Supplementary Information

Efficient cell surface labelling of live zebrafish embryos: wash-free fluorescence imaging for cellular dynamics tracking and nanotoxicity evaluation

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Experimental Details

Materials: Cholesterol−poly(ethylene glycol)$_{2k}$−NH_2 (Chol−PEG−NH_2) and cholesterol−poly(ethylene glycol)$_{2k}$−fluorescein (Chol−PEG−FITC) were obtained from Nanocs, Inc. (New York, NY). Cyanine5 *N*-hydroxysuccinimide ester (NHS-Cy5) was purchased from Nanjing Bioorth Biotech Co., Ltd. (Nanjing, China). Cholesterol−poly(ethylene glycol)$_{2k}$−nitrobenzoxadiazole (Chol−PEG−NBD) was ordered from Hunan Hua Teng Pharmaceutical Co., Ltd. DiD and sulforhodamine 101 sulfonyl chloride were bought from Fanbo Biochemicals Co., Ltd. (Beijing, China). CellMask Green plasma membrane stain was purchased from Invitrogen. SDS was purchased from Sigma-Aldrich. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-*sn*-glycero-3-phosphocholine (NBD-PC) were bought from Avanti Polar Lipids. Doxorubicin (Dox) hydrochloride was purchased from Beijing Huafeng United Technology Co., Ltd. (Beijing, China). Gold nanoparticles and graphene oxide nanosheets were ordered from Nanjing XFNANO Materials Tech Co., Ltd. Cell counting kit-8 (CCK-8) was bought from Beyotime Institute Biotechnology. Deionized water (18.2 MΩ·cm) was obtained from a Milli-Q system (Millipore, Billerica, MA).

Synthesis and Characterization of Chol−PEG−Cy5: The as-designed plasma membrane probe Chol−PEG−Cy5 was synthesized as follows: 4.5 mg of NHS-Cy5 and 8.9 mg of cholesterol−PEG2000−NH_2 were both dissolved in phosphate-buffered saline (PBS) solutions (pH = 7.4, 50 mM) and mixed together immediately with vigorous vortex for 2 min. Then, the mixture was kept reaction in the dark at room temperature under stirring for 4–6 h. The obtained solution was dialyzed (MWCO = 2 kDa) against deionized water for 1 day to

remove unconjugated Cy5 molecules, followed by lyophilization. To further purify the obtained products, we carried out thin-layer chromatography (TLC) using chloroform : methanol (18 : 2, *v*/*v*) as the solvent system (retention factor = 0.61). Subsequently, individual bands were scraped and the powders were dispersed in methanol. After centrifugation, the supernatant was collected for rotary evaporation to obtain purified Chol−PEG−Cy5. To validate the successful synthesis of this compound, the ^{1}H nuclear magnetic resonance (NMR) spectra of Chol−PEG−Cy5 were recorded using a Bruker Avance 500 MHz instrument. The prominent peak at 3.51 and the small peak at 5.34 were assigned to the proton signals of PEG chain and Cy5 moiety, respectively (Fig. 1b). The proton signals of cholesterol moiety could be observed 0.85 and 1.27 ppm (Fig. S1). The matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrum of Chol−PEG−Cy5 was measured by a Bruker UltrafleXtreme MALDI-TOF mass spectrometer using α-cyano-4-hydroxycinnamic acid (HCCA) as a matrix (Fig. 1c). Given that the average molecular weight of cholesterol−PEG2000−NH_2 is ~1950 Da as we previously reported,[1] a 671 Da increase (close to the molecular weight of Cy5) of Chol−PEG−Cy5 further demonstrated the successful conjugation of Cy5 to cholesterol−PEG2000−NH_2.

To determine the critical micelle concentration (CMC) of Chol−PEG−Cy5, we measured the surface tension values of Chol−PEG−Cy5 dispersed in PBS solutions at various concentrations using an optical tensiometer (Theta Lite, Biolin Scientific). The CMC value was recorded as the inflection point of the surface tension curve. The hydrodynamic diameter of Chol−PEG−Cy5 micelles was measured by dynamic light scattering (DLS) using a zetasizer instrument (Nano ZS, Malvern Instruments, UK). The size and morphology of

Chol−PEG−Cy5 micelles were observed by transmission electron microscopy (TEM) using a transmission electron microscope (JEM-2100, JEOL Ltd., Japan).

Synthesis and Characterization of Chol−PEG−Rhod: The control sample Chol−PEG−rhodamine (Chol−PEG−Rhod) was synthesized as follows: 3.9 mg of sulforhodamine 101 sulfonyl chloride and 9.1 mg of Chol−PEG−NH_2 were both dissolved in Na_2CO_3/$NaHCO_3$ buffer solutions (pH = 9.5) and then mixed together immediately with vigorous vortex for 2 min. Next, the mixture was kept reaction in the dark at room temperature overnight. The obtained solution was dialyzed (MWCO = 2 kDa) against deionized water for 3 days to remove unconjugated Rhod dyes, followed by lyophilization. The obtained products were further purified by TLC as described above using chloroform : methanol (20 : 1, *v*/*v*) as the solvent system.

Preparation of Liposomes: To prepare liposomes, 4 mg of POPC lipid was hydrated with 2 mL of PBS solution to a final concentration of 2 mg/mL. Then, the lipid suspension was vortexed for 2 min, followed by extrusion through a polycarbonate membrane (100 nm) for 21 times using an Avanti Mini-Extruder. The hydrodynamic sizes of liposomes before and after hydrophobic anchoring of Chol−PEG−Cy5 were measured by DLS.

Fluorescence and Absorbance Measurements: To compare the fluorescence properties of Chol−PEG−Cy5 before and after membrane anchoring, 1 mL of Chol−PEG−Cy5 solution (4 μM) was mixed with 1 mL of PBS solution or various concentrations of POPC liposomes to reach a final Chol−PEG−Cy5 concentration of 2 μM. After incubation for 30 min at room temperature, the fluorescence emission spectra of these solutions were recorded using a spectrofluorophotometer (RF-5301PC, Shimadzu, Japan). Similar studies on

Chol−PEG−FITC, Chol−PEG−Rhod and Chol−PEG−NBD were also carried out as described above. To investigate whether the interaction between Cy5 moieties could be dissociated by surfactants, we measured the fluorescence emission spectra of Chol−PEG−Cy5 in a PBS solution and 1% SDS solution, respectively. Besides, the ultraviolet–visible (UV–vis) absorbance spectra of Chol−PEG−Cy5 solutions (2 μM in PBS) in the absence and presence 1% SDS were measured using a Shimadzu UV-2600 spectrophotometer.

