSS buffer for 20 – 30 min. The concentration and labeling condition of each tracker was suggested by the manufacture. The cells were washed and replaced with fresh complete medium (phenol-red free) before confocal fluorescence imaging. Other cell lines, including C6, C6/LacZ7, and MDA-MB-231, were studied following the same protocol.

2.4. MTS cell viability assay

HeLa cells (5×10^3/well), seeded in wells of a 96-well plate, were treated with **AcQCy7** (1 μM) or MTG (250 nM) in complete medium for 30 min, washed, and cultured in complete medium for an additional 24 to 48 h. The medium was replaced with a fresh mixture containing DMEM and 20 μL Celltiter96 reagent solution (Promega, Wisconsin, WI). The cells were incubated for 3 h and the absorbance of each sample was measured at 490 nm to determine cell viability. The results are expressed as the mean percentage of cell viability relative to untreated cells. Each concentration was tested at least three times, and differences were deemed to be significant at a P value less than 0.1.

2.5. Images of washed vs. non-washed cells treated with AcQCy7 and MTG

C6/LacZ7 cells (5×10^3/well) were plated in wells of a 96-well plate for 20 h (37 °C, 5% CO_2). The cells were incubated with a solution of **AcQCy7** (1 μM) and MTG (0.5 μM) in DMEM (phenol-red free) for 40 min. For the washing experiment, the cells were washed with and maintained in DMEM (phenol-red free) for an additional 24 h and imaged with a fluorescence microscope. For non-washed visualization, the cells were left in the same **AcQCy7** and MTG containing DMEM for additional 24 h and imaged as-is.

2.6. Treatment with apoptosis inducer

HeLa cells (5×10^3/well) were incubated on a μ-Slide eight-well chamber in complete media for 20 h at 37 °C under humidified 5% CO_2. The cells were pre-incubated with **AcQCy7** (1 μM) for 40 min, followed by addition of Hoechst 33342 for an additional 20 min. The cells were washed and incubated in complete culture media (phenol-red free) at 37 °C under humidified 5% CO_2. Next, cells were treated with staurosporine (STS, 5 μM), and images were taken at 1.5, 3.0, and 5.5 h using a confocal microscope.

3. Results and discussion

3.1. Fluorogenic probe AcQCy7

The fluorescent dye **QCy7**, recently introduced by Karton-Lifshin *et al.*, has a π-conjugated system as other cyanine dyes, in which one positive charge delocalizes between the two nitrogen atoms through a heptamethine chain (Scheme 1) [18]. It emits red light with a peak at approximately 700 nm in phosphate-buffered saline (pH 7.4). Although **QCy7** is an excellent fluorophore, its application in cells is limited because of its poor membrane permeability. To overcome this shortcoming, a temporary lipophilicity-enhancing acetyl group is introduced (see structure **AcQCy7**) to increase its cell permeability. This acetyl group is expected to be removed quickly by intracellular esterase after the dye **AcQCy7** is internalized, as we previously demonstrated [19]. Another advantage of this acetylation is that the fluorescence emission property of **QCy7** is completely masked because its π-conjugated system is disrupted. Subsequent intracellular hydrolysis of the acetate ester of **AcQCy7** would restore the fluorescent property of **QCy7**.

3.2. Specific labeling of mitochondria in various types of cells

The preferential binding of intracellular organelles by the hydrolyzed **AcQCy7** was tested by co-staining with four known fluorescent organelle trackers in HeLa cells. MTG, Hoechst Blue, ER Tracker Blue-White, and Lyso Tracker™ Green were used for staining of mitochondria, the cell nucleus, ER, and lysosomes, respectively. Comparing the fluorescent images of the hydrolyzed **AcQCy7** with those of the known organelle trackers, a good co-registration was only observed between **AcQCy7-** and MTG-treated cells (Fig. 1a). The fluorescent patterns of hydrolyzed **AcQCy7** (red) and MTG (green) are near identical, as supported by the yellow staining in the merged image (Fig 1a). Poor co-localizations were observed when **AcQCy7**-treated cells were co-stained with Hoechst Blue, ER tracker, or Lyso tracker Green (Fig. 1b–d), indicating that the probe **AcQCy7** could not label these three organelles well. The positive charge on **QCy7** most likely facilitates quick translocation into mitochondria because of the negative membrane potential.

