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A two-photon fluorescent probe for real-time monitoring autophagy by ultrasensitive detection of the change in lysosomal polarity

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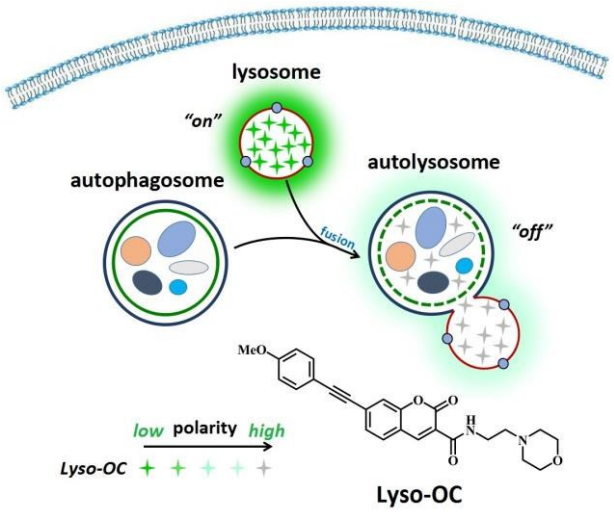
The first two-photon probe Lyso-OC for monitoring cell autophagy by detecting the change of lysosomal polarity during the membrane fusion process of autophagy was proposed. Lyso-OC exhibited desired optical properties and selective detection signal to the polarity change. More importantly, Lyso-OC displayed a real-time monitoring of autophagy in living cells.

Autophagy is a lysosomal degradation pathway that primarily serves as an adaptive agent for the protection of organisms against diverse pathologies, including infections, cancer, neurodegeneration, aging, and heart disease.[1-3] This special function makes the accurately monitoring technology of autophagy in highly demand. methods have been developed for monitoring autophagy including Transmission electron microscopy (TEM), Western Blotting (Atg8 / LC3) and GFP-Atg8 / LC3 fluorescence microscopy.[4, 5] However, these classical approaches are time consuming and financial costly, as well as lack of the autophagy information in a single living cell. Therefore, how to precisely and vividly monitoring autophagy in a simple and cost-effective manner is still under challenge.

Autophagy is initiated by the formation of autophagosomes, which are the organic complex that capable of engulfing cytosolic components both selectively and non-selectively. After the formation, autophagosomes grow and deliver sequestered components to lysosomes.[6] Lysosomes are membraneous acidic vesicles, which contain approximately 50 different degradative enzymes and proteins that are active at acidic pH.[7] The polarity of lysosome changed greatly during the member fusion process in autophagy due to the inner micro-environmental difference between lysosome and autophagosome.[8-10]

In the meantime, two-photon probes have become the leading bio-imaging tools thanks to their vast merits such as localized excitation, increased penetration depth, reduced tissue autofluorescence and photobleaching over the one-photon ones.[10, 11] Thus, we believe that detecting the change of lysosomal polarity during the member fusion in autophagy with novel two-photon fluorescent probes may be an efficient method for monitoring autophagy (Scheme 1).

Herein, we reported the first two-photon lysosome-targeting probe **Lyso-OC** (7-((4-methoxyphenyl) ethynyl)-N-(2-morpholinoethyl)-2-oxo-2H-chromene-3-carboxamide) for monitoring autophagy by detecting the change of lysosomal polarity during autophagy process. Coumarin was used as the fluorescent group for its solvatochromic property, i.e. it can responds fluorescently to the change of micro-environmental polarity for the variations in the molecular dipole upon excitation.[12-15] Morpholine was used as the lysosomal targeting group for its excellent lysosome targeting property.[16] To achieve the large two-photon absorption cross-section and Stokes shift, 1-ethynyl-4-methoxybenzene was grafted at the 7-position of the coumarin group (Scheme 1).[17, 18] We hope **Lyso-OC** will give



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Scheme 1. The structure of **Lyso-OC** and the mechanism of monitoring autophagy by **Lyso-OC** in living cells.

