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Photon-upconversion barcode for monitoring an enzymatic reaction with a fluorescence reporter in droplet microfluidics

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1 Chemicals

$YCl_3 \times 6\ H_2O$ (99.99%), $YbCl_3 \times 6\ H_2O$ (99.99%), $TmCl_3 \times 6\ H_2O$ (99.99%), $ErCl_3 \times 6\ H_2O$ (99.995%), Ho_2O_3 (99.999%), NH_4F (>98%), 1-octadecene (90%), oleic acid (90%), tetraethylorthosilicate (TEOS, ≥ 99%), 4-methylumbelliferyl β-D-galactopyranoside (≥99%), β-Galactosidase from Escherichia coli (powder, ~140 U/mg), 4-Methylumbelliferone (≥98%) and Igepal CO-520 were purchased from Sigma-Aldrich (www.sigmaaldrich.com), and used as starting materials without further purification. NaOH (p.a.), cyclohexane (p.a.), N,N-Dimethylformamide (p.a.), Methanol (p.a.), Acetone (p.a.), HCl 35% (p.a.) and NH_4OH 25% (p.a.) were purchased from PENTA (Czech Republic) and carboxyethylsilanetriol, sodium salt; 25% (w/v) in water was obtained from ABCR GmbH (www.abcr.de).

2 Apparatus

Transmission electron microscopy (TEM) images were taken with JEM2100F (JEOL, Tokyo, Japan; www.jeol.com) using a 200 kV electron beam. Dynamic light scattering measurement was performed on a Zetasizer (Malvern Instruments, www.malvern.com). The emission spectra from microfluidic chips were recorded by CCD array spectrometer Ocean Optics QE65 Pro (www.oceanoptics.com) connected by optical fiber (100 µm diameter).

3 Nanoparticle synthesis

3.1 UCNP1: $NaY_{0.80}Yb_{0.18}Er_{0.02}F_4$ [1,2]

$YCl_3 \times 6\ H_2O$ (1165 mg, 3.84 mmol), $YbCl_3 \times 6\ H_2O$ (335 mg, 0.864 mmol) and $ErCl_3 \times 6\ H_2O$ (36.6 mg, 0.096 mmol) were dissolved in methyl alcohol (20 mL) and added into a 100-mL three-neck round-bottom flask containing oleic acid (10.8 g) and 1-octadecene (23.6 g). The solution was heated to 160 °C for 30 min under an N_2 atmosphere and then cooled to 50 °C. Then, the protective atmosphere was disconnected, and the solution of NH_4F (711 mg, 19.2 mmol) and NaOH (480 mg, 12 mmol) in methyl alcohol (20 mL) was added to the intensively stirred solution. The N_2 atmosphere was reconnected, and the solution was stirred for 30 min. The temperature was carefully increased up to 150 °C, avoiding extensive boiling to ensure the evaporation of methyl alcohol. After that, the solution was rapidly heated using the rate of 10 °C per minute. At 300 °C, the heating was carefully regulated to 305 °C within two or three minutes. The flask was kept under N_2 flow at 305 °C for 150 min. The fluctuation of temperature was ± 3 °C during this time. Finally, the flask was placed on another stirrer and rapidly cooled to room temperature under air flux. The resulting nanoparticles were precipitated by adding isopropyl alcohol (40 mL) and collected by centrifugation (1,000 × *g*, 10 min). The pellet was washed with methyl alcohol (40 mL), centrifuged (1,000 × *g*, 10 min), and dispersed in cyclohexane (20 mL). By adding methyl alcohol (100 mL), the nanoparticles precipitated rapidly without the need for centrifugation. The precipitate was dispersed in cyclohexane (30 mL) and slowly centrifuged (50 × *g*, 20 min) to separate coarse particles from the final product.

