# Supplementary Information

# Highly fluorescent and bioresorbable polymeric nanoparticles with enhanced photostability for cell imaging

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**Supplementary Methods**

**Materials and reagents.** Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin /streptomycin mixture, phosphate buffered saline (PBS), Alexa Fluor® 633 phalloidin, TrypLE™ Express Enzyme (1×), Qtracker® 705 Cell Labeling Kit and PrestoBlue cell viability reagent were purchased from Life Technologies (Singapore). Other reagents including 4', 6-diamidino-2-phenylindole (DAPI), Bovine Serum Albumin (BSA), formalin solution were ordered from Sigma-Aldrich (Singapore). The synthetic details of DPPHT, PCL-DPP-PCL, PCL-QA-PCL and the polymer NPs are described in Supporting Information.

**General materials characterization**. Scanning electron microscopy (SEM) was performed on a field emission scanning electron microscopy JSM 6701F (JEOL, Japan) operated at 10 kV. Transmission electron microscope (TEM) measurements were carried out on a JEM-3010 (JEOL, Japan) electron microscope operating at an acceleration voltage of 120 kV. UV-vis transmission spectrum was recorded on a UV-2450 (SHIMADZU, Japan) spectrophotometer. Photoluminescence spectra were recorded with LS 55 (PerkinElmer, USA) fluorescence spectrometer. The particle size was measured with a dynamic light scattering (DLS) instrument, Zetasizer Nano ZS (Malvern, UK).

**Cell culture.** HuH-7 Cell Line (Hepato cellular carcinoma cells) were obtained from National Cancer Center (Singapore) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1% penicillin

/streptomycin mixture at 37 °C with 5% $CO_2$. Mesenchymal stem cells (MSCs) were isolated from porcine bone marrow aspirates following institutional guidelines, which demonstrated good purity and stability[33]. MSCs were also cultured in DMEM with 10% FBS and a penicillin (100 U/ml)/ streptomycin (100 μg/mL) mixture at 37 °C with 5% $CO_2$.

***In vitro* cellular uptake.** In order to explore the cellular uptake of PCL-DPP-PCL NPs and physically blended PCL/DPP NPs, HuH-7 or MSCs were seeded on Petri dish and cultured in 37 °C with 5% $CO_2$. After 12 h for attachment, NPs were added to the medium (0.2 mg/mL) and incubated with cells at 37 °C with 5% $CO_2$. After 24 h, excess NPs were washed with 1×PBS for three times. After that, cells were fixed by formalin solution for 20 min and then washed by 1×PBS extensively for three times. Then cells were permeabilized with 0.1% (vol/vol) Triton X-100 in 1×PBS for 5 minutes at room temperature. After washing twice by 1×PBS, cells were blocked for 30 min in 1×PBS containing 1% (wt/vol) BSA. Then Alexa Fluor® 633 phalloidin in 1×PBS was added to stain filamentous actin (F-actin) cytoskeleton for 1 h at room temperature. After washing three times, nucleus was stained by DAPI for 1 min at room temperature. Then samples were washed three times and then added in fresh 1×PBS. Lasers of 405, 561, and 633 nm were used to excite DAPI, NPs, and Alexa Fluor® 633 phalloidin, respectively. The corresponding fluorescence emissions were recorded through a confocal microscope (LSM 780, Carl Zeiss, Germany) using a band-pass filter combination including 421-481 nm, 569-621 nm, and 650-758 nm for imaging in three individual channels (Objective: LD Plan-Neofluar 20x/0.4 Korr M27). In order to observe cellular uptake of PCL-QA-PCL NPs, similar procedures were followed except that the lasers

of 405, 488, and 633 nm were used to excite DAPI, PCL-QA-PCL NPs, and Alexa Fluor® 633 phalloidin, respectively. The corresponding fluorescence emissions were recorded through a confocal microscope (LSM 780, Carl Zeiss, Germany) using a band-pass filter combination including 421-481 nm, 508-534 nm, and 650-758 nm for imaging in three individual channels (Objective: LD Plan-Neofluar 20x/0.4 Korr M27).

**Photobleach assay.** In order to perform the photobleach test of PCL-DPP-PCL NPs and physically blended PCL/DPP NPs, HuH-7 or MSCs were seeded on a Petri dish and cultured in 37 °C with 5% CO2. After 12 h for attachment, NPs were added to the medium and incubated with cells at 37 °C with 5% CO2. After 24 h, excess NPs were washed with 1×PBS for three times. After that, cells were fixed by formalin solution for 20 min and then washed by 1×PBS extensively for three times. Then cells were permeabilized with 0.1% (vol/vol) Triton X-100 in 1×PBS for 5 minutes at room temperature. After washing twice by 1×PBS, cells were blocked for 30 min in 1×PBS containing 1% (wt/vol) BSA. Then Alexa Fluor® 633 phalloidin in 1×PBS was added to stain filamentous actin (F-actin) cytoskeleton for 1 h at room temperature. After washing three times, nucleus was stained by DAPI for 1 min at room temperature. Then samples were washed three times and then added in fresh 1×PBS. After being multiply-stained with a cocktail of three dyes, both MSCs and HuH-7 were continuously exposed to 405 nm, 561 nm, and 633 nm laser irradiation with a higher intensity than that in cellular uptake assays and the fluorescence emissions from each dye were recorded at specific time points (Objective: LD Plan-Neofluar 20x/0.4 Korr M27). The nominal laser powers were calculated by multiplying the maximum laser power by the

percentage of intensity output during photobleaching. In Figure 3 and S5, nominal laser powers of 405 nm, 561 nm, and 633 nm were 20 mW, 20 mW, and 5 mW, respectively. For the photobleach test of PCL-QA-PCL NPs, 405 nm, 488 nm, and 633 nm lasers were used (Objective: LD Plan-Neofluar 20x/0.4 Korr M27). In Figure S6, nominal laser powers of 405 nm, 488 nm, and 633 nm were 25 mW, 25 mW, and 5 mW, respectively. Images were obtained in every scans and bleaches were repeated after every scans. The time in second indicated on images was the moments for scanning.

**Long term cell tracing.** HuH-7 tumor cells and MSC were seeded in a 6-well plate and cultured at 37 °C with 5% $CO_2$. After 12 h for attachment, 2 nM PCL-DPP-PCL NPs and Qtracker® 705 were added, respectively. After incubation for 48 h, the culture medium was removed and cells were washed three times by 1×PBS to remove excess NPs. Then the cells were detached by TrypLE Express and subcultured into another 6-well plate. After a number of days (Day 1, 3, 5, 7 for HuH-7 and Day 1, 7, 14 for MSC), cells were imaged by fluorescent microscopy (Olympus IX71, excited at 530-550 nm, emission filter 575IF). Then the cells were washed with 1×PBS and detached by TrypLE Express. After centrifugation (500 g, 5 min), supernatant were discarded and cell pellets were collected and resuspended in 1×PBS for flow cytometry (LSRII, BD Biosciences, Singapore). The fluorescence of PCL-DPP-PCL NPs and Qtracker® 705 were detected using PE (excitation: 561 nm, emission: 567 nm-597 nm) and PE-cy5 (excitation: 561 nm, emission: 660 nm-760 nm) channels, respectively. The cells without any fluorescence labeling were used as control. The flow cytometry data were analyzed using FlowJo software.

