SUPPORTING INFORMATION

Cell-penetrating nanobiosensors for pointillistic intracellular Ca^{2+}-transient detection

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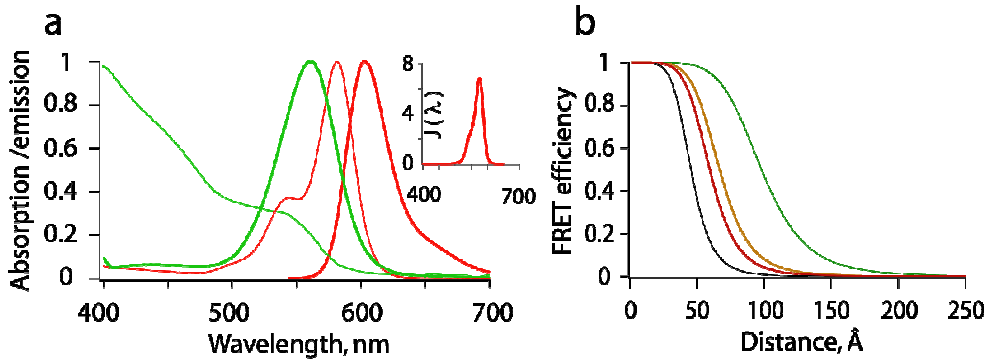


Fig. S1: **FRET characteristics of the donor and acceptor pair formed by CANdots and PEG5-CaRubyMe**. (a) Traces show normalized absorbance and emission spectra (thin and thick lines respectively) of QDs 565 (in red) and PEG5CaRubyMe (in green). QDs excitation between 407-450 nm will yield FRET emission of CaRubyMe due to overlap of the donor emission and acceptor excitation spectra (Inset shows the energy, J *vs* wavelength which peaks at $6.8e^{-13}$ M^{-1} cm^3 yielding a R_0 Förster radius of 45 Å). (b) FRET efficiency is here expressed as a function of the distance r donor to acceptor when the number n (or A/D) of CaRuby molecules attached per QD is varied. FRET efficacy, $E= nR_0^6 / (nR_0^6 + r^6)$, is given with A/D ratio equals from 1, 2, 5, 100 from left to right.

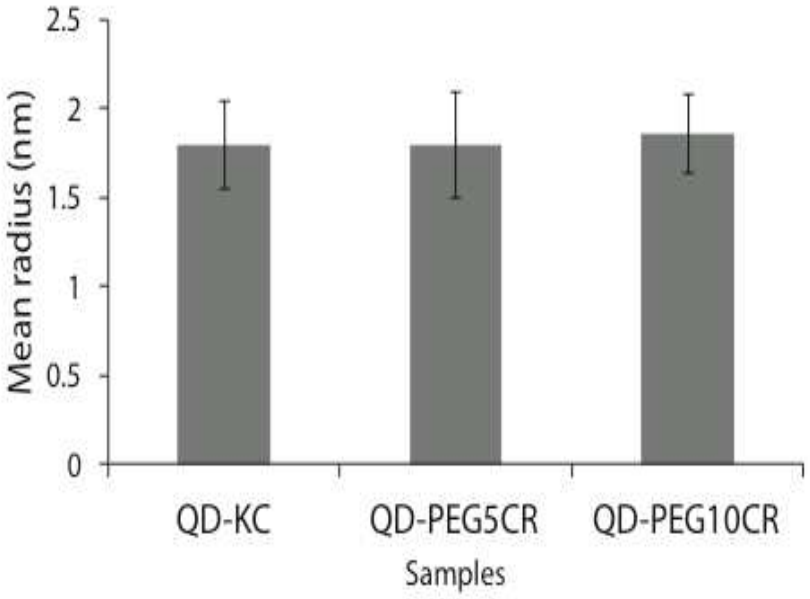


Fig. S2: Determination of the QDs core/2 shells radius by transmission electron microscopy. Statistical measurements yielded radius sizes of 1.8 ± 0.25 nm for QD-KC, 1.8 ± 0.3 nm for QD-PEG5CaRuby (CR) and 1.86 ± 0.22 nm for QD-PEG10CaRuby (CR) respectively (mean ± SD, 15-20 dots each)**.**