To determine its universal mitochondria-labeling capability, **AcQCy7** was further investigated in three additional cell lines, including MDA-MB231, C6, and C6/lacZ cells. As observed in Figure 2, the localization of the hydrolyzed **AcQCy7** (red signal) significantly overlaps with MTG (green signal) in all three lines. These results demonstrated that **AcQCy7** is a potential universal mitochondrial tracker for various live cells. As expected, when the polar un-acetylated **QCy7** probe was incubated with HeLa, MDA-MB231, C6, or C6/LacZ cells, no appreciable fluorescence was observed inside any cells under the microscope (data not shown).

3.3. Cytotoxicity of AcQCy7 and Mito Tracker MTG

The potential cytotoxicity of **AcQCy7** (1 μM) and MTG (250 nM) was first validated by incubating HeLa cells with either probe for 30 min, and imaging and performing assays at different time points. Using a fluorescence microscope, no immediate morphological changes were observed immediately after treatment. However, latent toxicity was found in MTG-treated cells after 24 or 48 h of incubation. Characteristic indications of apoptosis, such as a shrunken cytoplasm and condensed nuclei, were observed in MTG-treated cells 1 day after an initial pulse incubation (Figure 3). More apoptotic cells were observed when the incubation time was extended for another day. By contrast, HeLa cells remained healthy 1 or 2 days after the **AcQCy7** (1 μM) label (Figure 3a). The cytotoxicity of **AcQCy7** and MTG was further quantitated by MTS assays (Figure 3c). MTS assay is a common method used for measuring cytotoxicity of toxic materials. The assay uses a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) in conjunction with phenazine methosulfate, the intermediate electron acceptor. The MTS is reduced to its water soluble derivative formazan, which has an absorbance maximum at about 490 nm in phosphate-buffered saline, by the cellular NAD(P)H-dependent oxidoreductase enzymes in live cells. Therefore, the cellular oxidoreductase activity can be measured through the measurement of the absorbance of the formazan at 490 nm, which, under defined conditions, reflects the number of viable cells. The MTS assay results shown in Figure 3(c) indicated that only 70% of MTG-treated cells were viable after 2 days of incubation. These MTS cell viability results were consistent with the fluorescence imaging results, indicating that **AcQCy7** is non-toxic. Additionally, MTG caused significant damage to cells; even at a concentration of 250 nM, which was four times lower than that of **AcQCy7** (1 μM).

3.4. Cell images with AcQCy7 without washing

Because **AcQCy7** does not fluoresce in culture media until it is hydrolyzed to **QCy7** in cells, it is possible that a typical washing step could be skipped after labeling. The non-

washing process can circumvent some disadvantages caused by the conventional post-label washing process, such as cell intolerance and missing temporal information. Two parallel co-labeling experiments with **AcQCy7** and MTG were performed to test whether these probes are suitable for wash-free imaging. The first set of C6/LacZ7 cells were incubated with both probes for 40 min, washed, and supplied with fresh complete culture medium, and cells were cultured for an additional 24 h prior to imaging. The second set of cells was incubated with both probes for 24 h without washing and was imaged directly (Figure 4). Clearly, there is little difference between the washed and non-washed group using the **AcQCy7** fluorescence filter set. The fluorescence signals of **AcQCy7** (orange) were mainly confined inside mitochondria, and the background signal in the medium was negligible, even when the **AcQCy7** probe has been incubated for 24 h. However, non-washed MTG images (green) showed a strong background fluorescence signal in the cell culture media; therefore, extensive washes are needed to reduce the background MTG signal. These results indicate that mitochondria could be labeled by **AcQCy7** with a high signal to background ratio conveniently (wash-free imaging) because **AcQCy7** is non-fluorescent before hydrolysis, and its intracellularly hydrolyzed product is well retained in mitochondria.