Fluorescence Recovery after Photobleaching (FRAP) Analysis: We here employed FRAP analysis to confirm the lateral diffusion of anchored Chol−PEG−Cy5 in lipid membranes.[2] Before FRAP experiments, we first prepared supported lipid bilayers (SLBs) according to our previously reported methods.[3] Typically, POPC and NBD-PC dissolved in chloroform were mixed together at a molar ratio of 99.5 : 0.5. Next, chloroform was removed by nitrogen gas, followed by vacuum treatment overnight. The as-formed dry lipid films were then hydrated in deionized water to prepare liposomes as mentioned above. Subsequently, liposome solution was pipetted onto an ultraclean glass substrate (pretreated with oxygen plasma), and then incubated with a buffer solution (400 mM KCl, 4 mM Tris and 2 mM $CaCl_2$; pH 8.0) for 30 min, which allowed the liposomes to fuse together on the substrate for the final formation of SLBs. Finally, the SLBs were rinsed with PBS solution repeatedly to remove excess lipids.

The as-prepared SLBs were incubated with Chol−PEG−Cy5 (2 μM) for 30 min at room temperature. Then, the bilayers were rinsed with PBS solution for three times to remove unbound Chol−PEG−Cy5 molecules. To conduct FRAP analysis, a circular region of interest (ROI) with a diameter of 20 μm was selectively photobleached by a beam of intense 638 nm

laser for 3 s. Next, confocal fluorescence images were dynamically acquired with a predetermined time interval to track the fluorescence changes of the ROIs.

In Vitro Confocal Fluorescence Imaging: Typically, A549, MCF-7, AT II and HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 IU/mL penicillin−streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. U14 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS and 100 IU/mL penicillin−streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. To determine the staining time, A549 cells were incubated with 2 μM Cho−PEG−Cy5 for different time periods (5 min, 30 min, 2 h and 3 h), followed by confocal fluorescence imaging using an inverted confocal laser scanning microscope TCS SP8 (Leica, Germany) with a 63× oil immersion objective. The Cy5 fluorophore was excited by a 638 nm laser with an emission band from 650 to 700 nm. To optimize the staining concentration, A549 cells were incubated with various concentrations of Chol−PEG−Cy5 for 10 min, followed by confocal fluorescence imaging. To demonstrate that the plasma membrane labelling of Chol−PEG−Cy5 is cell type-independent, AT II, MCF-7, HepG2 and U14 cells were stained with this probe (2 μM) for 10 min, respectively, and their staining performance was evaluated by confocal fluorescence imaging. To study whether Chol−PEG−FITC, Chol−PEG−Rhod or Chol−PEG−NBD could achieve wash-free plasma membrane imaging, A549 cells were separately incubated with the three probes (2 μM) for 10 min. Before confocal imaging, PBS washing treatment was skipped. The S/B ratios of these confocal images were analyzed by the ImageJ software.

To compare with commercial plasma membrane stains, A549 cells were incubated with

DiD (1 μM) or CellMask Green (5 μg/mL) for different time periods. Then, the stained cells were washed with PBS for three times, followed by confocal fluorescence imaging. DiD was excited at 638 nm with an emission band from 650 to 700 nm, and CellMask Green was excited at 488 nm with an emission band from 500 to 540 nm.

To prepare fixed cells, A549 cells were treated with 2.5% glutaraldehyde for 15 min at room temperature. The fixed cells were washed with PBS for three times, and then stained by Chol−PEG−Cy5 (2 μM) for 10 min, followed by confocal fluorescence imaging. For cell permeabilization, the fixed cells were further treated with 0.2% Triton X-100 for 5 min at room temperature. After PBS washing for three times, the permeabilized cells were stained by Chol−PEG−Cy5 (2 μM) and observed as described above. To investigate the staining performance of Chol−PEG−Cy5 on apoptotic cells, A549 cells were pretreated with 5 μg/mL Dox (a clinical anticancer drug) for 24 h at 37 °C to induce cell apoptosis. Next, the treated cells were gently washed by PBS once, incubated with Chol−PEG−Cy5 for 10 min, and observed by confocal fluorescence imaging.

To compare the membrane binding abilities of the four probes (i.e., Chol−PEG−FITC/Cy5/NBD/Rhod), we employed A549 cells as the model. First, 500 μL of four probe solutions (2 μM) was prepared and their maximal fluorescence intensities were measured by fluorescence spectroscopy. Then, these probe solutions were separately incubated with 5×10^5 trypsinized A549 cells for 15 min at 37 ^{o}C, which enabled the plasma membrane binding of the probes. After incubation, the cell suspensions were centrifuged at 1000 rpm for 5 min and the supernatants containing unbound probes were collected for fluorescence spectroscopic measurements to record their maximal fluorescence intensities.

The percentage of membrane-bound probes was calculated as: 100% – (fluorescence intensity after cell incubation)/(fluorescence intensity before cell incubation) × 100%.

Cytotoxicity Evaluation: To study the cytotoxicity of Chol−PEG−Cy5, AT II cells were added to a 96-well plate at a density of 5×10^3 cells per well in 100 μL of cell culture medium. Next, cells were treated with different concentrations (1, 2, 5, 10 and 20 μM) of Chol−PEG−Cy5 probes for 24 h, respectively. For CCK-8 assay, the culture medium in each well was replaced by 100 μL of fresh DMEM and 10 μL of CCK-8 solution was then added into each well. After further incubation at 37 ^{o}C for 2 h, the absorbance of each well was measured by a microplate reader (Thermo-Scientific, Multiskan FC, USA) at 450 nm to determine the cell viabilities.

Zebrafish Imaging: Zebrafish embryos were provided by Nanjing EzeRinka Biotechnology Co., Ltd. Zebrafish were kept at 28 °C with a circadian cycle of 14 h of brightness and 10 h of darkness. Embryos were cultured in embryo media (EM; 150 mM NaCl, 0.5 mM KCl, 1.0 mM $CaCl_2$, 0.37 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 2.0 mM $MgSO_4$ and 0.71 mM $NaHCO_3$ in deionized water; pH 7.4). To avoid the interference of pigmentation toward confocal imaging, the EM were supplemented with 75 μM 1-phenyl 2-thiourea (PTU) for the generation of body transparency.