3.2 UCNP2: $NaY_{0.80}Yb_{0.18}Ho_{0.02}F_4/NaYF_4$ core/shell [1,2]

Step 1: core nanoparticles $NaY_{0.80}Yb_{0.18}Ho_{0.02}F_4$.

$YCl_3 \times 6\ H_2O$ (971 mg, 3.2 mmol), $YbCl_3 \times 6\ H_2O$ (279 mg, 0.72 mmol) and $HoCl_3$ (0.08 mmol, prepared by dissolving Ho_2O_3 – 0.04 mmol, 15.1 mg – in 15% HCl) were dissolved in methyl alcohol (20 mL) and added into a 100-mL three-neck round-bottom flask containing oleic acid (9 g) and 1-octadecene (19.7 g). The solution was heated to 160 °C for 30 min under an N_2 atmosphere and then cooled to 50 °C. Then, the protective atmosphere was disconnected, and the solution of NH_4F (593 mg, 16 mmol) and NaOH (400 mg, 10 mmol) in methyl alcohol (20 mL) was added to the intensively stirred solution. The N_2 atmosphere was reconnected, and the solution was stirred for 30 min. The temperature was carefully increased up to 150 °C, avoiding extensive boiling to ensure the evaporation of methyl alcohol. After that, the solution was rapidly heated using the rate of 10 °C per minute. At 300 °C, the heating was carefully regulated to 305 °C within two or three minutes. The flask was kept under N_2 flow at 305 °C for 150 min. The fluctuation of temperature was ± 3 °C during this time. Finally, the flask was placed on another stirrer and rapidly cooled to room temperature under air flux. The resulting nanoparticles were precipitated by adding isopropyl alcohol (40 mL) and collected by

centrifugation (1,000 × *g*, 10 min). The pellet was washed with methyl alcohol (40 mL), centrifuged (1,000 × *g*, 10 min), and dispersed in cyclohexane (20 mL). By adding methyl alcohol (100 mL), the nanoparticles precipitated rapidly without the need for centrifugation. The precipitate was dispersed in cyclohexane (30 mL) and slowly centrifuged (50 × *g*, 20 min) to separate coarse particles from the final product.

Step 2: core/shell $NaY_{0.80}Yb_{0.18}Ho_{0.02}F_4/NaYF_4$.

$YCl_3 \times 6\ H_2O$ (607 mg, 2.0 mmol) was dissolved in methyl alcohol (10 mL) and added into a 100-mL three-neck round-bottom flask containing oleic acid (9 g) and 1-octadecene (19.7 g). The solution was heated to 160 °C for 30 min under an N_2 atmosphere and then cooled to 50 °C. Then, the protective atmosphere was disconnected, and the solutions of (A) NH_4F (296 mg, 8 mmol), NaOH (200 mg, 5 mmol) in methyl alcohol (20 mL), and (B) 310 mg of core nanoparticles $NaY_{0.80}Yb_{0.18}Ho_{0.02}F_4$ in cyclohexane (10 mL) were added to the intensively stirred solution. The N_2 atmosphere was reconnected, and the solution was stirred for 30 min. The temperature was carefully increased up to 150 °C, avoiding extensive boiling to ensure the evaporation of methyl alcohol. After that, the solution was rapidly heated using the rate of 10 °C per minute. At 300 °C, the heating was carefully regulated to 305 °C within two or three minutes. The flask was kept under N_2 flow at 305 °C for 90 min. The fluctuation of temperature was ± 3 °C during this time. Finally, the flask was placed on another stirrer and rapidly cooled to room temperature under air flux. The resulting nanoparticles were precipitated by adding isopropyl alcohol (40 mL) and collected by centrifugation (1,000 × *g*, 10 min). The pellet was washed with methyl alcohol (40 mL), centrifuged (1,000 × *g*, 10 min), and dispersed in cyclohexane (20 mL). By adding methyl alcohol (100 mL), the nanoparticles precipitated rapidly without the need for centrifugation. The precipitate was dispersed in cyclohexane (20 mL) and slowly centrifuged (50 × *g*, 20 min) to separate coarse particles from the final product.