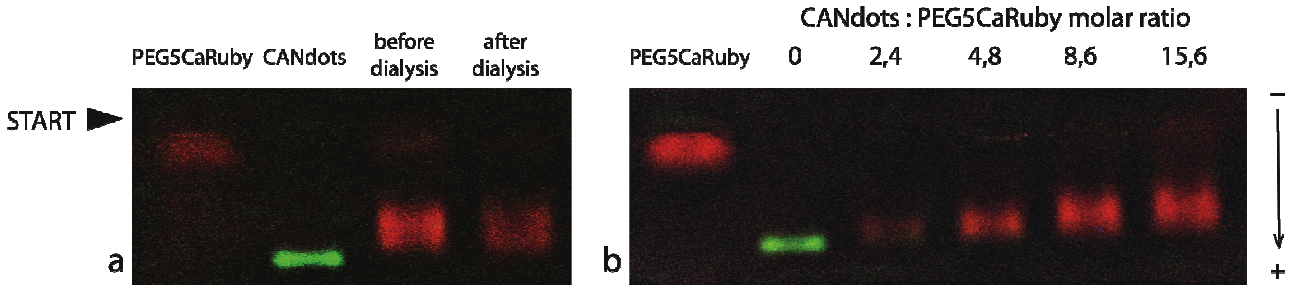


Fig. S3: Electrophoretic mobility of QD-PEG5CaRubyMe complexes in 0.5% agarose gel. (a) Gel electrophoresis of free PEG5-CaRuby, CANdots after ligand exchange, CANdots-PEG5-CaRuby complexes in ratio 1:12, illustrating efficiency of 48 hours dialysis purification. (b) Gel electrophoresis of free PEG5-CaRuby, and CANdots-PEG5-CaRuby complexes in different molar (A/D) ratios, as indicated at top of each lane. Samples show decrease of electrophoretic mobility with increased number of fixed dye molecules on the quantum dots**.**

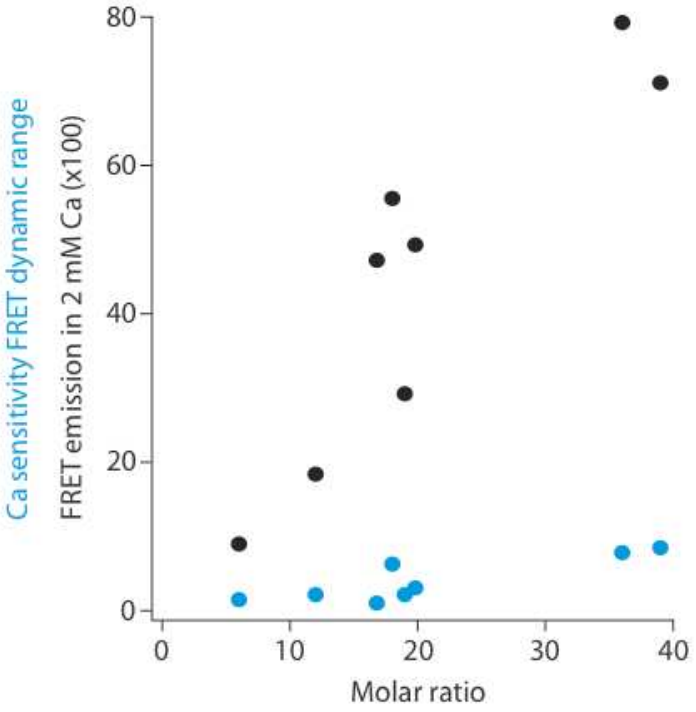


Fig. S4: Ca^{2+} sensitivity of QD-PEG10CaRuby. Graph displaying FRET absolute value increase (in 2 mM Ca^{2+}; black circles) whereas DR (blue dots) is constant when A/D is varied between 0 and 40. All data refer to the same QDs concentration.