For confocal imaging, zebrafish embryos at different developing stages were incubated with EM containing Chol−PEG−Cy5 (2 μM) for 15 min. Then, the stained embryo was transferred to a 35 mm confocal dish and observed using an inverted confocal laser scanning microscope TCS SP8 (Leica, Germany) with a 10× objective under 638 nm excitation. During the process of confocal imaging, the embryo was anesthetized by 0.003% tricaine

methanesulfonate (MS222). To acquire three-dimensional (3D) imaging of the stained embryo, a sequence of optical sections (z-series) were collected by coordinating step-by-step changes in the fine focus of the microscope with sequential image acquisition at each step. The step size was predetermined as 4.0 μm and the programmed scanning distance varied according to the thickness of different specimens. Then, the collected z-series were processed into a 3D representation of the embryo using a Leica Application Suite X software platform. After confocal imaging, the embryo was transferred to fresh EM.

The staining performance of DiD and CellMask Green on zebrafish embryos was evaluated as follows: To begin with, embryos at hatching period were bathed in EM containing 1 μM DiD or 5 μg/mL CellMask Green. After incubation for 30 min or 3 h, the stained embryos were washed with fresh EM for three times, and then observed via confocal fluorescence imaging as described above.

To investigate the toxicity of nanomaterials on the epidermis of zebrafish, embryos at gastrula period (7 hpf) were cultured in EM containing various concentrations of Au NPs (2, 5, 10 and 20 μg/mL) or GO nanosheets (10, 20, 50 and 100 μg/mL) under standard culture conditions. Then, the treated embryos were collected at 72 hpf and washed with fresh EM for three times. For epidermis fluorescence imaging, the embryos were stained by Chol−PEG−Cy5 (2 μM) for 15 min and observed via confocal fluorescence imaging as described above.

Toxicity Evaluation on Zebrafish Embryos: Zebrafish embryos at gastrula period were randomly divided into several groups with different treatments and each group contained 7 embryos. For the control group, embryos were incubated with fresh EM (control) at 28 °C

with a circadian cycle of 14 h of brightness and 10 h of darkness. For experimental groups, embryos were treated with various concentrations of Au NPs (2, 5, 10 and 20 μg/mL) or GO nanosheets (10, 20, 50 and 100 μg/mL) at the same culture condition. Next, the survival rates of these embryos were recorded every day for a week.

Supporting figures

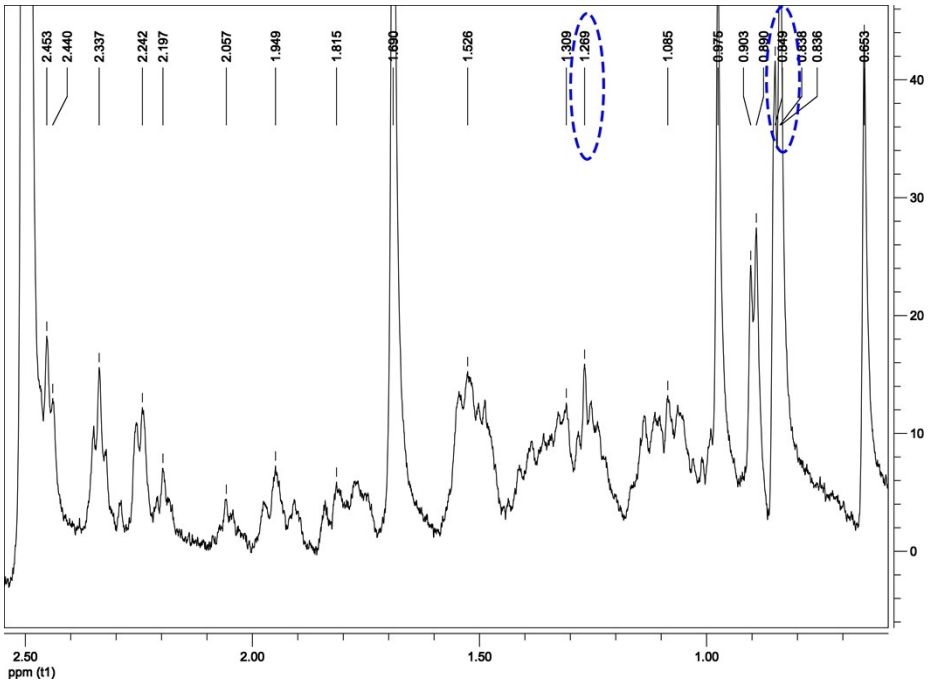

Fig. S1. Magnified ^{1}H NMR spectrum of Chol−PEG−Cy5 corresponding to Fig. 1b. The blue dotted circles indicate the proton peaks of the cholesterol moiety.

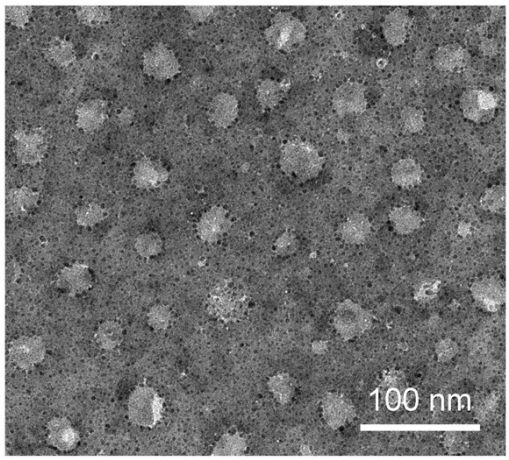

Fig. S2. TEM image of Chol−PEG−Cy5 (200 μg/mL) in a PBS solution.