3.3 UCNPRef: $NaY_{0.80}Yb_{0.18}Tm_{0.02}F_4$ [1,2]

$YCl_3 \times 6\ H_2O$ (971 mg, 3.2 mmol), $YbCl_3 \times 6\ H_2O$ (279 mg, 0.72 mmol) and $TmCl_3 \times 6\ H_2O$ (30.7 mg, 0.08 mmol) were dissolved in methyl alcohol (20 mL) and added into a 100-mL three-neck round-bottom flask containing oleic acid (9 g) and 1-octadecene (19.7 g). The solution was heated to 160 °C for 30 min under an N_2 atmosphere and then cooled to 50 °C. Then, the protective atmosphere was disconnected, and the solution of NH_4F (593 mg, 16.0 mmol) and NaOH (400 mg, 10 mmol) in methyl alcohol (20 mL) was added to the intensively stirred solution. The N_2 atmosphere was reconnected, and the solution was stirred for 30 min. The temperature was carefully increased up to 150 °C, avoiding extensive boiling to ensure the evaporation of methyl alcohol. After that, the solution was rapidly heated using the rate of ~10 °C per minute. At 300 °C, the heating was carefully regulated to 305 °C within two or three minutes. The flask was kept under N_2 flow at 305 °C for 150 min. The fluctuation of temperature was ± 3 °C during this time. Finally, the flask was placed on another stirrer and rapidly cooled to room temperature under air flux. The resulting nanoparticles were precipitated by adding isopropyl alcohol (30 mL) and collected by centrifugation (1,000 × *g*, 10 min). The pellet was washed with methyl alcohol (30 mL), centrifuged (1,000 × *g*, 10 min), and dispersed in cyclohexane (20 mL). By adding methyl alcohol (100 mL), the nanoparticles precipitated rapidly without the need for centrifugation. The precipitate was dispersed in cyclohexane (30 mL) and slowly centrifuged (50 × *g*, 20 min) to separate coarse particles from the final product.

3.4 Nanoparticle coating with a carboxylated silica shell

UCNPs were coated with a carboxylated silica shell using a microemulsion technique.[1,3] The protocol was adjusted to account for different sizes of UCNPs (as measured by DLS in cyclohexane dispersion), as shown in Table S1. UCNPs were diluted in cyclohexane with Igepal CO-520 and tetraethyl orthosilicate and stirred with high intensity for 10 min. The microemulsion was created after adding an aqueous ammonium hydroxide 12 % (w/v). The resulting mixture was slowly stirred for 18 hours. Another volume of tetraethyl orthosilicate was added, and the microemulsion was slowly stirred for 4 hours. Carboxyethylsilanetriol sodium salt (25 % w/v in water) was added, and the cloudy emulsion was sonicated for 15 min and further stirred for 60 min. Carboxylated UCNPs were

extracted by adding dimethylformamide and washed five times with acetone and three times with water. Carboxylated UCNPs were stored at laboratory temperature as aqueous dispersions.

Table S1. Silica coating of photon-upconversion nanoparticles.

	UCNP1	UCNP2	UCNPRef
UCNPs	10 mg	10 mg	10 mg
Igepal CO-520	412 mg	241 mg	359 mg
Cyclohexane (including UP dispersion volume)	5.23 mL	3.06 mL	4.55 mL
Tetraethyl orthosilicate (the first addition)	23.0 µL	13.4 µL	20 µL
NH_4OH 12% (w/v)	25.3 µL	14.8 µL	22 µL
Tetraethyl orthosilicate (the second addition)	5.8 µL	3.4 µL	5 µL
Carboxyethylsilanetriol, sodium salt; 25% (w/v) in water	11.6 µL	6.8 µL	10 µL

4 Nanoparticle characterization

4.1 Estimation of UCNP mass concentration

For estimating UCNP mass concentrations, the volume of 200 µL of nanoparticle dispersion in water or cyclohexane was dispensed into glass vials. The vials were placed first on a heater to evaporate dispersant and then for 3 hours in an oven (450 °C). Estimated UP masses were used for estimating mass concentrations.