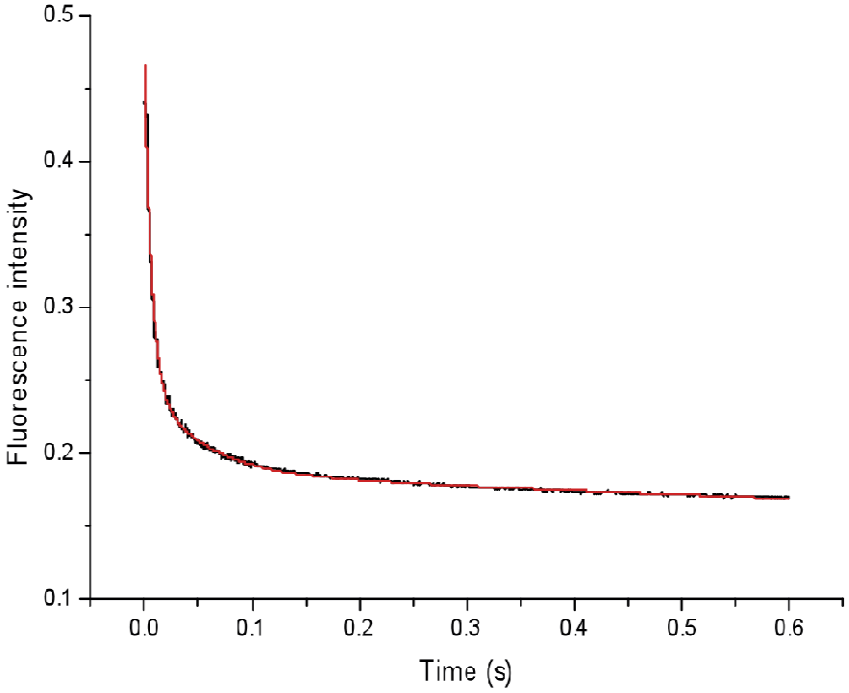


Fig. S5: Kinetics of Ca^{2+} binding to the QD-PEG5-CaRubyMe complexes. Addition of 5 mM EGTA displaced Ca^{2+} from saturated 0.5 µM CaRubyMe complexes. Emission was observed using an OG 590 nm long pass filter (excitation at 546 nm). A bi-exponential function (red line) fitted to the average (black line) of 4 experiments yielded two dissociation constants of 155 s-1 and 19.4 s-1 (and respective amplitudes 0.227 and 0.064). Similar experiments with 0.5 µM CaRubyMe yielded a koff of 300s-1 and 9.3 s-1 (of respective amplitudes 0.262 and 0.0528). From their respective K_D (2.9 and 3. 4 µM), estimated Ca^{2+} binding kon to the the nanoprobe and free CaRubyMe were 0.5 10^8 and 10^8 M^{-1} s^{-1}**.**