***In vitro*** **cytotoxicity.** PrestoBlue assay was used to evaluate the effect of different concentrations of PCL-DPP-PCL NPs on cell viability in HuH-7 and MSCs. Both cells were seeded on a 96-well plate (10000 cells per well) and cultured at 37 °C with 5% $CO_2$. After 12 h for attachment, PCL-DPP-PCL NPs at four indicated concentrations were added to the medium. After incubation for 24 and 72 h, respectively, the culture medium was removed and cells were washed three times by 1×PBS to remove excess NPs. Then, PrestoBlue reagent diluted by DMEM were added to each wells and incubated at 37 °C with 5% $CO_2$. At the same time, PrestoBlue reagents diluted by DMEM were also added to blank wells without cells as control. After 1 h incubation, supernatant in each wells were transferred into another new 96-well plate in order to reduce the absorbance background of NPs. The absorbance at 571 nm (reference wavelength is 600 nm) was detected by Plate Reader (Tecan Infinite M200 series Pro, Tecan Asia, Singapore). Percentage reduction of PrestoBlue reagent of each sample was calculated according to the following formula provided in manufacturer's protocol.

% Reduction of PrestoBlue Reagent = $[(117216\times A_1) - (80586\times A_2)] / [(155677\times N_2) - (14652\times N_1)] \times 100$, where $A_1$ is absorbance of test wells at 570 nm, $A_2$ is absorbance of test wells at 600 nm, $N_1$ is absorbance of media only wells at 570 nm, and $N_2$ is absorbance of media only wells at 600 nm. Cells without treatment by NPs were used as control and corresponding cell viability was set as 100%. Cell viability treated with a specific concentration of NPs = (% reduction of PrestoBlue reagent at this concentration) / (% reduction of PrestoBlue reagent of cells not treated by NPs). Statistics analysis of different

groups was conducted by the paired Student's t-test using online calculator (http://www.physics.csbsju.edu/stats/t-test.html). All experiments were repeated in three times. p value of <0.05 was considered to be statistically significant.

***2,5-bis(6-hydroxyhexyl)-3,6-di(thiophen-2-yl)pyrrolo[3,4-c]pyrrole-1,4(2H,5H)-dione* (DPPHT).** Potassium carbonate (8.28 g, 60 mmol) and DPP-monomers (*3,6-di(thiophen-2-yl)pyrrolo[3,4-c]pyrrole-1,4(2H,5H)-dione)* (6 g, 20 mmol) were mixed in 200 mL of dimethylformamide under stirring in a 250 mL three-neck round bottom flask under nitrogen protection. The mixture was heated up to 140 °C followed by addition of 6-bromo-1-hexanol (10.88 g, 60 mmol) dissolved in 25 mL of dimethylformamide dropwise over 1 hour. The reaction mixture was stirred at 140 °C overnight. Then the flask was allowed to cool to room temperature and rinsed with chloroform. The product was washed by saturated brine, and extracted by dichloromethane twice. The organic phase was dried with $MgSO_4$. The crude product was further purified by silica chromatography (dichloromethane: tertahydrofuran = 1:1) to obtain dark purple solid DPPHT (1.02 g, 10%)

**Synthesis of PCL-DPP-PCL and PCL-QA-PCL.** The DPP or QA initiator were added into a sealed Schlenk tube with nitrogen protection, and dissolved in anhydrous toluene under magnetic stirring. Stannous octoate as the catalyst was injected through the rubber seal with a syringe. ε-Caprolactone was injected into the reaction mixture. The mixture was degassed with $N_2$-bubbling before the Schlenk tube was submerged in an oil bath and the reaction

mixture was heated up to 110 °C and kept at this temperature overnight. Crude product was purified by dissolved in tetrahydrofuran and precipitated in cold methanol twice.

**General procedure for the synthesis of the PCL-DPP-PCL nanoparticles**. In a typical procedure, 6 mL of PCL-DPP-PCL (3 mg/mL in THF) was injected into 30 mL of vigorously stirred solution of Pluronic® 127 (8.3 g/L in water) at room temperature. After being stirred for 3 mins, the sample was left for stabilization for about 2 hours. Then the sample was subjected to dialysis against di-water using a 4 kDa membrane. The resulting NPs were separated by centrifugation at 11,500 g for 15 mins, and the sediments were redispersed in di-water before characterization or dispersed in 1×PBS buffer for cell experiments.

**Supplementary Figures**

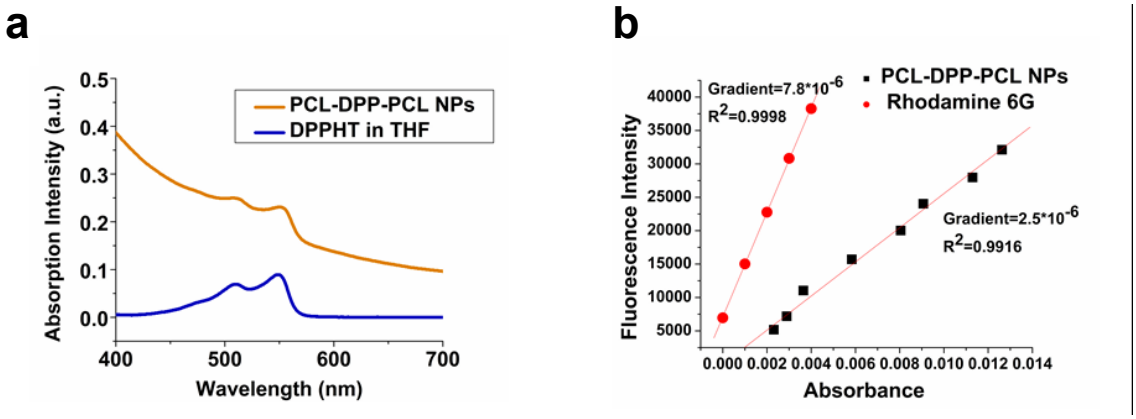
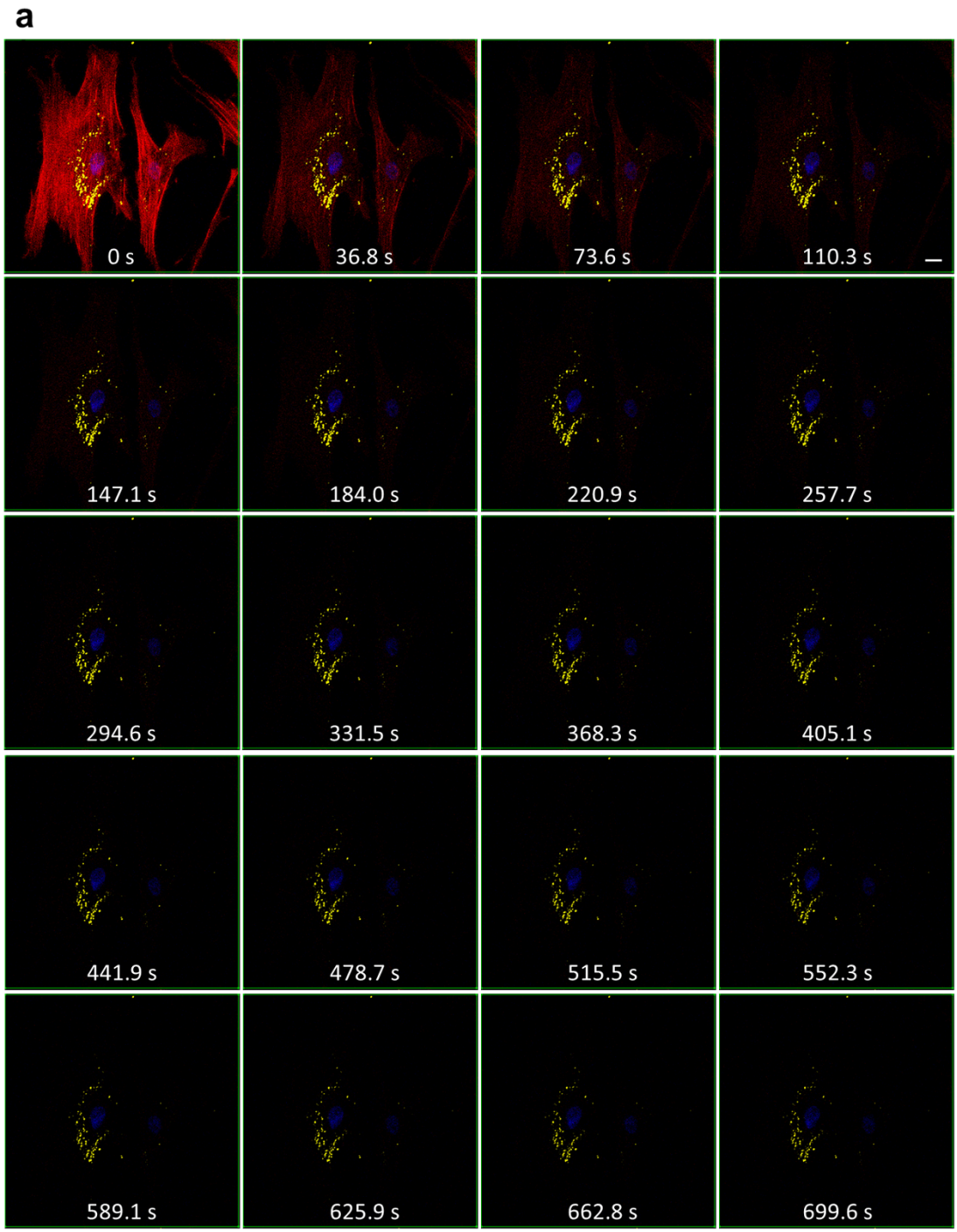