4.2 Transmission electron microscopy (TEM)

Copper grids coated with formvar were first modified by adsorption of cBSA. A drop of 6 µL of cBSA in water (1 mg mL^{-1}) was placed on the grid and incubated at room temperature for 5 min. After 10 washing steps with 10 µL of water, a 6-µL drop of the nanomaterial dispersion was deposited on the grid surface and incubated for 5 min at room temperature. To facilitate the adsorption on a cBSA-coated surface, nanomaterials were diluted in 10 mM MES with pH 6.1 set by NaOH. Finally, the grid was washed 10 times with 10 µL of water. Dry grids were imaged using a TEM microscope.

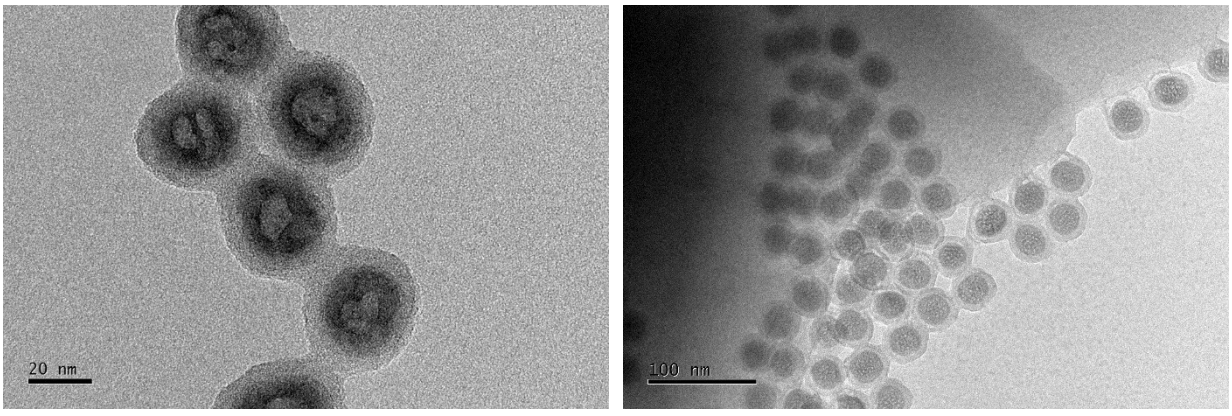

Figure S1. TEM images of UCNP1. The diameter of UCNP1 without the silica shell was 27.1 ± 1.5 nm (RSD 5.7%). The thickness of the carboxylated silica shell was 5.9 ± 0.8 nm (RSD 14%).

