Supplementary Information

Targeted and efficient activation of channelrhodopsins expressed in living cells via specifically-bound upconversion nanoparticles

Kanchan Yadav,†,‡,Δ Ai-Chuan Chou,†Δ Rajesh Kumar Ulaganathan,†,‡,§ Hua-De Gao,†,± Hsien-Ming Lee,‡,* Chien-Yuan Pan,†,¶,* and Yit-Tsong Chen†,†,*

†Department of Chemistry, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 106, Taiwan
‡Nanoscience and Nanotechnology Program, TIGP, Institute of Physics, Academia Sinica, P.O. Box 23-166 and National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 106, Taiwan
§Institute of Atomic and Molecular Sciences, Academia Sinica, P.O. Box 23-166, Taipei 106, Taiwan
ΔDepartment of Life Science, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 106, Taiwan
‡Institute of Chemistry, Academia Sinica, Nankang, Taipei, 11529, Taiwan
¶Graduate Institute of Brain and Mind Sciences, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 106, Taiwan
ΔThese authors equally contributed to this work.
*E-mail: ytcchem@ntu.edu.tw, cypan@ntu.edu.tw, leehm1@gate.sinica.edu.tw

† Electronic supplementary information (ESI) available: Electron microscopy and X-ray diffraction characterizations of UCNPs, A ninhydrin assay for the presence of amine groups on UCNPs, A Bradford assay for NAv-UCNP, The zeta potentials on different surfaces of UCNPs, Hydrodynamic sizes of UCNPs with different surface modifications, Localization of V5-ChR2m on a cell membrane, Specific binding of NAv-UCNP to V5-ChR2m in cell lysate, Specific binding of NAv-UCNPs to the V5-ChR2m on a cell membrane, and Experimental Section.
S1. Electron microscopy and X-ray diffraction characterizations of UCNPs

Fig. S1 Characterization of the UCNPs of NaYF₄:30 % Yb³⁺, 1 % Tm³⁺. (a–b) TEM images of bare UCNPs without any surface modification. (c) A TEM image of Silica-UCNP. (d) A comparison was made for the powder X-ray diffraction patterns between bare UCNPs (upper panel) and the standard hexagonal reference (lower panel).
S2. A ninhydrin assay for the presence of amine groups on UCNPs

Fig. S2 A ninhydrin assay was conducted to confirm the presence of amine groups on the UCNP surface. The color change from (a) colorless to (b) purple indicates the presence of amine groups on the UCNP surface.
S3. A Bradford assay for NAv-UCNP

**Fig. S3** A Bradford assay was applied to monitor the presence of protein (NeutrAvidin) on the UCNP surface (NAv-UCNP). The NAv-UCNPs were centrifuged at 10,000× g for 10 min. The samples presented are (a) an HBSS buffer at pH 7.4 as a control test, (b) the NAv-UCNP pellets resuspended in HBSS, (c) the supernatant, and (d) bPEG-UCNP in HBSS buffer.
S4. Zeta potentials on different surfaces of UCNPs

Fig. S4 The measured zeta potentials indicate different surface charges on (a) Silica-UCNP, (b) NH$_2$-UCNP, (c) bPEG-UCNP, and (d) NAv-UCNP.
S5. Hydrodynamic sizes of UCNPs with different surface modifications

**Fig. S5** Dynamic light scattering measurements show the hydrodynamic sizes of UCNPs with different surface modifications: Silica-UCNP (red), NH$_2$-UCNP (blue), bPEG-UCNP (pink), and NAv-UCNP (green). The measured hydrodynamic sizes are presented in the parentheses.
S6. Localization of V5-ChR2m on a cell membrane

**Fig. S6** The localization of V5-ChR2m on a cell membrane. The HEK293T cells expressing V5-ChR2m or ChR2m were stained first with an antibody against the V5 epitope and then an Alexa Fluor-488-conjugated secondary antibody. (a) Live cells. (b) Permeabilized cells. For permeabilization, cells were fixed and treated with Triton X-100 before staining. The images were taken in a confocal microscope. Scale bar: 10 µm (applicable to each panel).
**S7. Specific binding of NAv-UCNP to V5-ChR2m in cell lysate**

To evaluate the binding specificity of NAv-UCNPs to V5-ChR2m, we expressed V5-ChR2m or ChR2m (as a control) in HEK293T cells and collected the lysates for immuno-dot and pull-down assays. For the immuno-dot assay, lysates were immobilized on a nitrocellulose membrane (i.e., NCM) followed by the addition of bV5-Ab and incubation with NAv-UCNPs. As shown in Fig. S7a, the V5-ChR2m lysate interacted with NAv-UCNPs via bV5-Ab to form the NAv-UCNP/V5-ChR2m complex, which emitted blue light (from UCNPs) upon NIR illumination. In contrast, the ChR2m lysate control (instead of V5-ChR2m) emitted no blue light, because ChR2m did not bind with bV5-Ab and NAv-UCNP, which were washed out before imaging. For the other control test without lysate, no upconverted blue emission was observed because NAv-UCNPs were not bound on the NCM.