**Figure S1.** (**a**) Absorbance spectra of PCL-DPP-PCL NPs and DPPHT in THF and (**b**) linear plots for standard samples and PCL-DPP-PCL NPs.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184.0 s
220.9 s
257.7 s
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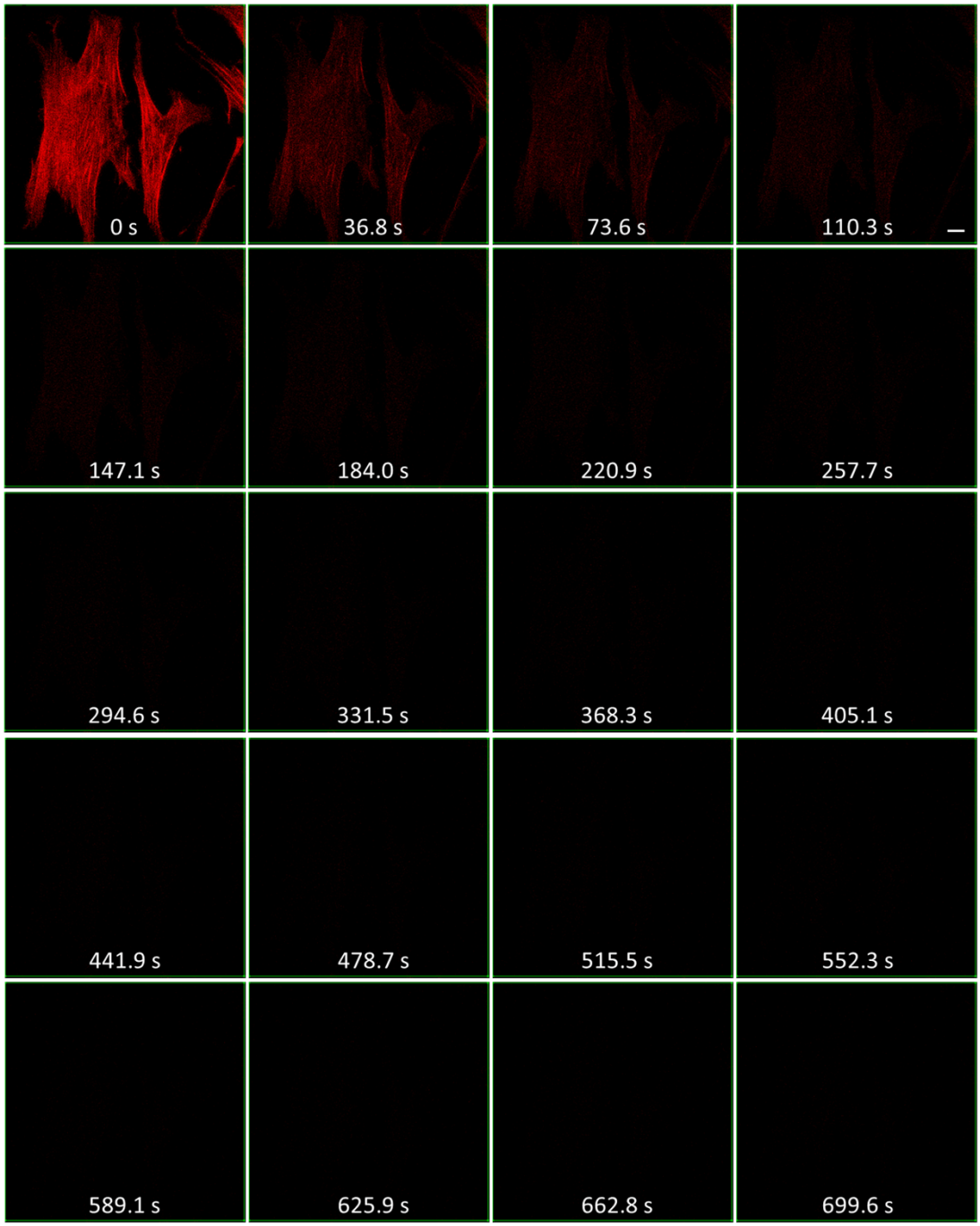
**b**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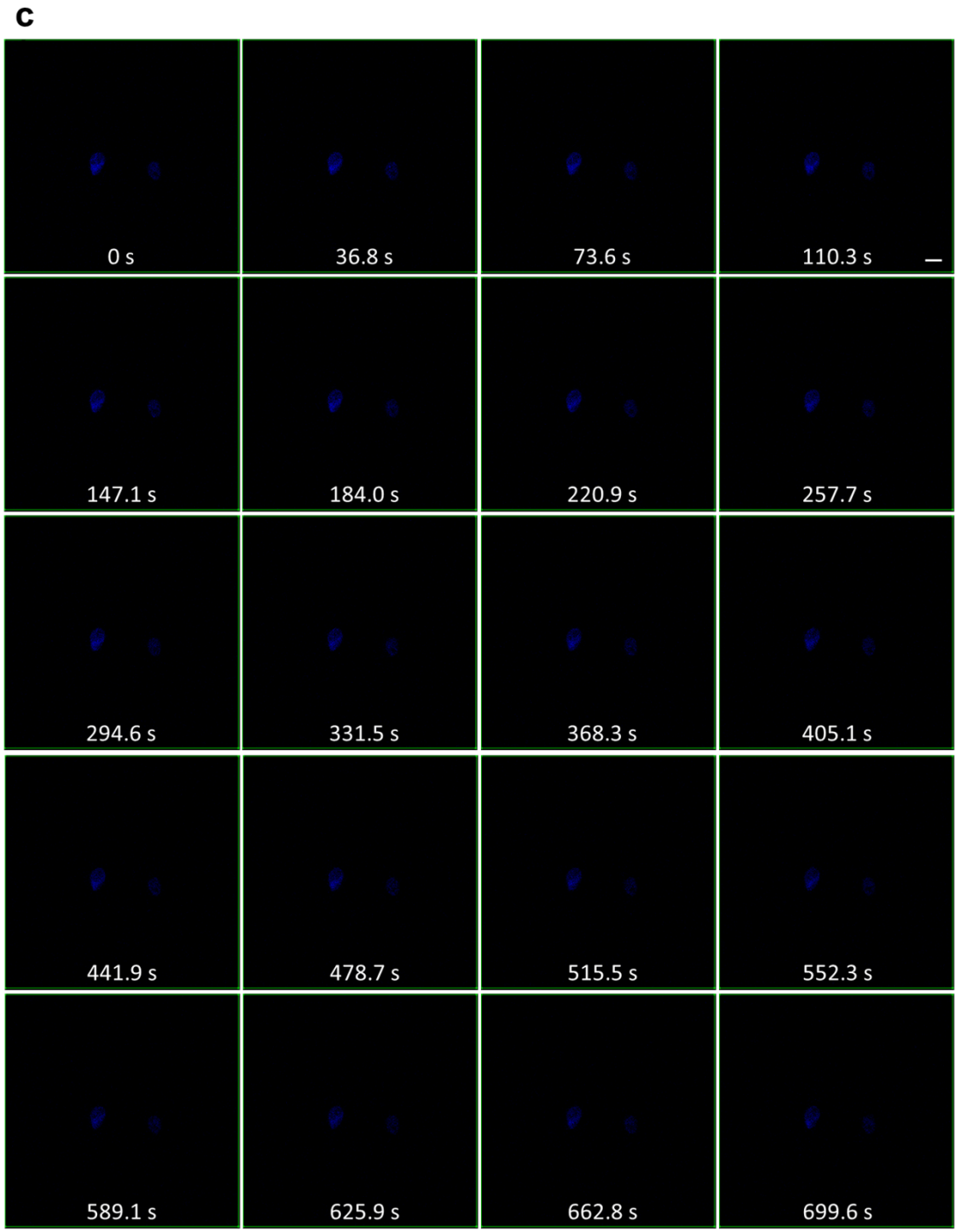
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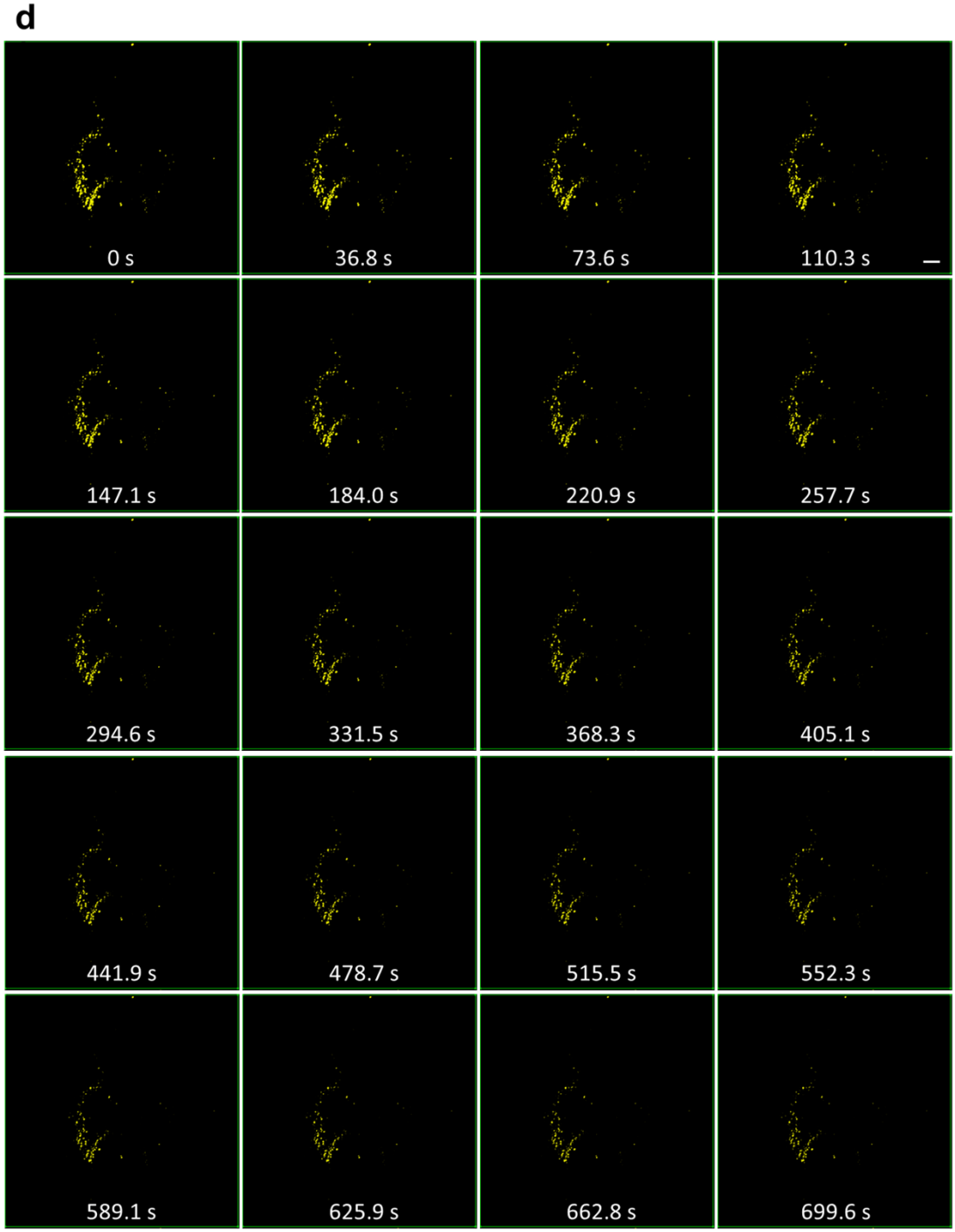