3.5. Imaging of morphological changes during apoptosis

Mitochondria, a cellular apoptosis regulator, plays central roles in both extrinsic and intrinsic apoptotic pathways [20, 21]. Fragmentation of mitochondria and opening of the mitochondrial permeability transition pore to release apoptogenic factors, such as cytochrome c and apoptosisinducing factors, are critical and characteristic events in apoptosis [22, 23]. Therefore, imaging of mitochondria during apoptosis is of utmost important because it can detect the response of mitochondria to the stimuli and reveal the process of apoptosis. This is often accomplished with commercially available Mito Trackers, such as MTG, CTMRos, and CMXRos [17]; however, the process could be misinterpreted. Detection errors are usually observed because these trackers are also toxic to cells. It is reasoned that the new tracker **AcQCy7** could avoid this disadvantage of current mitochondria trackers because of its lack of cytotoxicity. The potential usage of **AcQCy7** was thus demonstrated by monitoring the specific mitochondria changes during apoptosis. **AcQCy7**-stained HeLa cells were treated with staurosporine (STS, 5 μM), a known apoptosis inducer for various cell types [11, 24]. In healthy cells, mitochondria were in a filamentous shape (Figure 5, 0 h). After 1.5 h of STS treatment, the mitochondria were shrunken to rounded structures. Extending the treatment time caused the mitochondria to further shrink, and some of them were completely disrupted as indicated by the loss and the presence of the red fluorescence in the cytosol and nucleus, respectively (Figure 5; 3 h and 5.5 h). Obviously, chromatin condensation and nucleus shrinkage were also observed in this apoptosis process as indicated by the blue fluorescence nuclear stain. The combination of the mitochondria-selective probe **AcQCy7** and nucleus stain Hoechst 33324 clearly captured the characteristics of apoptosis.

4. Conclusion

In summary, **AcQCy7** showed high selectivity for mitochondria over other subcellular organelles in cells. Clear mitochondrial images could be obtained conveniently after simple addition of the probe (1 μM) into the culture medium. Bypassing the routine cell-washing step, mitochondria images could be acquired without delay, and fragile cells could also be studied immediately. The probe is not toxic to cells; it persisted in mitochondria for more than 2 days. By contrast, the commercially available mitochondria tracker, MTG, caused significant cell death. Our validation results suggest that **AcQCy7** is an excellent fluorogenic probe for mitochondria imaging.

Acknowledgments

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Highlight

- A fluorogenic dye for mitochondrial labeling
- Long lasting nontoxic mitochondria stain
- A simple add-and-read dye, cell washing is not needed.