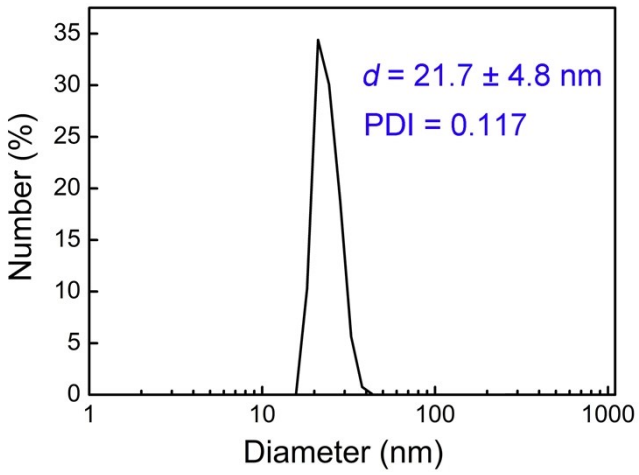

Fig. S3. Hydrodynamic size of Chol−PEG−Cy5 in PBS solution as measured by DLS.

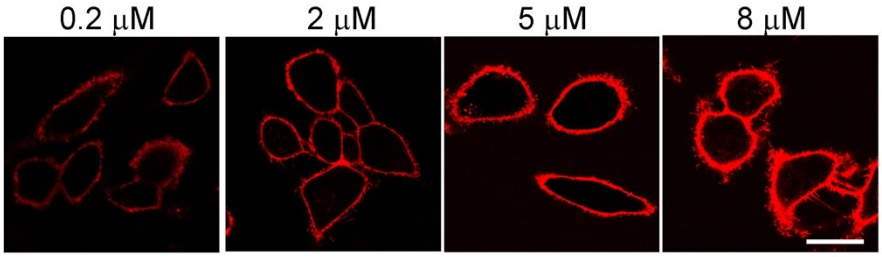

Fig. S4. Confocal fluorescence images of A549 cells stained with various concentrations of Chol−PEG−Cy5 for 10 min. Scale bar = 25 μm.

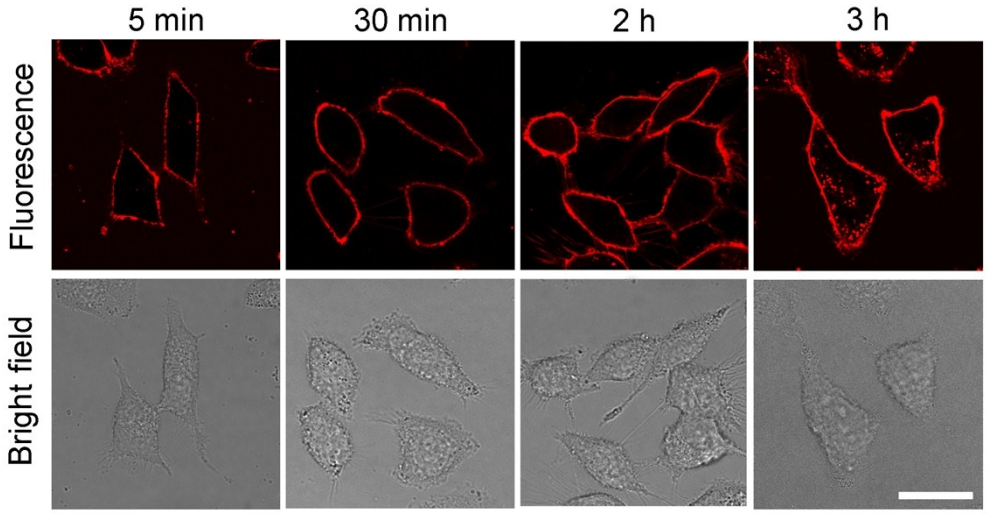

Fig. S5. Confocal images of A549 cells after incubation with Chol−PEG−Cy5 (2 μM) for

different time periods as indicated. Scale bar = 25 μm.

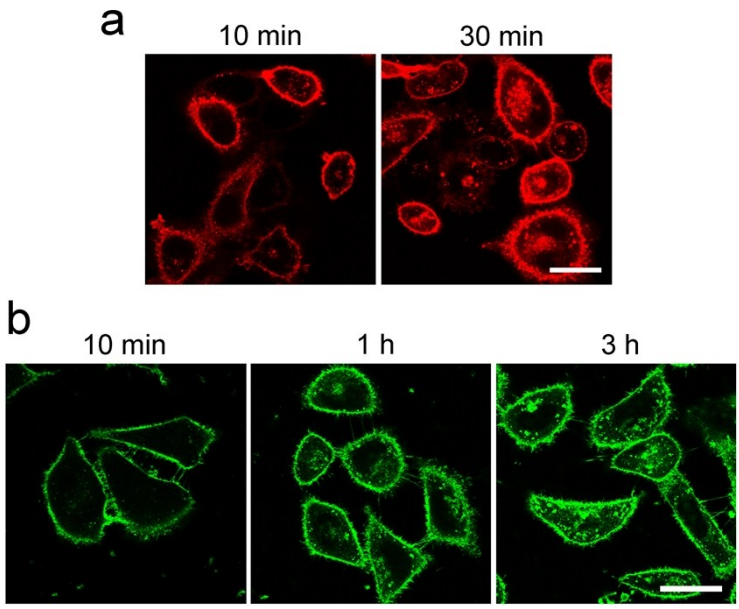

Fig. S6. Confocal fluorescence images of A549 cells after incubation with 1 μM DiD (a) or 5 μg/mL CellMask Green (b) for different time periods. Before imaging, the stained cells were washed by PBS solution for three times. Scale bars = 25 μm.

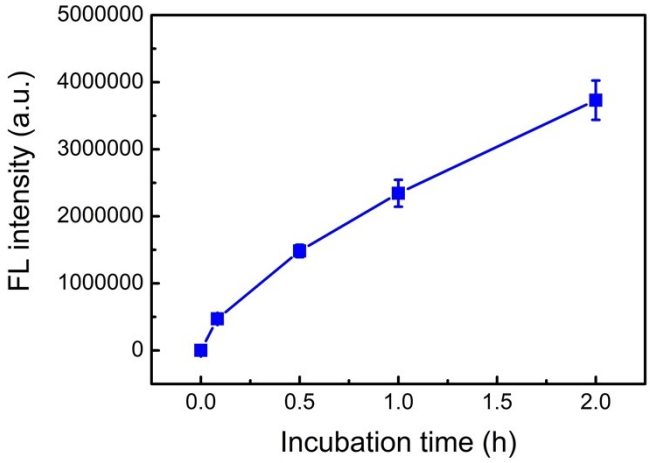

Fig. S7. Fluorescence intensities of A549 cells treated with Chol−PEG−Cy5 (2 μM) for different incubation time periods as measured by flow cytometry.