**Figure S2.** More detailed chronological decay (at 53 s intervals) of fluorescence intensity of merged images (**a**) and those from three individual channels: (**b**) Alexa Fluor® 633 phalloidin, (**c**) DAPI and (**d**) NPs. The fluorescences of DAPI, NPs, and Alexa Fluor® 633 phalloidin are pseudo-labeled with blue, yellow, and red, respectively. Scale bar: 20 μm.

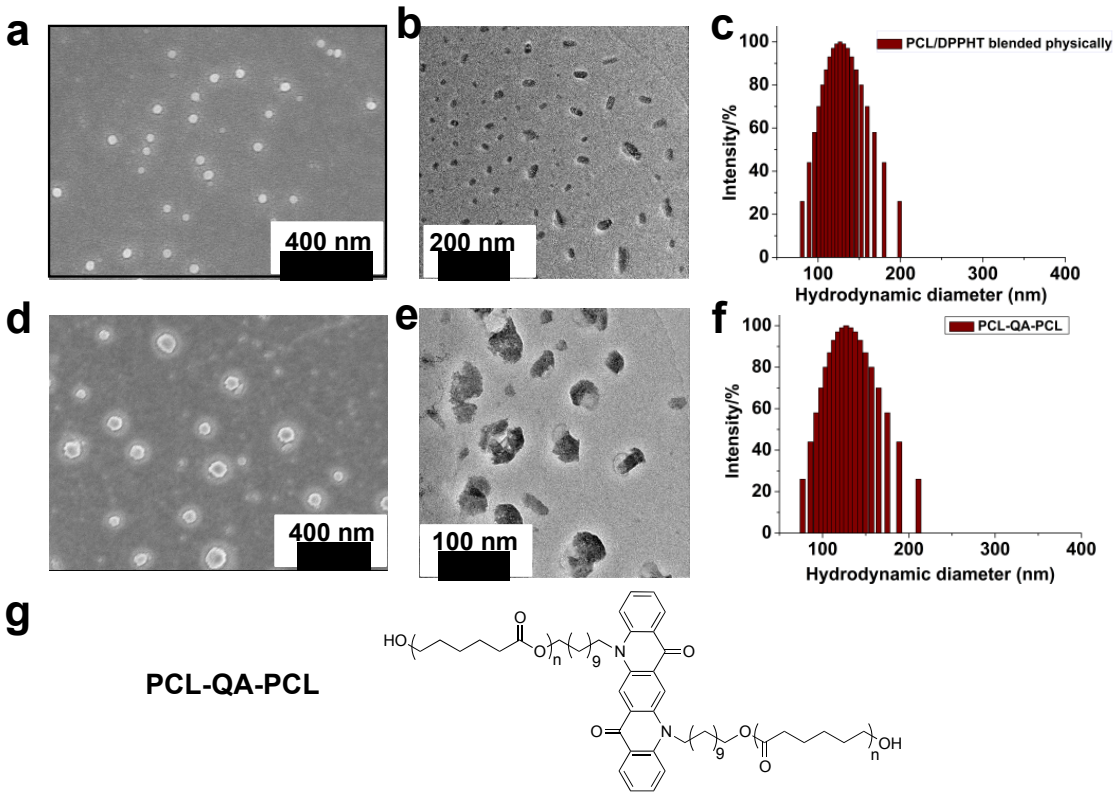


**Figure S3**. SEM (**a, d**), TEM (**b, e**) and DLS (**c, f**) characterization of physically blended DPPHT/PCL NPs (**a-c**) and PCL-QA-PCL NPs (**d-f**). (**g**) The chemical structure of PCL-QA-PCL.

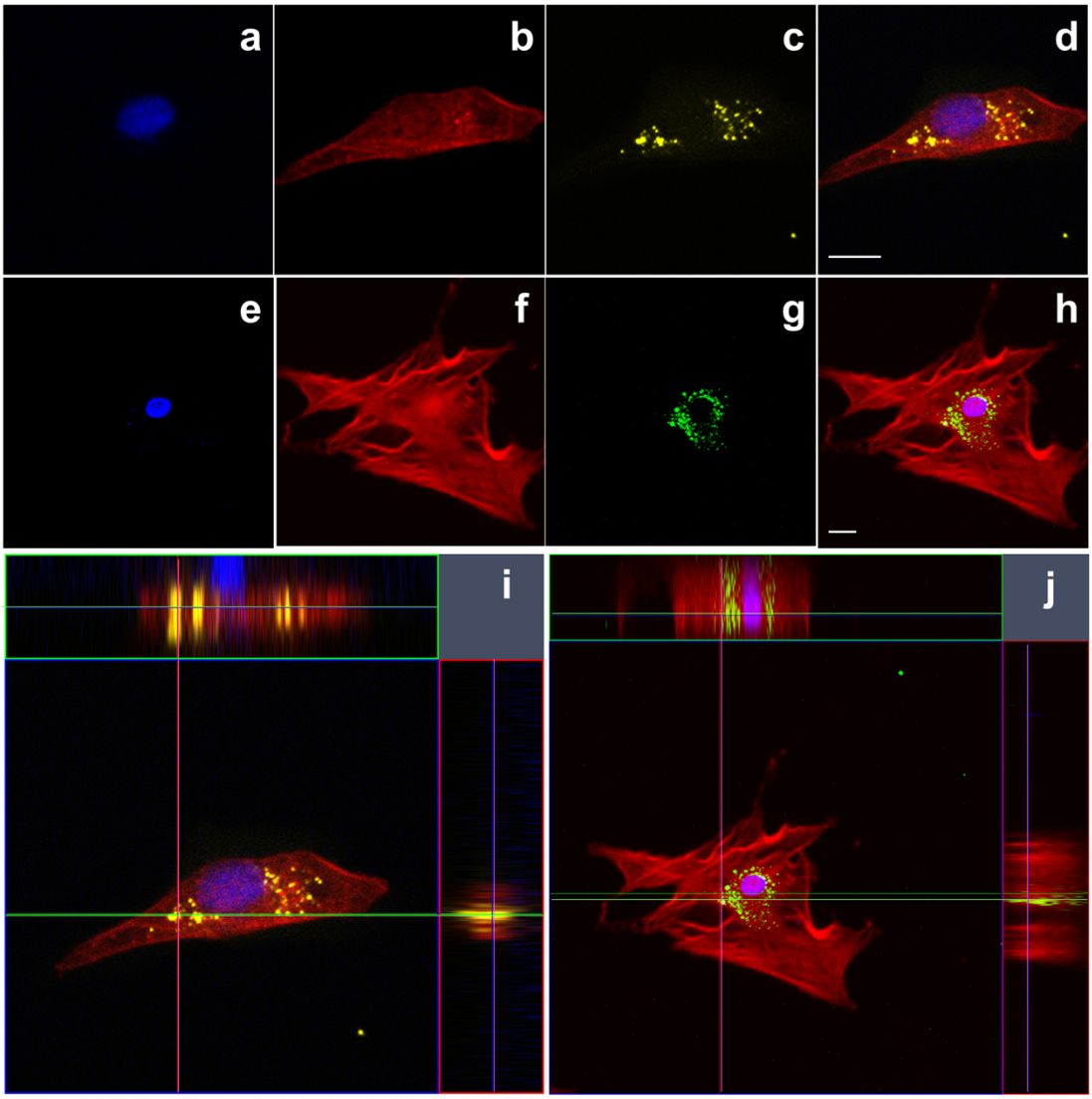


**Figure S4.** Cellular uptake of physically blended PCL/DPPHT NPs (**a-d and i**) and PCL-QA-PCL nanoparticles (**e-h and j**) in MSCs imaged by confocal laser scanning microscopy. The fluorescence of DAPI, Alexa Fluor® 633 phalloidin, physically blended PCL/DPPHT NPs, and PCL-QA-PCL nanoparticles are pseudo-labeled with blue (**a, e**), red (**b, f**), yellow (**c**), and green (**g**), respectively. Merged images of MSCs from different channels are shown in (**d**) and (**h**), respectively. (**i**) and (**j**) are ortho-view images of z-stack, showing they could be uptaken by the cells. Scale bars: 20 μm. (For a-d and i, pixel dwell: 1.27 μs, frame size: 512×512; for e-h, and j, pixel dwell: 0.63 μs, frame size: 1040×1040).