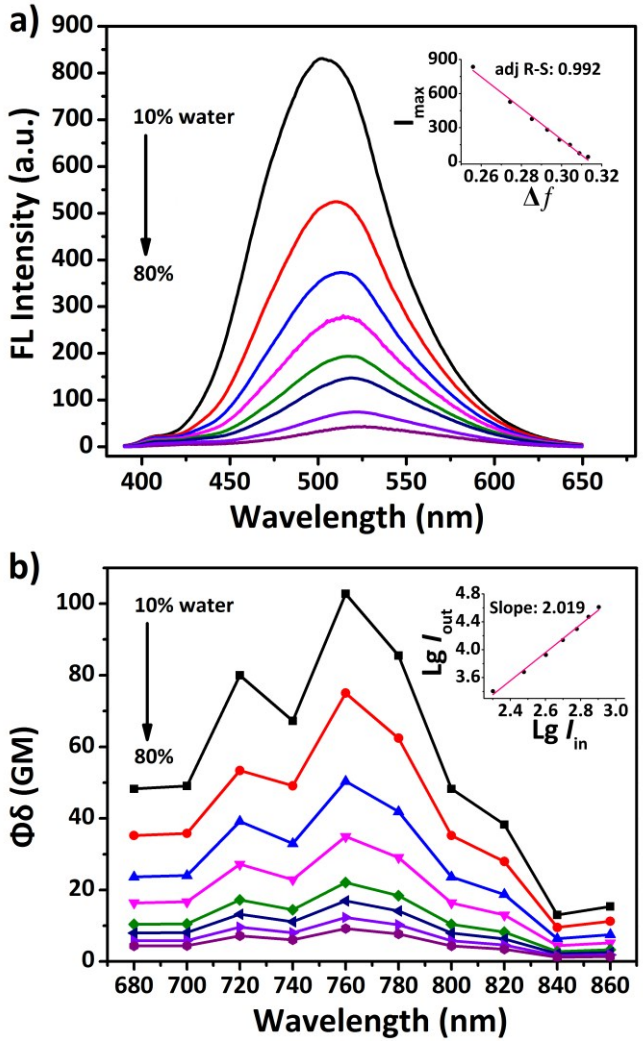

Figure 2. a) Fluorescence emission spectra of **Lyso-OC** (10 μM) in different H_2O/THF mixtures (water from 10 % to 80 %). Insert: Linearity of I_{max} versus the solvent parameter *Δf*. b) Two photon action cross sections of **Lyso-OC** in different H_2O/THF mixtures (water from 10 % to 80 %). Insert: The square relationship of two-photon excited fluorescence intensity (I_{out}) of **Lyso-OC** with the addition of input power (I_{in} = 300-800 mW). λ_{ex} = 375 nm.

good two photon fluorescent detection signal to the change of lysosomal polarity during the autophagy process.

The emission of **Lyso-OC** under different solvent polarity was tested in H_2O/THF system. Lippert Mataga polarity parameter Δf was used to indicate the polarity of the solution.[19, 20] As shown in Figure 1a, when the solvent changed from 10% water (Δf ≈ 0.256) to 80% water (Δf ≈ 0.313) in H_2O/THF mixtures, the fluorescence intensity of **Lyso-OC** decreased about 40-fold along with a red shift (~ 30 nm) of the emission spectra. A good linear correlation between the fluorescence intensity maximum and solvent polarity (Δf) is obtained. Optical properties of **Lyso-OC** in different solvents also confirmed that fluorescence intensity of **Lyso-OC** was depended on solvent polarity (Figure S1, S2 and Table S1). However, the change of