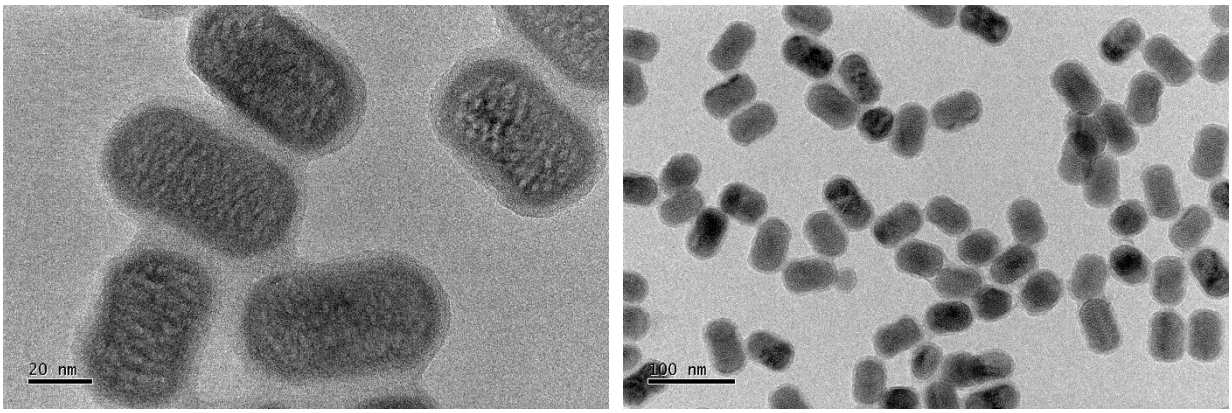

Figure S2. TEM images of UCNP2. The length of UCNP2 without the silica shell was 54.1 ± 4.3 nm (RSD 8.0%). The length of UCNP2 without the silica shell was 33.7 ± 1.8 nm (RSD 5.3%). The thickness of the carboxylated silica shell was 3.9 ± 0.6 nm (RSD 16%).

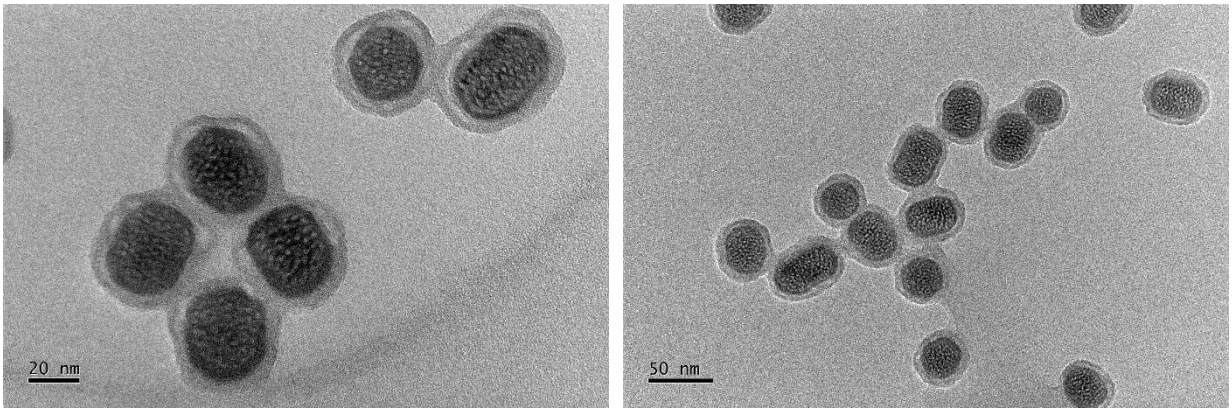

Figure S3. TEM images of UCNPRef. The length of UCNPRef without the silica shell was 39.0 ± 3.5 nm (RSD 9.1%). The length of UCNP2 without the silica shell was 29.0 ± 1.8 nm (RSD 6.1%). The thickness of the carboxylated silica shell was 5.3 ± 0.9 nm (RSD 17%).

4.3 Dynamic light scattering

Hydrodynamic diameters were estimated by dynamic light scattering. Dynamic light scattering was measured in a quartz cuvette with a 10 mm optical path on a Zetasizer (Malvern Instruments). The volume of dispersion was 1000 µL and sample mass concentration was 0.5 mg mL^{-1} in water or cyclohexane.

Table S2. Hydrodynamic diameters of UCNPs.

	Oleic acid-coated in cyclohexane		Carboxylated silica-coated in water	
	Hydrodynamic diameter (nm)	Polydispersity index	Hydrodynamic diameter (nm)	Polydispersity index
UCNP1	24.7	0.058	34.3	0.046
UCNP2	42.2	0.332	56.5	0.128
UCNPRef	28.4	0.151	39.4	0.146

4.4 Emission spectra

Table S3. Emission maxima of photon-upconversion nanoparticles.