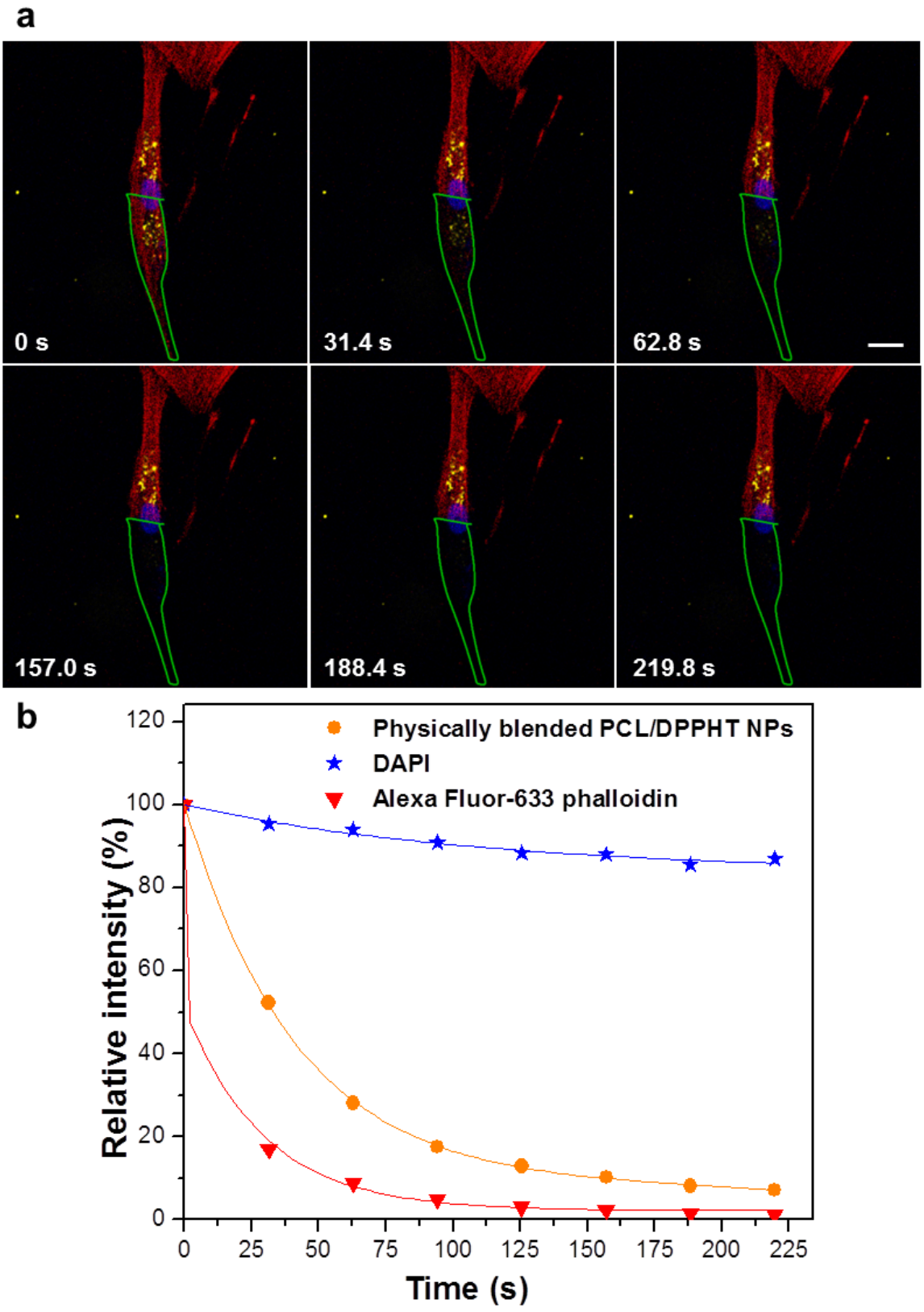


**Figure S5.** Photostability of physically blended PCL/DPPHT NPs compared to Alexa Fluor® 633 phalloidin and DAPI in a MSC. The fluorescence of DAPI, physically blended

PCL/DPPHT NPs, and Alexa Fluor® 633 phalloidin are pseudo-labeled with blue, yellow and red, respectively. (**a**) Chronological decay of fluorescence intensity of a multiply-stained MSC at different time points imaged by confocal laser scanning microscopy. The green polygon indicates the region photobleached. Pixel dwell time: 1.27 μs; frame size: 512×542; pixel size: 0.37 μm. Scale bar: 20 μm. (**b**) Relative intensity (instantaneous intensity / initial intensity) change of three fluorophores in the photobleached region. Curves are fitted using mono-exponential (DAPI) or bi-exponential functions (physically blended PCL/DPPHT NPs and Alexa Fluor® 633 phalloidin). The nominal powers of 405 nm, 561 nm, and 633 nm lasers were 20 mW, 20 mW, and 5 mW, respectively.