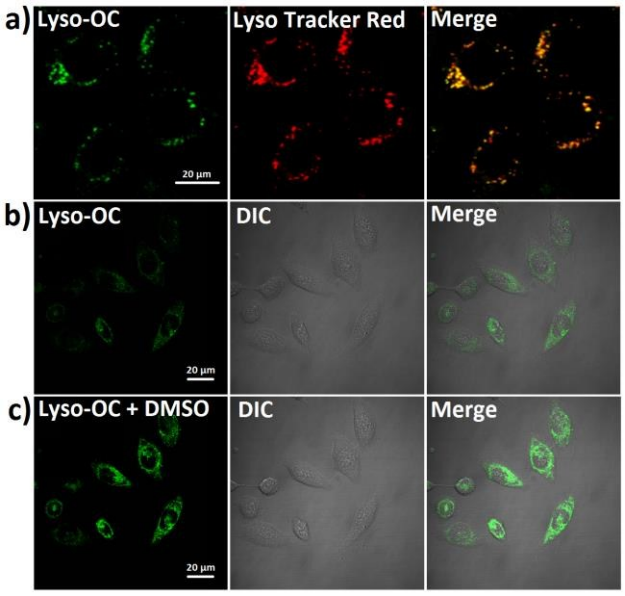

Figure 2. a) Fluorescence imaging of MCF-7 cells co-stained with **Lyso-OC** (10.0 μM) and Lyso Tracker Red (0.5 μM) for 45 min. b) Fuorescence imaging of MCF-7 cells stained with **Lyso-OC** (10 μM) for 45 min. c) Fluorescence imaging of MCF-7 cells in b) further treated with DMSO (10.0 μL) for 60 min, λ_{ex} = 760 nm, λ_{em} = 490-550 nm.

the solvent polarity caused negligible effect on the absorption of the probe (Figure S3). The calculation results also confirmed the observation data (Figure S4 and S5). These results suggest that the fluorescence of **Lyso-OC** is highly sensitive to the change of micro-environmental polarity. We further examined the effect of solvent polarity on the quantum yield and lifetime of **Lyso-OC** in H_2O/THF mixtures. The quantum yield and lifetime values showed remarkable linearity with Δf from 0.256 to 0.313 (Figure S6 and S7), in good accordance with the emission behavior. Similar behavior of **Lyso-OC** was observed in THF/MeOH mixtures (Figure S8). These data confirmed that the fluorescence of **Lyso-OC** was regulated by polarity of the solvent.[21-23]

The TP cross sections of **Lyso-OC** were determined by the two-photon induced fluorescence technique.[24, 25] As shown in Figure 1b, the two-photon action cross-section (Φδ) of **Lyso-OC** at 760 nm decreased gradually from 102 GM (10% water) to 4.4 GM (80% water) in H_2O/THF mixtures. Furthermore, with the increase of laser power from 0.2 W to 0.8 W at 760 nm, a square relationship of two-photon excited fluorescence intensity is observed with a reasonable linearity, indicating the two-photon excitation nature of the probe.

In the pH titration and viscosity titration experiments, the fluorescence intensity of **Lyso-OC** is found to be pH-insensitive in the lysosomal pH range (Figure S9) and viscosity-insensitive (Figure S10). MTT assay results indicated that the new probe is low cytotoxicity and suitable for detection polarity change in living cells (Figure S11).[26] To further investigate the subcellular

localization of **Lyso-OC**, co-localization experiment with Lyso Tracker Red was carried out in MCF-7 and HeLa cells. As shown in Figure 2 and Figure S12, the images of **Lyso-OC** and Lyso Tracker Red overlapped very well with each other. co-localization coefficient was calculated to be 0.91 and 0.89 in MCF-7 and HeLa cells respectively which suggested that **Lyso-OC** localized in lysosomes in living cells.[27] The three-dimensional fluorescence imaging of rat liver tissue slices incubated with **Lyso-OC** indicated that the penetrative depth of the probe was 120 μm under the two-photon fluorescence excitation (Figure S13).[28]

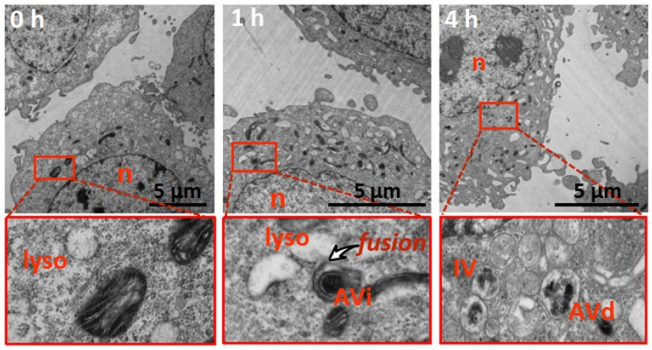

Figure 3. Representive electron microscopic images of the cytoplasmic regions of MCF-7 cells. Cells were cultured in starvation for different times and stained with osmium tetroxide. n = nucleus, lyso = lysosome, AVi = autophagosome, AVd = degradative autophagic vacuole, IV = internal vacuoles.