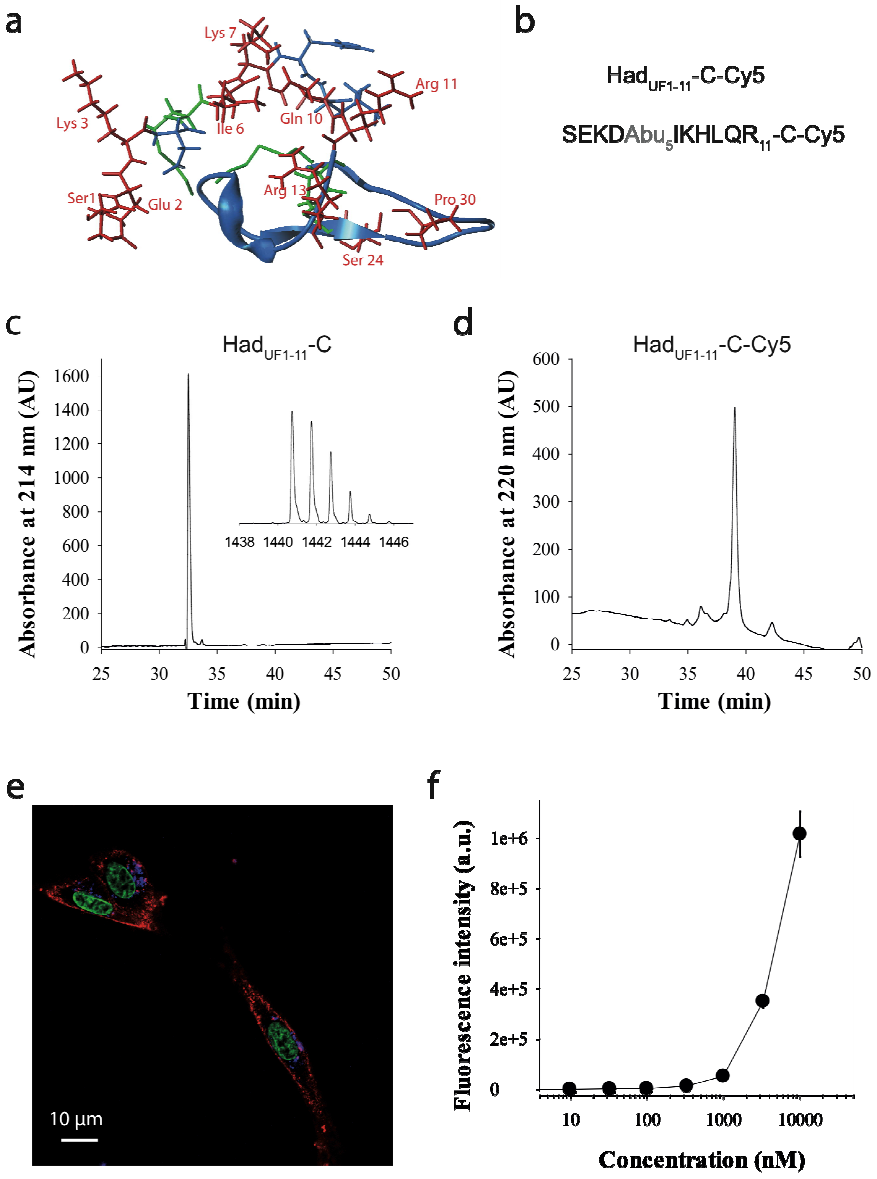


Fig. S6: Characterization of the cell penetration properties of HadUF1-11-C-Cy5 in cultured BHK cells. (a) Modeled 3D structure hadrucalcin (based on maurocalcine 3D structure, PDB access code 1C6W). Red residues are amino acids that differ from the amino acid sequence of maurocalcine. Residues in green are cystine residues. The peptide used for cell penetration Had$_{UF1-11}$ goes from Ser^{1} to Arg^{11} and forms one upper face of hadrucalcin. (b) Single letter code amino acid sequence of Had$_{UF1-11}$-C-Cy5. The fifth Cys residue is replaced by Abu in our synthetic peptide and is represented in grey color. (c) Analytical C18 reversed phase HPLC profile of purified Had$_{UF\ 1-11}$-C peptide. Insert: mass spectra of Had$_{UF1-11}$-C. (d) Analytical C18 reversed phase HPLC profile of purified Cy5-labeled Had$_{UF\ 1-11}$-C-Cy5 peptide. Note the shift in elution time indicating greater hydrophobicity of the conjugate. (e) Confocal microscopy image of 1 µM Had$_{UF1-11}$-Cy5 (blue) penetration in BHK cells. Cell membranes are labelled with concanavalin A-rhodamin (red), while nuclei are stained with Hoechst (green). (f) Quantitative assessment of cell penetration of Had$_{UF1-11}$-C-Cy5 by flow cytometry.

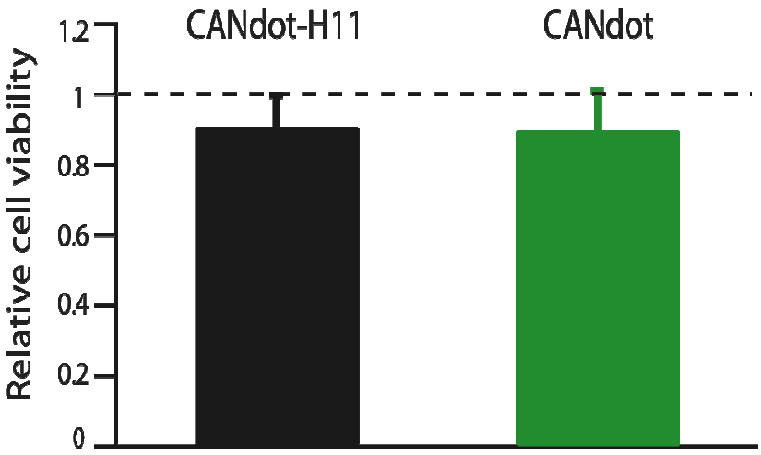


Fig. S7: Cell viability upon addition of QD functionalized with CPPs as assessed by the MTT toxicity test. 0.25 µM QDs without and with liganded CPPs (molar ratio 1:5) were incubated for 24 hours on BHK cells in culture at 80% confluence. Values are relative to control values (wells with solution exchange but no addition of QDs), 3 experiments, 4-6 wells each. Independent experiments with addition of 10 µM $Had_{UF1\text{-}11}$ did not reveal any toxicity of the CPPs**.**