In another test, a pull-down assay was carried, where the cell lysates were incubated with the NAv-UCNP associated with bV5-Ab (i.e., bV5-Ab/NAv-UCNP). The pull-down NAv-UCNPs showed the fluorescence signals of UCNP and mCherry, only when they formed the NAv-UCNP/V5-ChR2m complex (Fig. S7b). These results demonstrated the capability of NAv-UCNP to bind specifically to the V5-ChR2m.
Fig. S7 Specific binding of NAv-UCNP to V5-ChR2m in cell lysate. (a) An immuno-dot assay shows the specific binding of NAv-UCNP to V5-ChR2m. The cell lysate isolated from the cells expressing V5-ChR2m or ChR2m was immobilized on a nitrocellulose membrane (NCM) and incubated in a buffer containing bV5-Ab. NCM (with V5-ChR2m, ChR2m, or no lysate) was then incubated with NAv-UCNPs and was illuminated with NIR at 980 nm. Scale bar: 200 μm (applicable to each panel). (b) A pull-down assay shows the specific binding of NAv-UCNP to V5-ChR2m. The cells expressing V5-ChR2m or ChR2m were then lysed to collect the membrane fractions. The fractions were mixed with bV5-Ab/NAv-UCNPs; after centrifugation, the pellets containing the pull-down complexes were spread on a coverslip and the fluorescence images were observed in a confocal microscope with different excitation wavelengths. Left to right: bright field, mCherry under 560 nm excitation, UCNP under 980 nm excitation, and the merged pictures. The insets show the amplified images as indicated by the dashed squares. Scale bar: 50 μm (applicable to each panel).
S8. Specific binding of NAv-UCNPs to the V5-ChR2m on a cell membrane

**Fig. S8** Two more representative images show the specific binding of NAy-UCNPs to the V5-ChR2m on a cell membrane (a continuation of Fig. 2). In the bright field images, the yellow circles indicate the cells without mCherry expression, representing the absence of NAy-UCNP binding. Scale bar: 20 µm (applicable to each panel).
S9. Experimental Section

**Synthesis of UCNPs:** Oleate-capped UCNPs (NaYF₄: 30 % Yb³⁺, 1 % Tm³⁺) were synthesized following the procedures reported previously with minor modifications. Y(CH₃CO₂)₃ hydrate (99.9 %, 0.69 mmol), Yb(CH₃CO₂)₃ hydrate (99.9 %, 0.3 mmol), and Tm(CH₃CO₂)₃ hydrate (99.9 %, 0.01 mmol) were added to a 100-mL three-necked round-bottom flask containing octadecene (15 mL) and oleic acid (6 mL). To remove residual water and oxygen, the solution was stirred and heated slowly to 120 °C in a vacuum for 30 min. The temperature was then lowered to 50 °C and the reaction flask was placed under a gentle flow of N₂ gas. Meanwhile, a methanol (10 mL) solution containing ammonium fluoride (4 mmol) and sodium hydroxide (2.5 mmol) was prepared via sonication and added into the reaction flask. The resulted cloudy mixture was stirred at 50 °C for 30 min and then at 75 °C followed by increasing the N₂ flow to enhance the methanol evaporation from the reaction mixture. Subsequently, the temperature was increased and maintained at 300 °C for 60 min under a gentle N₂ flow. Meanwhile, a slightly yellowish solution was obtained and allowed to cool to room temperature. The as-synthesized UCNPs were precipitated by adding ethanol and isolated via centrifugation. The resultant pellets were dispersed in a minimal amount of hexane and then precipitated with an excess of ethanol. The UCNPs were again isolated via centrifugation (8000× g, 15 min) and then dispersed in cyclohexane for the subsequent silica coating. TEM images show that UCNPs were highly monodispersed and possessed a nearly hexagonal morphology with a diameter of 30 ± 1.5 nm (Fig. S1a,b). XRD pattern of the UCNPs shows well-defined sharp peaks and matches fairly well with that of the hexagonal β-NaYF₄ reference, indicating their high crystallinity (Fig. S1d).