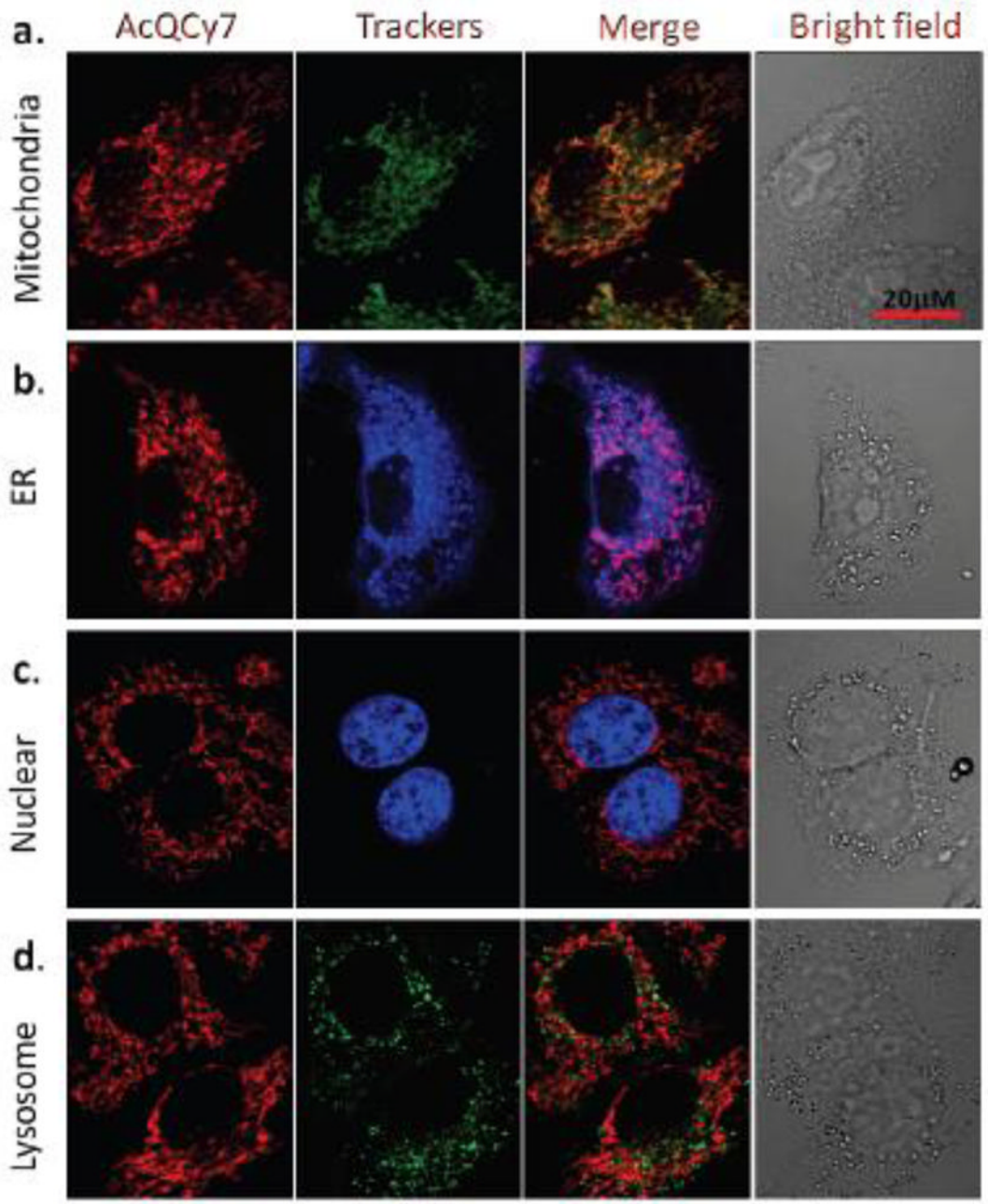


Fig. 1.

The subcellular fluorescence signals produced by the hydrolyzed **AcQCy7** probe. HeLa cells were co-incubated with **AcQCy7** (1 μM) and representative organelle trackers (a) MTG (250 nM) for mitochondria, (b) ER Tracker™ Blue-white (500 nM) for the ER, (c) Hoechst 33324 (200 nM) for the nucleus, and (d) Lyso Tracker™ Green (500 nM) for lysosomes. The pictures from left to right in each panel are the hydrolyzed **AcQCy7** fluorescence images, and organelle trackers images, as well as merged fluorescence images and bright field images, respectively.