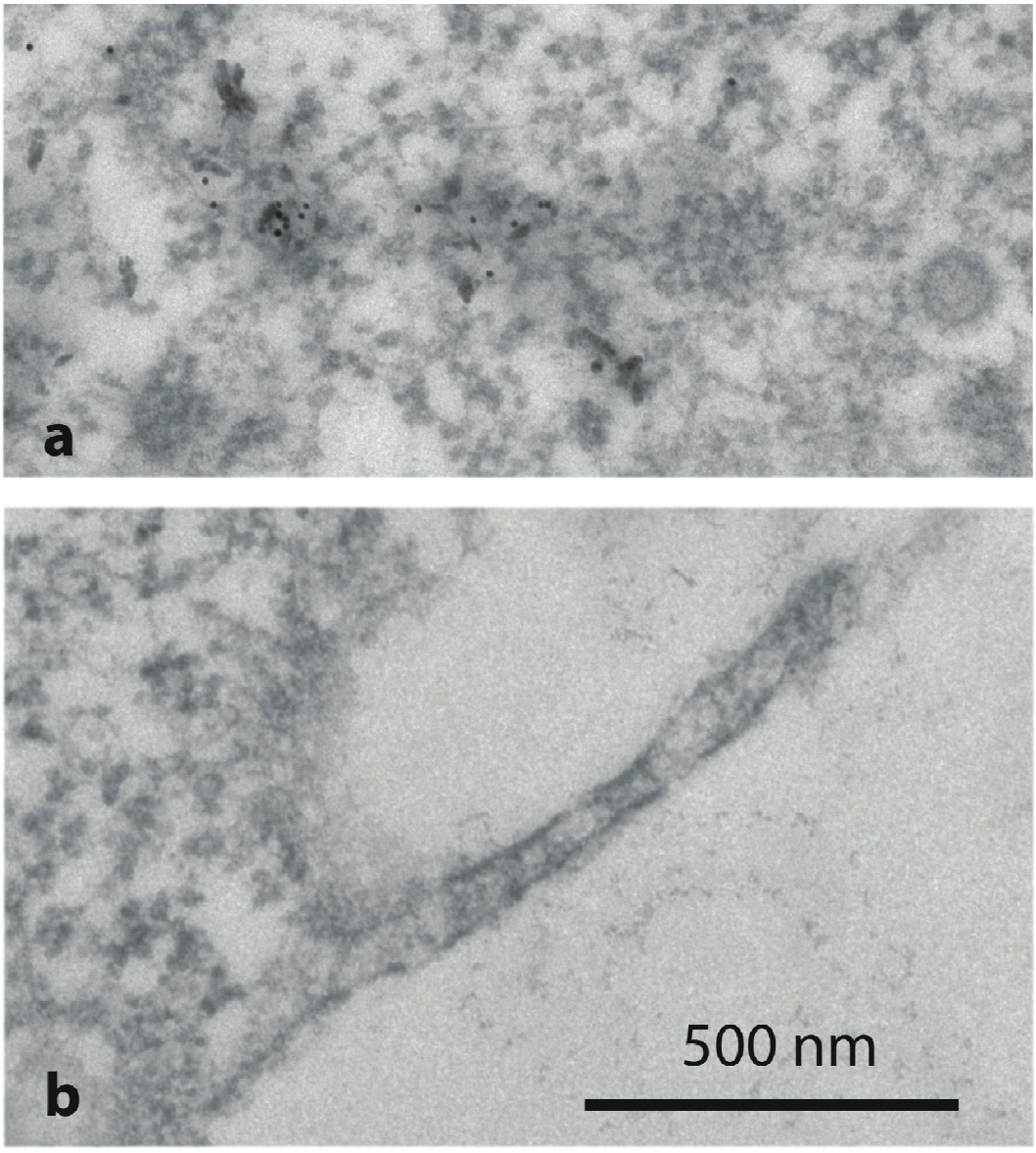


Fig. S8: EM micrograph of internalized QD-CaRuby-H11 complexes applying strong staining. A reinforced staining (with 5% uranyl acetate in 70 % methanol) was used for improved membrane visualization. In this condition, membranes are well preserved, see a likely coated-vesicule at bottom right in (a) and a filopode of the same cell in (b). However, importantly in (a) no membrane can be detected in a region where grouped QDs can be observed constituting a figure similar to that seen in Figure 7. Note the presence of deposits characteristic of uranyl acetate in the regions where QDs are present.

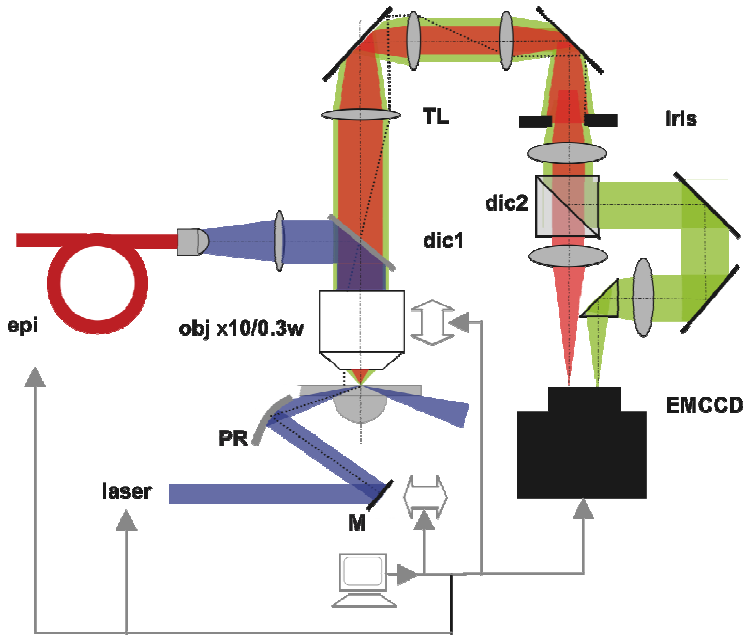


Fig. S9: Schematic optical layout of the variable-angle prism-based TIRF microscope used for imaging individual nanobiosensors in live cells. Obj: objective lens, TL: tube lens, dic: dichroic mirror, EMCCD: electron-multiplying charge-coupled device. Depending on the excitaton wavelength (shown here: 405 nm, blue) the beam angle of the totally reflected light was adjusted by sliding a small mirror (M). The parabolic reflector (PR) guaranteed that the beam always hit the