The ability of **Lyso-OC** to detect the change of lysosomal polarity was further tested. We examined **Lyso-OC** staining in multiple cell lines including MCF-7 cells, HeLa cells, HELF cells and CHO cells treated with dimethyl sulfoxide (DMSO), which can be absorbed by cells in a short time.[17] As shown in Figure 2 and Figure S14, it was found that the fluorescence of cells has increased obviously after adding 10 μL DMSO to the cell culture medium within 60 minutes. The sensitivity to polarity of **Lyso-OC** in lysosome encourages us to monitor cell autophagy by detection of the change of lysosomal polarity during the autophagy process with **Lyso-OC**.

Hank's Balanced Salt Solution (HBSS), a medium without nutrient, was used to induce autophagy of MCF-7 cells by starvation. Transmission electron microscopy (TEM) was eventually adopted to monitor the autophagy induced by starvation at subcellular level.[29, 30] As shown in Figure 3, in cells with rich nutrient, the normal cytoplasm and lysosomes can be observed vividly. However, after incubated with HBSS for 1 hour, the formation of autophagosomes (AVi) was clearly observed and the membrane fusion between lysosomes and autophagosomes can already been observed. Then, autolysosomes (AVd) and the region filled by small internal vesicles could be observed at 4 hours. Western blotting, another classical way for detecting autophagy, was carried out simultaneously. The expression level of LC3-II protein in cells is regarded as marker of autophagy.[4, 5] GAPDH, one of the key enzymes involved in glycolysis, is kept in the original quantity in the autophagy process. The rate of GAPDH and LC3-II could be used to reflect the level of autophagy. As shown in Figure S13, the level of LC3-II has increased obviously during the early stages of autophagy in MCF-7 cells and reached the maximum after incubating for 1 h. The results demonstrate that the autophagy of MCF-7 cells could be induced by starvation and the membrane fusion process began within one hour.

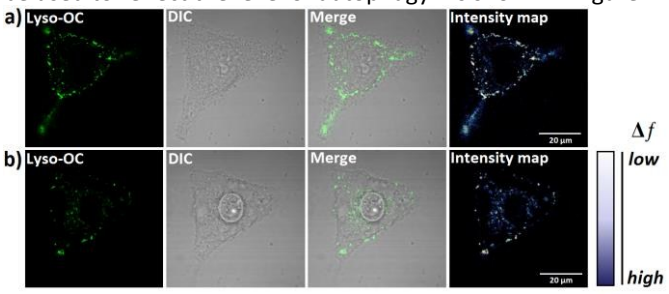

Figure 4. a) Fluorescence confocal imaging of MCF-7 cells stained with **Lyso-OC** before be induced. b) Fluorescence confocal imaging of MCF-7 cells stained with **Lyso-OC** in autophagy. λ_{ex} = 760 nm, λ_{em} = 490-550 nm. The scale bar represents 20 μm.