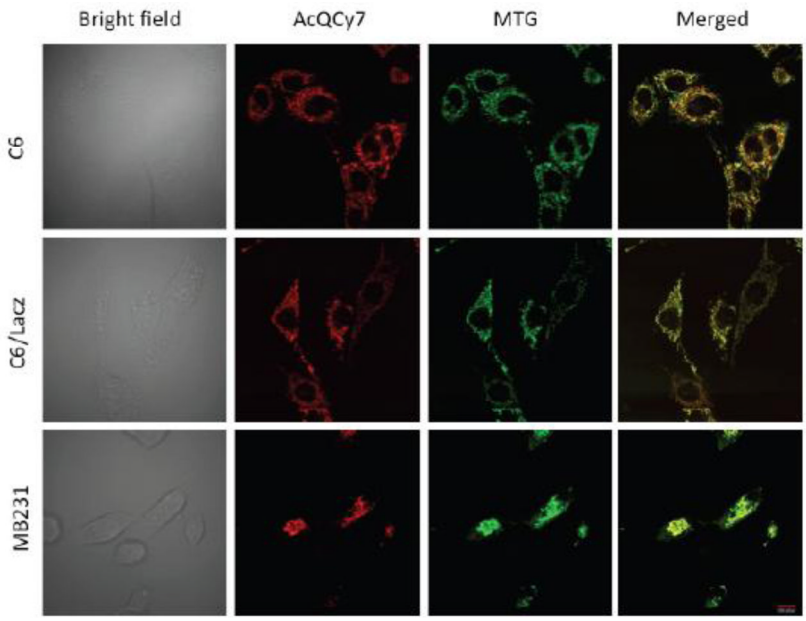


Fig. 2.

The subcellular fluorescence signals of **AcQCy7** in C6, C6/LacZ and MB231 cells. All cells were co-treated with **AcQCy7** (1 μM) and MTG (250 nM). From left to right in each panel are the bright field images, fluorescence images of **AcQCy7** (red), MTG (green), and merged images, respectively.

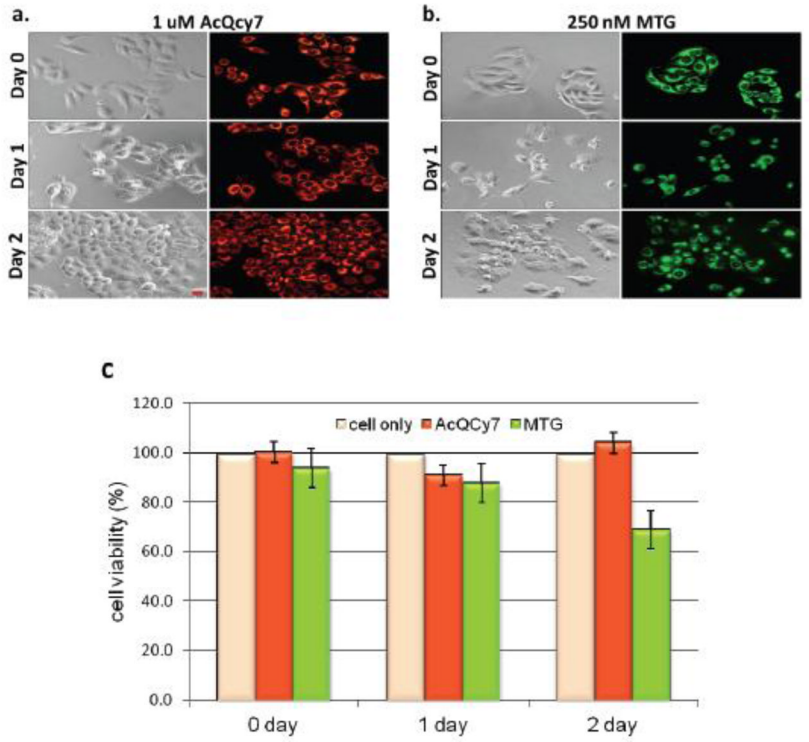


Fig. 3.

Cell toxicity of **AcQCy7** or MTG studied by real-time fluorescence imaging of (a) **AcQCy7**-treated, (b) MTG-treated HeLa cells and (c) quantitative MTS assays. The left and right panel in figure a and b are the bright field and fluorescence images respectively. The cells were treated with either **AcQCy7** (1 μM) or MTG (250 nM) for 30 min and then cultured with fresh complete media until fluorescent imaging or the MTS assay. Scale bar = 20 μm.