**Synthesis of Silica-Coated UCNPs (Silica-UCNPs):** The oleate-capped UCNPs were subsequently coated with tetraethyl orthosilicate (TEOS) by a microemulsion method with slight modifications to obtain a thin, uniform silica layer on the UCNPs (referred to as Silica-UCNP) to improve the solubility and biocompatibility of UCNPs. This coating provided the silanol groups (Si–OH) on the UCNP surface for the next modification of the silane-based functionalization. About 25 mg of UCNPs were dissolved in 7 mL of cyclohexane and sonicated for 30 min. At the same time, 500 µL of Igepal dissolved in 2 mL of cyclohexane was stirred for 30 min and then added into the solution containing UCNPs. Next, 80 µL of ammonium hydroxide was added to the mixture and sonicated for another 30 min, followed by adding 25 µL of TEOS and stirring the mixture for 18 hr. Finally, the solution was precipitated by acetone and isolated via centrifugation. The pellets were washed twice with an
ethanol:water (1:1) mixture to remove impurities. TEM image of the as-synthesized Silica-UCNPs shows the average diameter to be 35 ± 1 nm (Fig. S1c).

**Surface modification for APTES-Conjugated UCNPs (NH$_2$-UCNPs):** Silica-UCNPs were functionalized with aminopropyltriethoxysilane (APTES) to introduce amine groups (–NH$_2$) on the UCNP surface (referred to as NH$_2$-UCNPs) using a previously reported method with some modifications.\textsuperscript{S3} Silica-UCNPs (25 mg) were added into 15 mL of water:ethanol (5:95 %) solution containing (25 μL) APTES. The solution was transferred to a three-necked round-bottom flask (50 mL), followed by stirring and heating at 60 °C for 12 hr under a N$_2$ atmosphere. Finally the solution was precipitated by acetone (20 mL) and isolated via centrifugation. The pellets were washed twice with an ethanol:water (1:1) mixture to remove impurities. A ninhydrin assay was conducted for the amine quantification, where the successful surface modification of amine groups was confirmed by the presence of purple color (Fig. S2) and the substitutional level of amino groups on the Silica-UCNP was estimated to be 84 nmol/mg of UCNPs.

**Surface modification for NAv-Conjugated UCNPs (NAv-UCNPs):** The NH$_2$-UCNPs were further functionalized with NeutrAvidin (NAv) following a previous method with some modifications.\textsuperscript{S4} First, NH$_2$-UCNP reacted with N-hydroxysuccinimide-(polyethylene-glycol)$_4$-biotin (denoted by NHS-(PEG)$_4$-Biotin) for the surface biotinylation and was then incubated with excess NAv to ensure the maximal NAv binding. In the sample preparation, NH$_2$-UCNPs (1 mg) were suspended in 1 mL of dimethylformamide (DMF), to which 2.5 mM of NHS-(PEG)$_4$-Biotin was added and stirred for 12 hr at room temperature. The bPEG-modified UCNPs (referred to as bPEG-UCNPs) were isolated by centrifugation and washed with water four times. The bPEG-UCNPs were then dissolved in 10 mM of HEPES buffer (pH 8.0) to have a concentration of 1.0 mg/mL and mixed with 9.5 μM of NAv to be stirred for 1 hr at room temperature. The resultant NAv-UCNPs were isolated by centrifugation and washed with water three times. The pellets were resuspended in HBSS buffer overnight and then centrifuged. Both supernatant and pellets were examined by the Bradford assay to check if any NAv protein had detached from the bPEG-UCNP surface. The substitution level was analyzed to be ~9.2 nmol NAv/mg of UCNPs and the appearance of purple color suggested the successful and stable protein conjugation (Fig. S3). TEM images of NAv-UCNPs show the average diameter to be 39 ± 1.5 nm (Fig. 1b).
**Immuno-dot Assay:** Cell lysates were dotted on a nitrocellulose membrane (NCM) and incubated in a buffer containing bV5-Ab at 4 °C overnight. After PBS wash and the addition of NAv-UCNPs, the fluorescence was visualized under NIR illumination.

**Pull-down Assay:** We mixed the bV5-Ab/NAv-UCNPs with cell lysate at room temperature for 1 hr in PBS containing a Protease Inhibitor Cocktail (Set V, 1:100 dilution, CalBiochem, La Jolla, California, USA). The UCNPs were centrifuged and washed with PBS three times and the pellet was placed on an inverted microscope (Zeiss LSM 510) for fluorescence imaging.
References