	Activator	Composition	Emission maxima (nm)[a)]
UCNP1	Er^{3+}	$NaY_{0.80}Yb_{0.18}Er_{0.02}F_4$	520, 528, 540, 550, 654, 660, 698, 842
UCNP2	Ho^{3+}	$NaY_{0.80}Yb_{0.18}Ho_{0.02}F_4$ @$NaYF_4$	485, 538, 578, 644, 749
UCNPRef	Tm^{3+}	$NaY_{0.80}Yb_{0.18}Tm_{0.02}F_4$	450, 474, 802

[a)]Maxima for nanoparticles coated by carboxylated silica shell in water

5 Microfluidics

5.1 Chip design

Microchannel architecture with three aqueous phase inputs, an oil phase input, a flow-focusing device (droplet formation), passive mixer, and incubation loops was designed in AutoCAD® software (Autodesk 2015).[4] The detection was carried out from a part of the channel, which was approximately 2-3 mm out of the incubation part. The depth of 100 μm was constant for all channels. The width of channels varied from 100 μm in the aqueous phase confluence to 125 μm in the mixing, incubation, and detection part of the chip. Chip design was photolithographically transferred onto Photomask Blank Plates (Nanofilm, USA) using a high-resolution laser printer (Heidelberg μPG 101 Laser Writer, Heidelberg Instruments Mikrotechnik GmbH, Germany).

5.2 SU-8 mold development

The negative photoresist SU-8 3050 (MicroChem Corp., USA) was coated on a glass substrate (Boro Glass Slides, 4 × 4 × 0.06”; MB Whitaker & Associates, Germany), which was cleaned with *piranha* solution (H_2SO_4:H_2O_2 3:1 (v/v)). The photoresist was spin-coated by ramping from speed 500 rpm to the final speed of 3000 rpm within 10 s and then holding at this speed for 30 sec. The substrate bearing the layer of photoresist was baked at 65 °C for 10 min, at 95 °C for 30 min, and lastly at 65 °C for 5 min. After cooling, the photomask was placed on the SU-8 coated substrate and exposed to the UV light with an exposure energy of 250 mJ cm^{-2}. After exposure, the substrate was baked for 1 min at 65 °C for 10 min at 95 °C and for 1 min at 65 °C. Finally, the mold was developed by ma-600 SU-8 developer (MicroChem Corp.) and washed with isopropyl alcohol.

5.3 Polydimethylsiloxane chip fabrication

SYLGARD 184 kit (Dow Corning, Midland, MI, USA) was used for polydimethylsiloxane (PDMS) chip fabrication with 1/10 (w/w) ratio of curing agent. PDMS mixture was poured over the SU-8 master mold and cured for 2 hours at 65°C. After cooling, holes for channels inlets were punched by biopsy punch (Miltex, 1.5 mm diameter), and the PDMS chip was attached on a cleaned microscope slide (76 × 26 mm, Corning Glass) by plasma bonding. Immediately, the chip was manually flushed with a mixture of a fluorinated oil FC-40 (3M FluorinertTM FC-40, Sigma Aldrich) supplemented with 2% 1H,1H,2H,2H-Perfluorododecyltrichlorosilane (Sigma Aldrich) for 5 minutes followed by 1 hour of washing (250 μL h^{-1}) with the pure fluorinated oil FC-40.