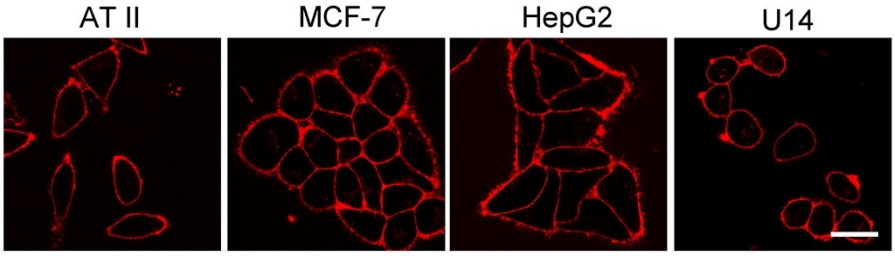

Fig. S8. Confocal fluorescence images of AT II, MCF-7, HepG2 and U14 cells stained with 2 μM Chol−PEG−Cy5 for 10 min. Scale bar = 25 μm.

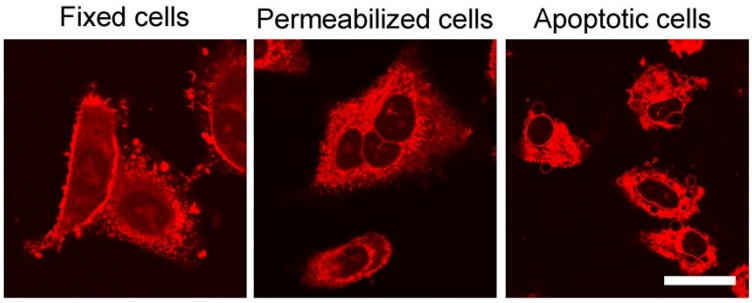

Fig. S9. Confocal fluorescence images of fixed, permeabilized and apoptotic cells after being stained with Chol−PEG−Cy5 (2 μM) for 10 min. Scale bar = 25 μm.

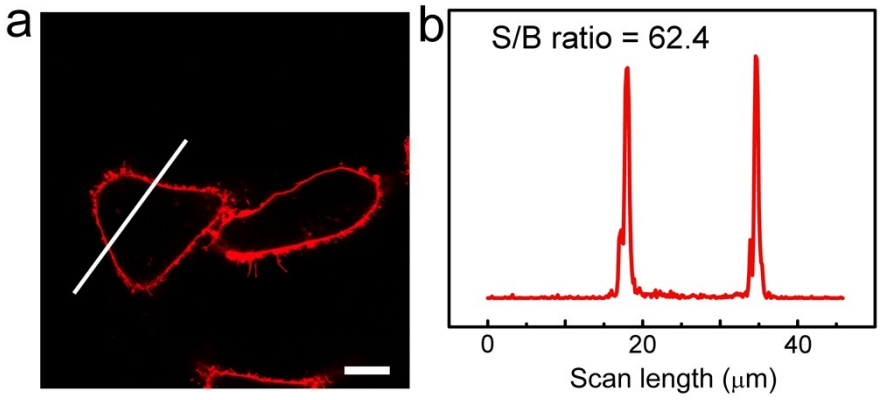

Fig. S10. (a) Confocal fluorescence image of Chol−PEG−Cy5-stained A549 cells. Before imaging, the cells were washed by PBS for three times. Scale bar = 10 μm. (b) Corresponding fluorescence intensity profile of the marked white line in (a).

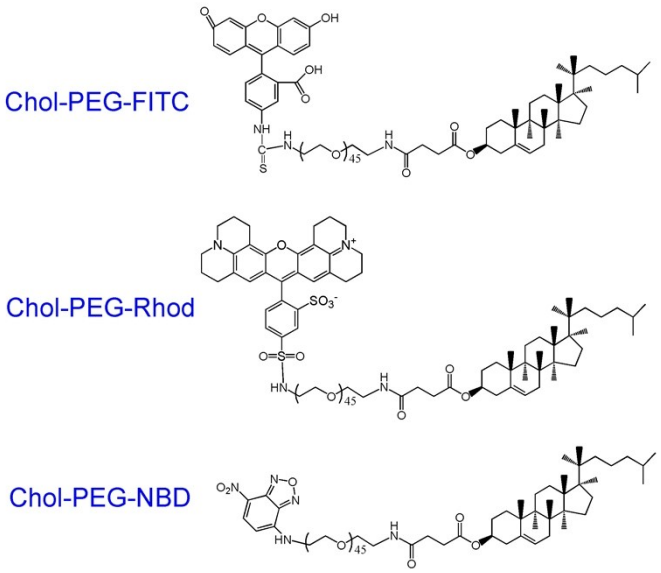

Fig. S11. Molecular structures of Chol−PEG−FITC, Chol−PEG−Rhod and Chol−PEG−NBD.

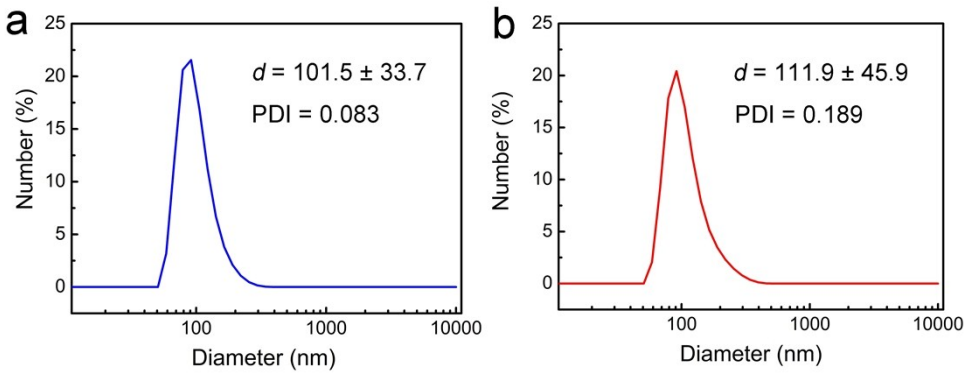

Fig. S12. Hydrodynamic sizes of POPC liposomes (200 μg/mL) before (a) and after (b) incubation with Chol−PEG−Cy5 (2 μM).