The change of lysosomal polarity in autophagy was further investigated. As shown in Figure 4a, **Lyso-OC** distributes in the

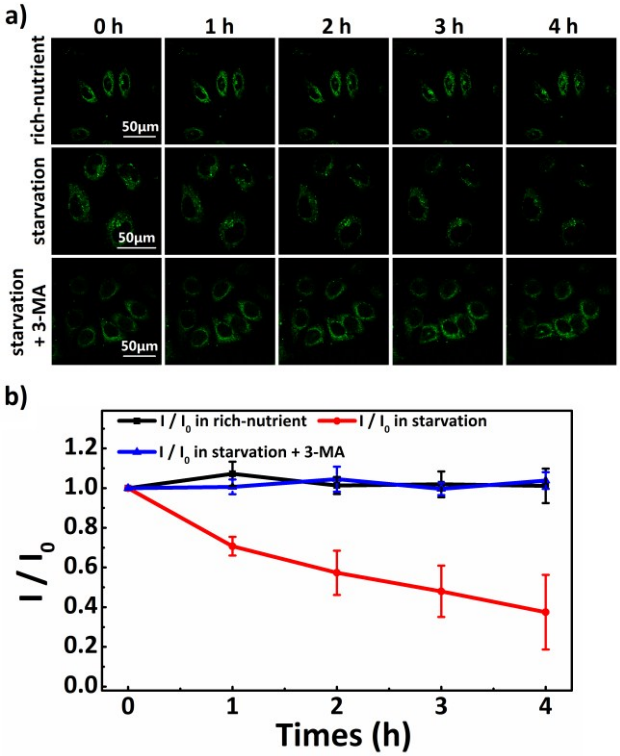

Figure 5. a) Real-time confocal imaging of MCF-7 cells in rich-nutrient (control), starvation (autophagy) and starvation + 3-MA (autophagy inhibited), respectively. Cells were stained with **Lyso-OC** (10.0 μM) for 45 min before imaging. b) Fluorescence intensity ratio (I / I_0) of **Lyso-OC** in rich-nutrient (black line), starvation (red line) and starvation + 3-MA (blue line) at each time nodes. I represents the intensity at each

time nodes, I_0 represents the intensity at 0 h. λ_{ex} = 760 nm, λ_{em} = 490-550 nm.

MCF-7 cell regularly before be induced by HBSS, the lysosomes could be observed clearly by the fluorescence of **Lyso-OC**. when induced lysosomes evolve into autolysosomes, **Lyso-OC** exhibits weak fluorescence simultaneously (Figure 4b). Therefore, we deduced that the lysosomal polarity has been increased in the membrane fusion between lysosomes and autophagosomes.

With the above data in hand, we tried to use **Lyso-OC** to monitor autophagy with fluorescent signal. The con-focal imaging of the MCF-7 cells stained with **Lyso-OC** was recorded at different time (0-4 hours) under different cell culture conditions (normal, autophagy and autophagy inhibited). As shown in figure 5, the fluorescence of cells in rich nutrient (normal) has kept constant at different time dues (Video S1) suggesting probe **Lyso-OC** is photo resistance at this experimental circumstance. However, the fluorescence of cells in starvation decreased to 40% gradually with time increase (Video S2). Furthermore, cells were incubated in HBSS in the presence of 3-Methyladenine (3-MA, autophagy inhibitor), the fluorescence in these cells was found to be remained unchanged with the incubation time increasing (Video S3). From Figure 5 and Video S2, we can also see that the fluorescence change of a single cell under autophagy condition could be recorded vividly. Similar results were found in HeLa cells (figure S16).Thus, the fluorescent change of **Lyso-OC** could be used as an efficient detection signal for monitoring autophagy process in living cells.

Conclusions

In conclusion, we have developed a novel two-photon probe **Lyso-OC**, the first ultrasensitive fluorescent probe for detecting lysosomal polarity and real-time monitoring autophagy in living cells. **Lyso-OC** exhibits a modest Stokes shift and excellent linear relationship in optical measurements when the polarity of micro-environment changes, as well as favorable two-photon action cross-sections and low cytotoxicity. It can selec-tively stain lysosomes in live cells and tissues. Fluores-cence data of multiple cell lines including MCF-7 cells, HeLa cells, HELF cells and CHO cells demonstrates the detecting ability and wide applicability of **Lyso-OC** to lysosomal polarity. More significantly, **Lyso-OC** can monitor autophagy of a single cell and multiple cells by detecting the change of lysosomal polarity efficiently and economically. These results provide useful guide-lines for using **Lyso-OC** as a novel method for real-time monitoring autophagy from chemical overview.

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