same excitation spot. Fluorescence was extracted by a first dichroic (dic 1) that also permitting epifluorescence excitation. A custom dual viewer device separated red (CaRuby) and green (QD) emissions with a secondary dichroic (dic2) and appropriate emission filters (not shown). A green-light emitting LED below the quartz prism permitted bright field imaging (not shown)**.**

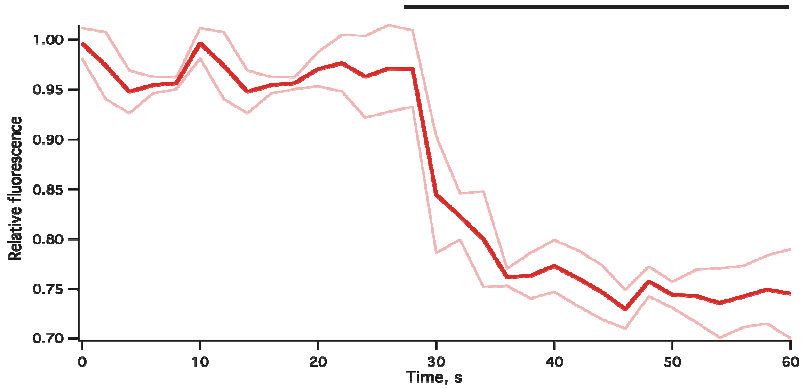


Fig S10: TIRF imaging of copper quenching of the extracellular nanobiosensors. A 40 µM CuSO4 bath solution quenches both QDs and CaRuby (see main text). Here with a preparation of QD-CaRuby loaded cells, then trypsinized and replated, copper application - horizontal black line - reduces basal cell fluorescence by 24% (average normalized fluorescence of 3 cells ± S.D., in red and light red respectively). Effect ranged from 10% to 25%, which implies that after trypsinization most imaged QDs were intracellular. After a first copper application, absolute remaining fluorescence did not change after wash nor upon a second CuSO4 application (not shown). 250 ms long TIRF frames taken for one minute at 0.5 Hz to minimize photobleaching**.**