5.4 Device integration

The set of silica capillaries with a length of 50 cm and 30 μm inner diameters (Molex) were used for connecting aqueous phases. For connecting the oil phase, a capillary with a length of 50 cm and 100 μm inner diameter was used. The ends of capillaries (~2 cm length) were tightly fitted with polytetrafluoroethylene tubes, which served as robust connectors for PDMS chip (1/16" outer diameter and 0.010" inner diameter; Alltech). The flow rates were regulated by the laboratory-made pressure controller.[5]

5.5 Time traces of concentrations of UCNPs and 4-methylumbelliferone in a single droplet

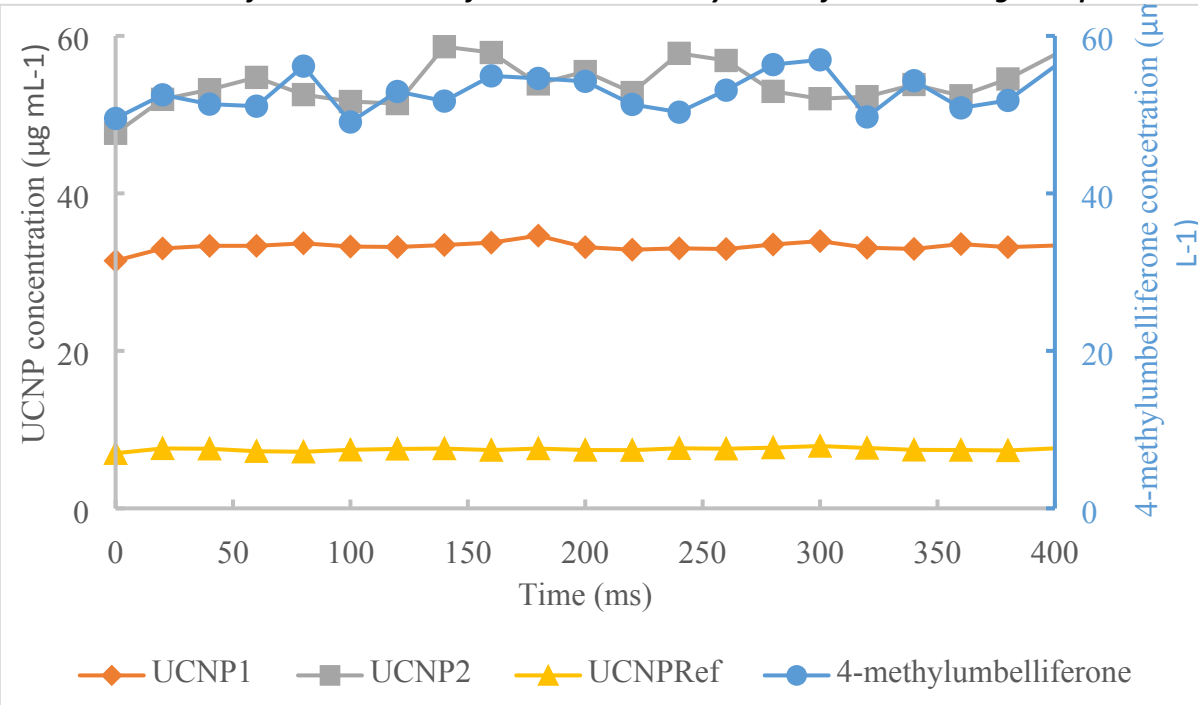

Figure S4. Time traces of concentrations of UCNPs (µg mL^{-1}) and 4-methylumbelliferone (µmol L^{-1}) in a single droplet. The droplet contained 33 µg mL^{-1} of UCNP1, 53 µg mL^{-1} of UCNP2, 7 µg mL^{-1} of UCNPRef, and 50 µmol L^{-1} of 4-methylumbelliferone. The number of 22 emission spectra were collected with an integration time of 20 ms under combined 364 and 979 nm excitation. The emission spectra were analyzed by the non-negative least-squares algorithm as described in the main text (equation 1). Average estimated values were 33.1 ± 0.88 µg mL^{-1} of UCNP1 (relative standard deviation (RSD) 2.7%), 53.5 ± 3.1 µg mL^{-1} of UCNP2 (RSD 5.9%), 7.4 ± 0.27 µg mL^{-1} of UCNPRef (RSD 3.7%) and 52.7 ± 2.4 µmol L^{-1} of 4-methylumbelliferone (RSD 4.6%).

6 References

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