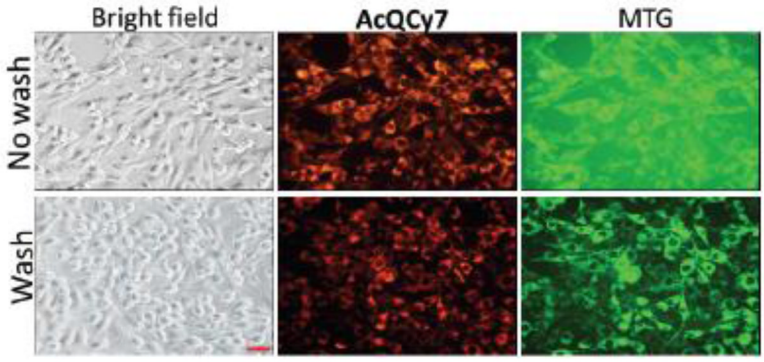


Fig. 4.

Live-cell imaging of mitochondria with the **AcQCy7** (1 μM) probe and Mito Tracker MTG (0.5 μM) without (top raw) and with (bottom raw) washing procedures. The left, middle, and right panels are bright field, **AcQCy7**, and MTG fluorescence images.

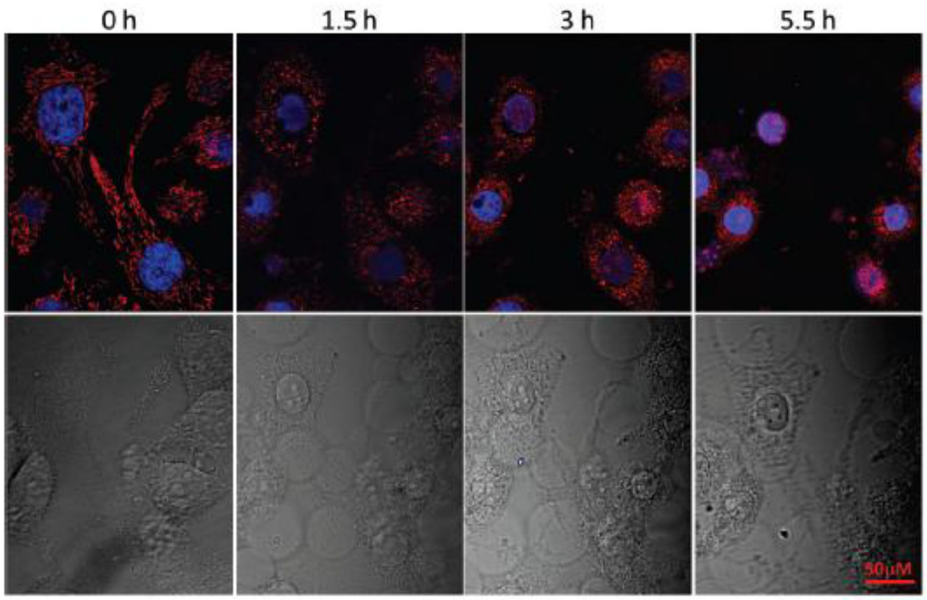
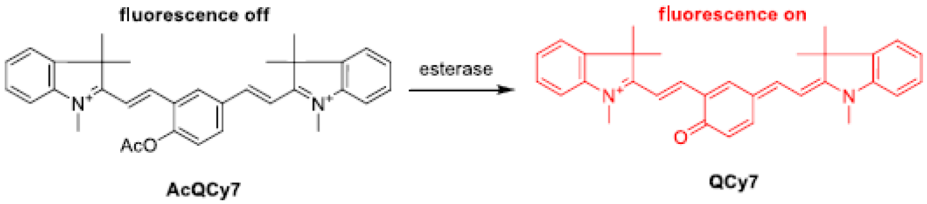


Fig. 5.

Progression of apoptosis in STS (5 μM)-treated HeLa cells. The cell morphology was captured using a fluorescence confocal microscope. Mitochondria and nuclei were visualized with red (**AcQCy7**) and blue (Hoechst 33324) fluorescence, respectively. Scheme



Scheme 1.
The fluorogenic probe **AcQCy7** and proposed hydrolysis inside live cells. Graphical abstract