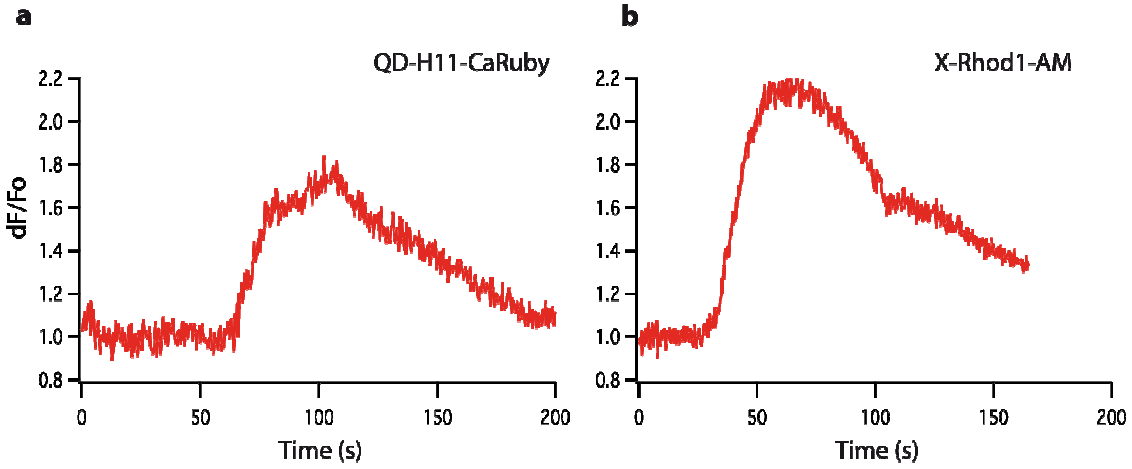


Fig. S11: Averaged Ca2+ transient recorded with QD-H11-CaRuby loaded cell (a) and X-Rhod1 loaded cell (b) have similar kinetics. (a) is the average of the signals evoked at 12 red-emitting points from top cell presented in Fig. 8a. In (b), cells were loaded by 5 min exposure to 1 µM X-Rhod1-AM (+ pluronic acid) before recording the response to bath applied NMDAR agonists at the same saturating concentration as in (a).

MATERIAL and METHODS

Drugs and chemicals:
ACN (Sigma-Aldrich, 271004); APV (Tocris, 0106); 1M CaCl2 (Fluka, 21115); Blasticidine S hydrochloride (Sigma-Aldrich, 15205); Coumarin 314 (Sigma-Aldrich, 392995); DIEA (Sigma-Aldrich, 387649); DMEM (Invitrogen, 31885); Ethanol (Merck, 100983); HEPES (Sigma-Aldrich, H3375); geneticine (Gibco, 10131); glycine (Tocris, 0219); glutamate (Tocris, 0218); HOBt (Iris-Biotech, RL-1035); Methanol (Merck, 106009); MOPS (Sigma-Aldrich, M-1254); MTT (Sigma-Aldrich, M-5655); NTA (Sigma-Aldrich, N-9877); TFA (Sigma-Aldrich, T6508); water (Fluka, Cat No. 95305).

Quantum dots (QDs): Hydrophobic TOP/TOPO coated CdSe/CdS/ZnS QDs (CANdots, Hamburg, Germany ; λEm=565 nm) sold in hexane were transferred to a toluene medium by slowly evaporating hexane under reduced pressure and kept as a µM stock solution at 4°C.