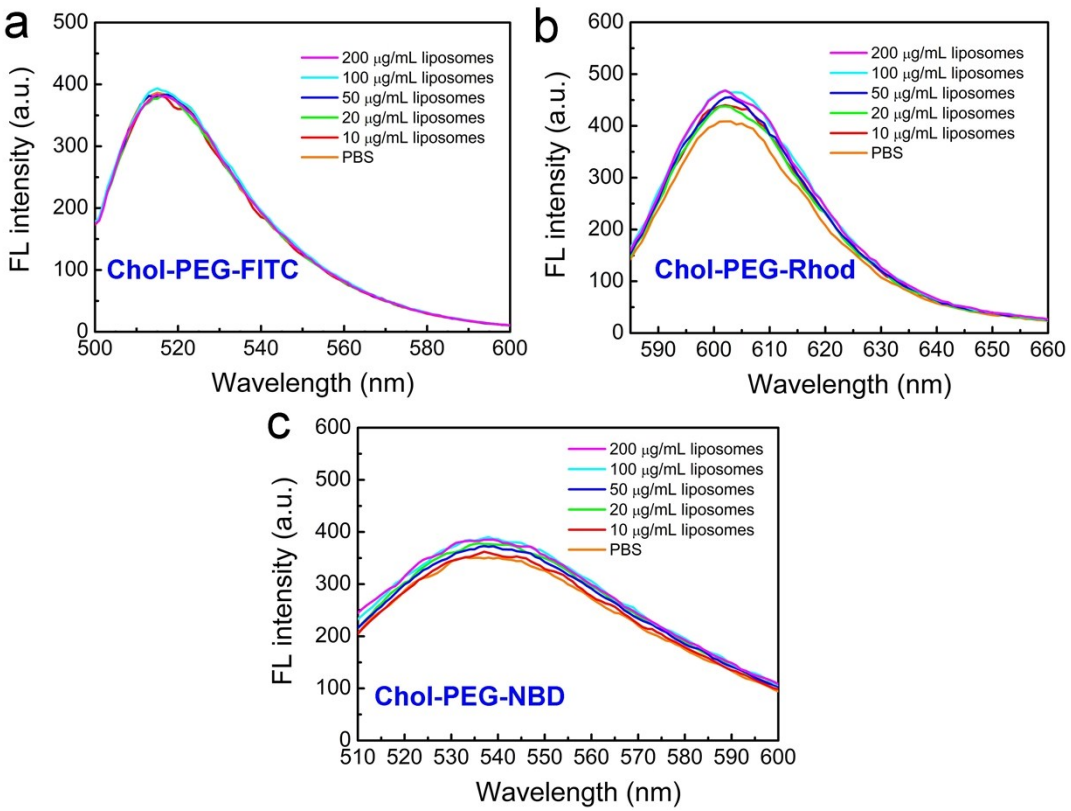

Fig S13. Fluorescence emission spectra of 2 μM Chol−PEG−FITC (a), Chol−PEG−Rhod (b) and Chol−PEG−NBD (c) in aqueous solutions containing different concentrations of POPC liposomes as indicated.

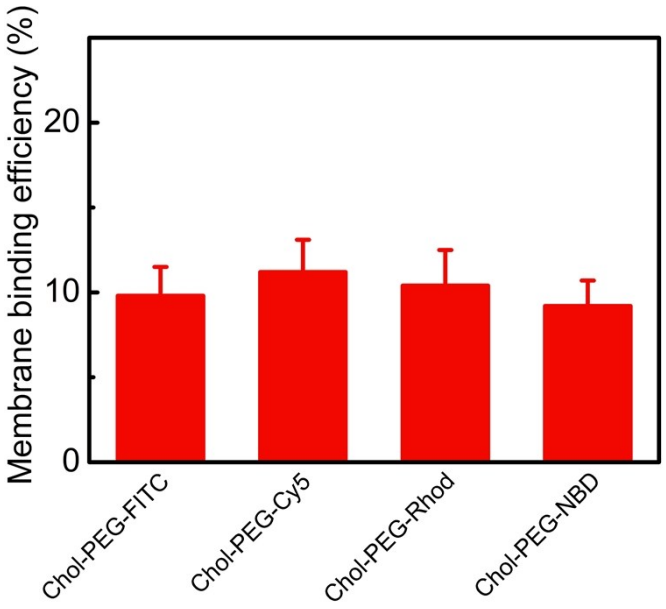

Fig. S14. Relative binding efficiencies of Chol−PEG−FITC, Chol−PEG−Cy5,

Chol−PEG−Rhod and Chol−PEG−NBD probes toward the plasma membranes of A549 cells.

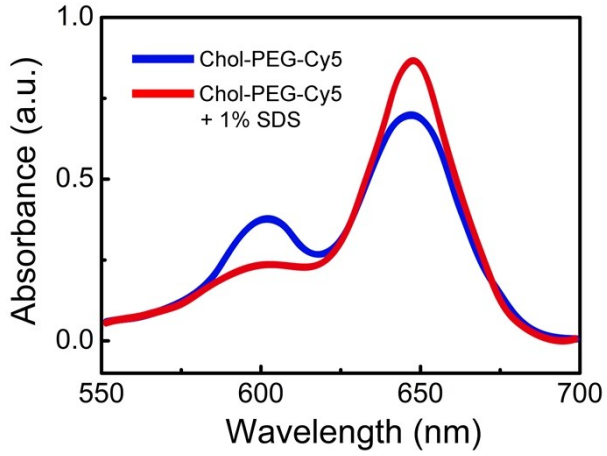

Fig. S15. UV–vis spectra of Chol−PEG−Cy5 (2 μM in PBS) in the absence and presence of 1% SDS.