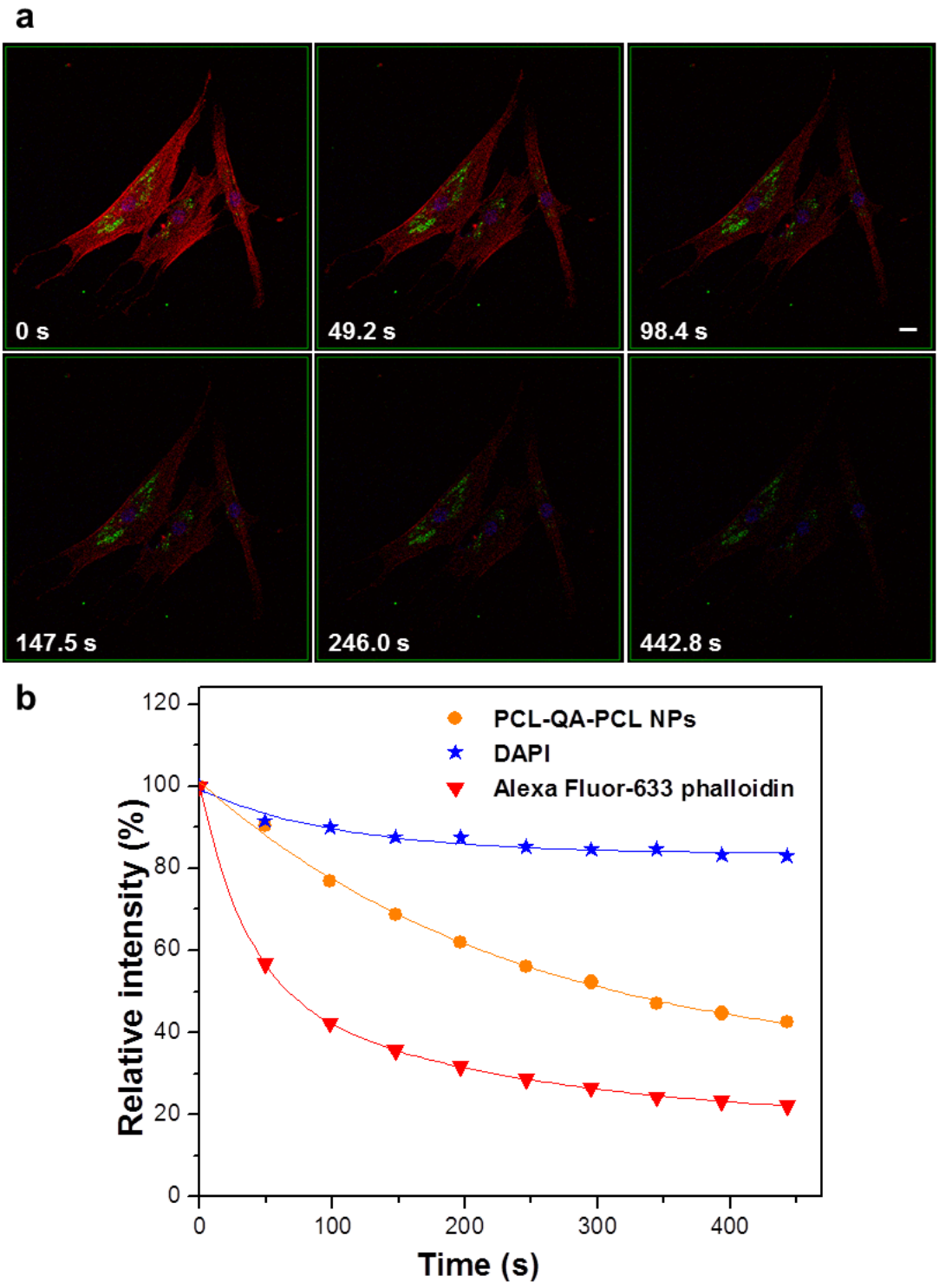


**Figure S6.** Photostability of PCL-QA-PCL NPs compared to Alexa Fluor® 633 phalloidin and DAPI in a MSC. The fluorescence of DAPI, PCL-QA-PCL NPs, and Alexa Fluor® 633 phalloidin are pseudo-labeled with blue, green and red, respectively. (**a**) Chronological decay of fluorescence intensity of a multiply-stained MSC at different time points imaged by

confocal laser scanning microscopy. The whole image field was photobleached. Pixel dwell time: 0.64 μs; frame size: 1024×1024; pixel size: 0.42 μm. Scale bar: 20 μm. (**b**) Relative intensity (instantaneous intensity / initial intensity) change of three fluorophores in the photobleached region. Curves are fitted using mono-exponential (DAPI) or bi-exponential functions (PCL-QA-PCL NPs and Alexa Fluor® 633 phalloidin). The nominal powers of 405 nm, 488 nm, and 633 nm lasers were 25 mW, 25 mW, and 5 mW, respectively.

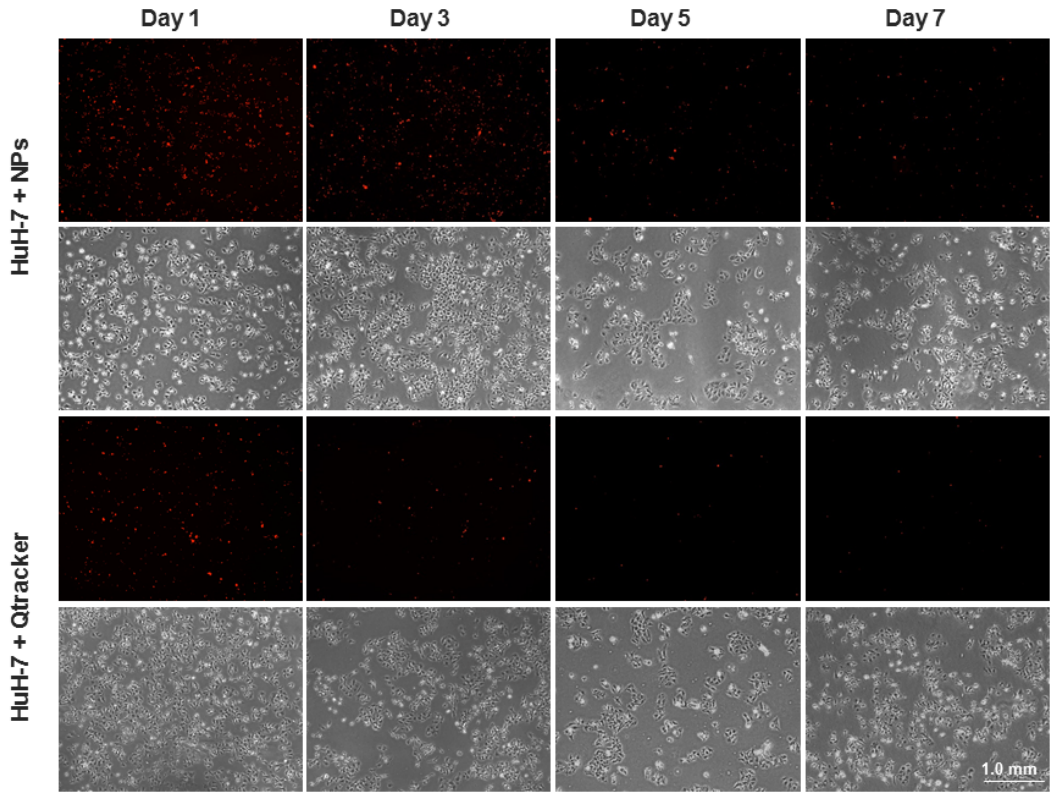


**Figure S7.** Long-term tracing of HuH-7 tumor cells using PCL-DPP-PCL NPs and Qtracker®. The fluorescence intensity change of the labeled cells is characterized by optical contrast and fluorescence microscopy measured at different time points.