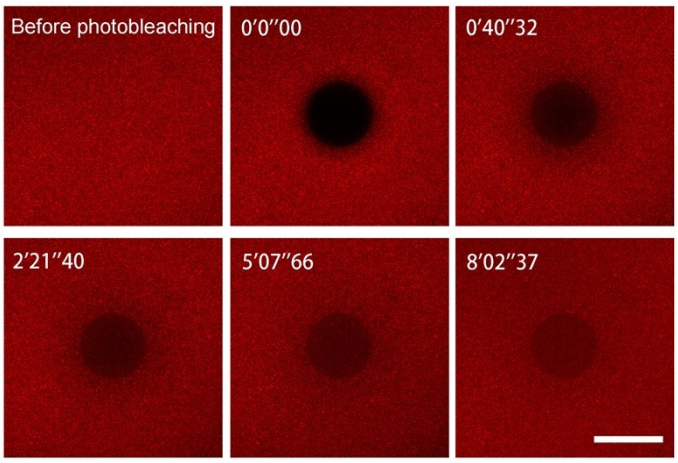

Fig. S16. Confocal fluorescence images of a Chol−PEG−Cy5-anchored supported lipid bilayer before and after photobleaching at different time points. The dark circular fields represent the photobleached ROIs. Scale bar = 25 μm.

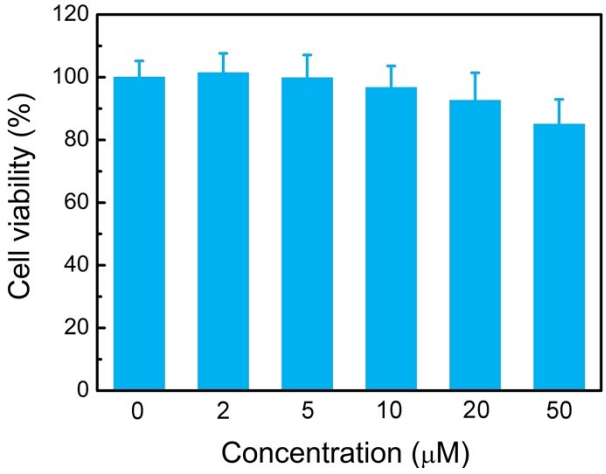

Fig. S17. Relative cell viabilities of AT II cells after incubation with different concentrations of Chol−PEG−Cy5 for 24 h.

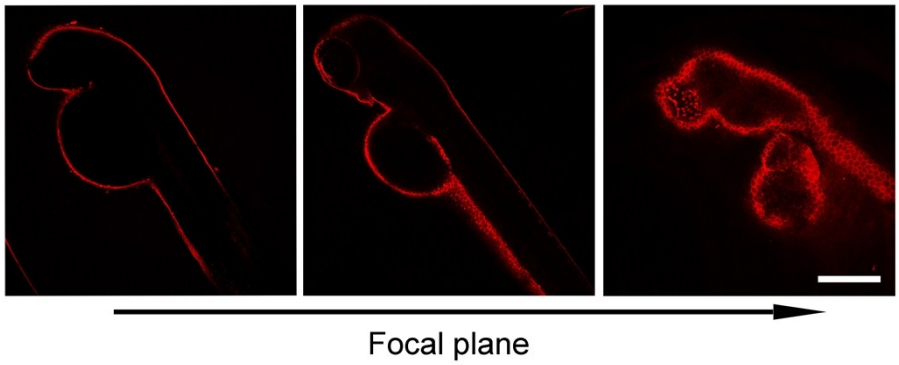

Fig. S18. Confocal fluorescence images of the Chol−PEG−Cy5 (2 μM)-stained zebrafish embryo at different focal planes. Scale bar = 250 μm.

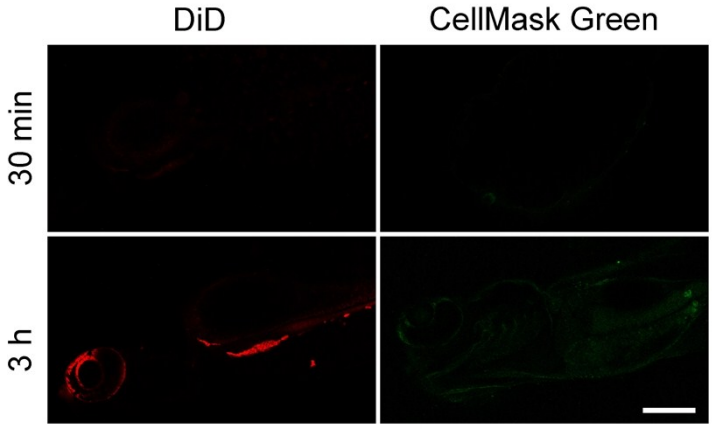

Fig. S19. Confocal fluorescence images of zebrafish embryos stained by DiD (1 μM) or CellMask Green (5 μg/mL) for different incubation periods as indicated. Scale bar = 250 μm.

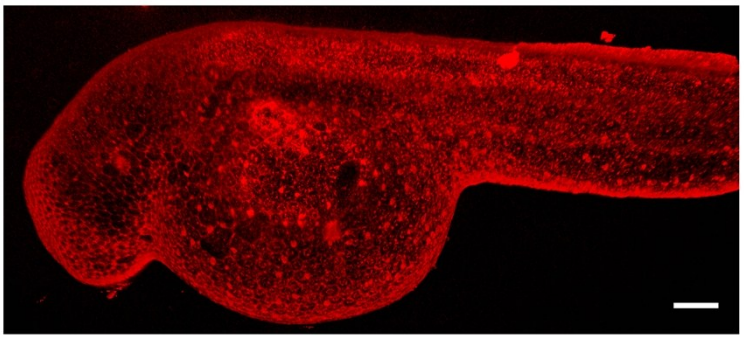

Fig. S20. Reconstructed 3D confocal fluorescence image of a zebrafish embryo after being stained by Chol−PEG−Cy5 (2 μM) for 4 h. Scale bar = 100 μm.

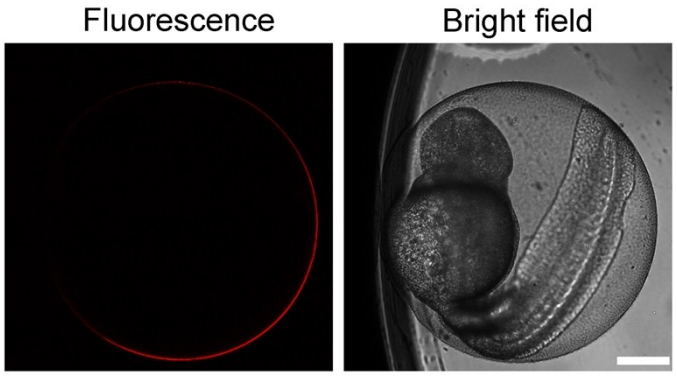

Fig. S21. Confocal images of unhatched embryo (24 hfp) stained by Chol−PEG−Cy5 (2 μM) for 15 min. Scale bar = 250 μm.

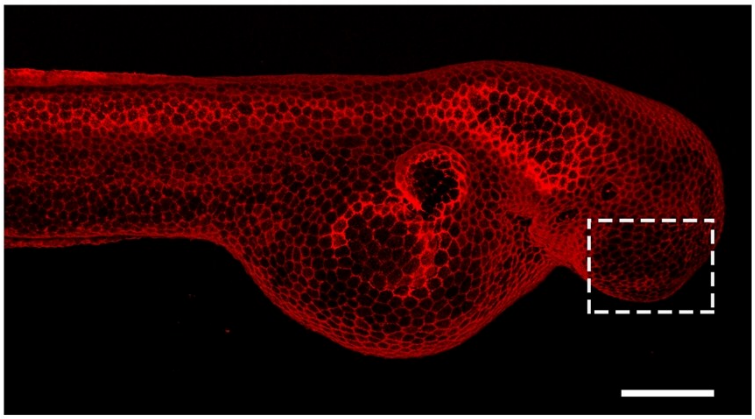

Fig. S22. Large-scale 3D confocal fluorescence image corresponding to Fig. 4b. The marked white rectangle represents the eye area. Scale bar = 200 μm.

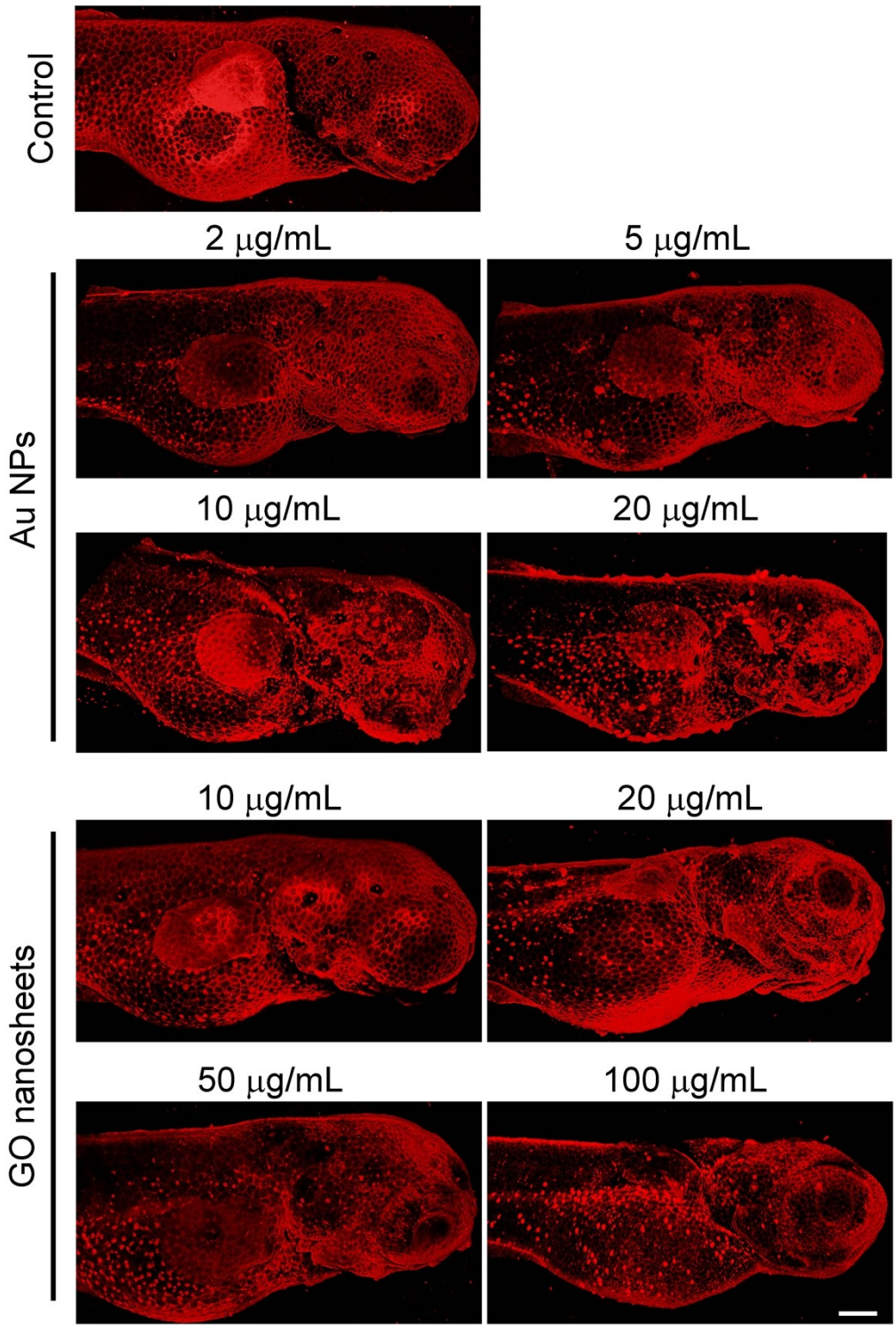

Fig. S23. Representative 3D confocal fluorescence images of zebrafish embryos (at 72 hpf) cultured in fresh EM (control) and EM containing various concentrations of Au NPs or GO nanosheets. Before imaging, the treated embryos were stained by Chol−PEG−Cy5 (2 μM) for 15 min to visualize the epidermis. Scale bar = 100 μm.

References

1 H. R. Jia, Y. X. Zhu, K. F. Xu, X. Y. Liu and F. G. Wu, *J. Controlled Release*, 2018, **286**, 103–113.

2 E. S. Wu, K. Jacobson and D. Papahadjopoulos, *Biochemistry*, 1977, **16**, 3936–3941.

3 H. R. Jia, Y. W. Jiang, Y. X. Zhu, Y. H. Li, H. Y. Wang, X. F. Han, Z. W. Yu, N. Gu, P. D. Liu, Z. Chen and F. G. Wu, *J. Controlled Release*, 2017, **255**, 231–241.