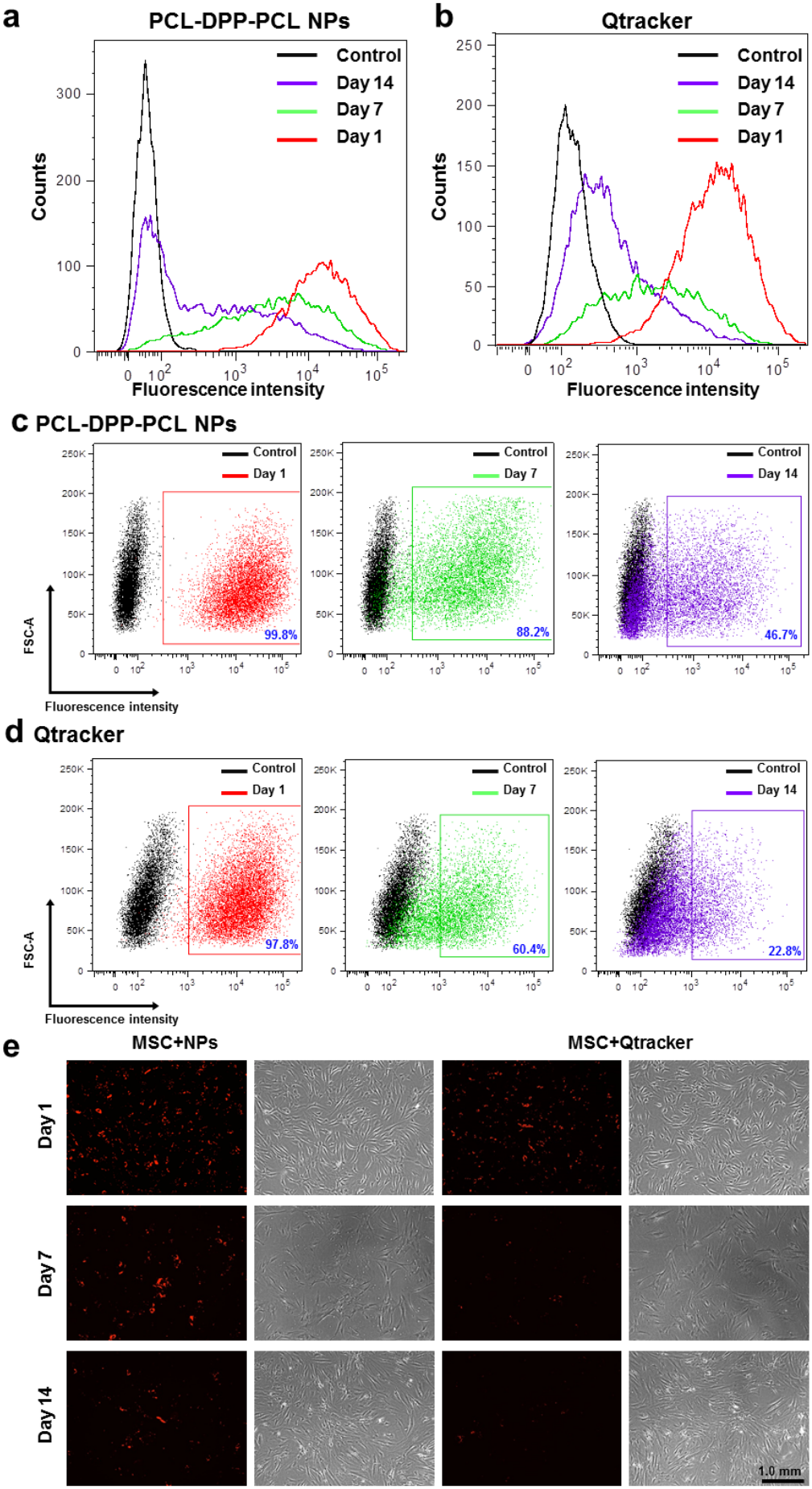
a
PCL-DPP-PCL NPs
Control
Day 14
Day 7
Day 1
Counts
Fluorescence intensity
b
Qtracker
Control
Day 14
Day 7
Day 1
Counts
Fluorescence intensity
c PCL-DPP-PCL NPs
Control
Day 1
99.8%
Control
Day 7
88.2%
Control
Day 14
46.7%
FSC-A
Fluorescence intensity
d Qtracker
Control
Day 1
97.8%
Control
Day 7
60.4%
Control
Day 14
22.8%
FSC-A
Fluorescence intensity
e
MSC+NPs
MSC+Qtracker
Day 1
Day 7
Day 14
1.0 mm

**Figure S8.** Long-term tracing of MSCs using PCL-DPP-PCL NPs and Qtracker®. The fluorescence intensity change of the labeled cells is characterized by (**a**) flow cytometry histograms with PCL-DPP-PCL NPs, (**b**) flow cytometry histograms with Qtracker, (**c**) flow cytometry dot plots with PCL-DPP-PCL NPs, (**d**) flow cytometry dot plots with Qtracker, (**e**) optical contrast and fluorescence microscopy measured at different time points.

## Supplementary Tables

Table S1. Parameters for fitting relative intensity change of PCL-DPP-PCL NPs in MSC (Figure 3b) using $y=A_1*\exp(-t/\tau_1)+y_0$

| | Value | Standard Error |
|---|---|---|
| $y_0$ | 84.99 | 0.32 |
| $A_1$ | 14.97 | 0.28 |
| Mean lifetime $\tau_1$(s) | 331.82 | 17.28 |
| Adj. R-square | 0.9967 | |
| Half-life ($t_{1/2}=\ln2*\tau_1$, s) | 230.00 | |

Table S2. Parameters for fitting relative intensity change of DAPI in MSC (Figure 3b) using $y= A_1*\exp(-t/\tau_1)+y_0$

| | Value | Standard Error |
|---|---|---|
| $y_0$ | 79.64 | 0.80 |
| $A_1$ | 17.17 | 1.09 |
| Mean lifetime $\tau_1$ (s) | 190.62 | 32.56 |
| Adj. R-square | 0.9288 | |
| Half-life ($t_{1/2}^*=\ln2*\tau_1$, s) | 132.13 | |

Table S3. Parameters for fitting relative intensity change of Alex Fluor® 633 in MSC (Figure 3b) using $y=A_1*\exp(-t/\tau_1)+A_2*\exp(-t/\tau_2)+y_0$

| | Value | Standard Error |
|---|---|---|
| $y_0$ | 2.80 | 0.06 |
| $A_1$ | 56.34 | 1.27 |
| Mean lifetime $\tau_1$ (s) | 14.28 | 0.68 |
| $A_2$ | 40.86 | 1.23 |
| Mean lifetime $\tau_2$ (s) | 84.56 | 2.02 |
| Adj. R-square | 0.9999 | |
| Half-life ($t_{1/2}^*=\ln2*\tau_2$, s) | 58.61 s | |

Note: In this bi-exponential model, the half-life ($t_{1/2}^*$) was calculated by $\ln2*\tau_2$.

## Supplementary Videos

Supplementary video 1
Photostability of fluorescent PCL-DPP-PCL nanoparticles (NPs) compared to Alexa Fluor® 633 phalloidin and DAPI in a mesenchymal stem cell (MSC) (Figure 3). This movie shows the merged time-lapse images of a multiply-stained MSC with internalized PCL-DPP-PCL NPs after continuous exposure with laser irradiation by confocal microscopy (DAPI: blue; NPs: yellow; Alexa Fluor® 633 phalloidin: red).

Supplementary video 2
Photostability of fluorescent PCL-DPP-PCL nanoparticles (NPs) compared to Alexa Fluor® 633 phalloidin and DAPI in a mesenchymal stem cell (MSC) (Figure 3). This movie shows the time-lapse images of individual channels (DAPI: blue; NPs: yellow; Alexa Fluor® 633 phalloidin: red).