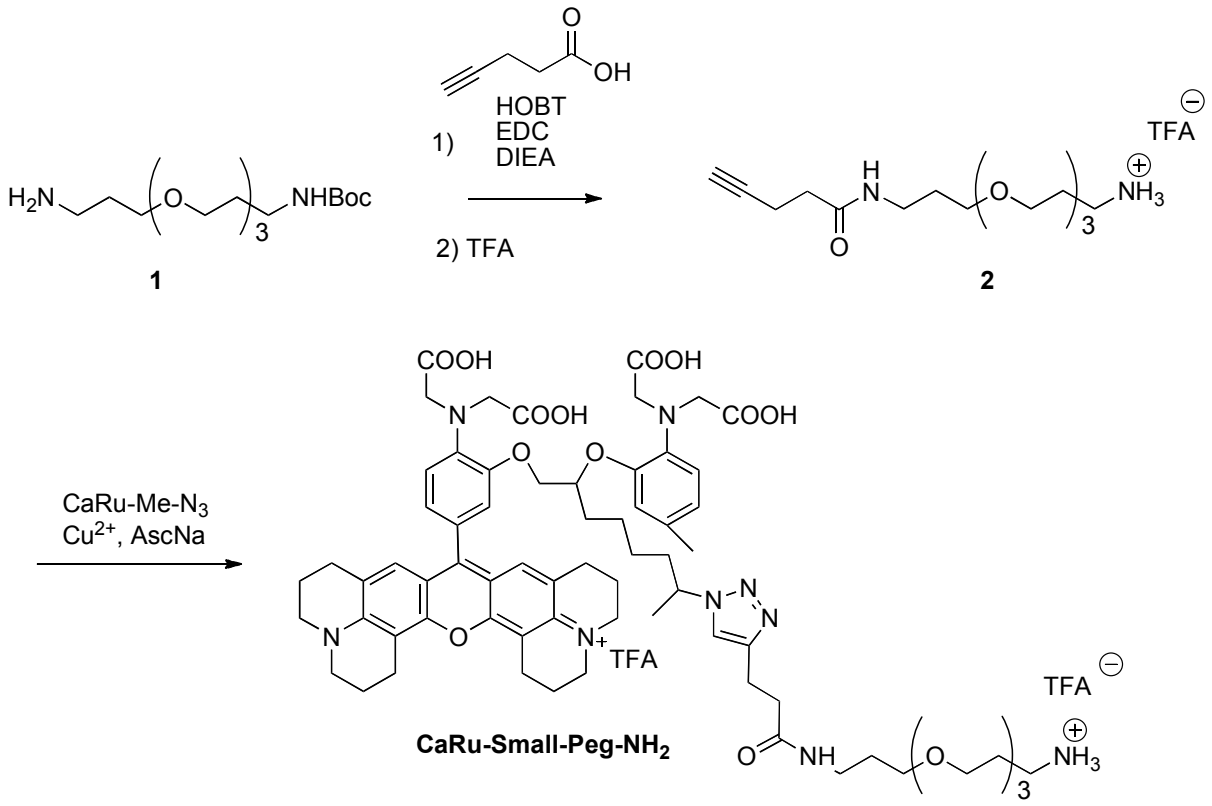
Synthesis of peptide-coated QDs: Hydrophobic QDs were coated with peptides as previously described.[1] Briefly, 1 nmole of the QDs stock solution in toluene was heated to 120°C to remove the solvent and redispersed in 450 µL of pyridine. Separately, 2 mg of each following peptides (New England Peptide, USA) were dissolved in 25 µL dimethyl sulfoxide (DMSO) before mixing at the indicated ratio: Ac-CGSESGGSESG$(FCC)_3$F-amide and NH2-KGSESGGSESG$(FCC)_3$F-amide. The QDs in pyridine and peptides in DMSO were mixed. Surfactant exchange and peptide binding was initiated by rising pH by addition of 12 µL of tetramethylammonium hydroxide (25% w/v in methanol). Following centrifugation, the QDs formed a pellet that was redissolved in 100 µL of DMSO. Next, the resulting peptide-coated QDs (pQDs) in DMSO were eluted through G25 Sephadex column equilibrated with twice its volume with a HEPES-buffered solution (HBS, in mM: 140 NaCl, 2.5 KCl, 10 HEPES, pH 7.2). To remove excess of peptides, the pQDs were purified using desalting column (Macrospin™, Harvard Apparatus). The pQDs were stored in HBS at 4°C until further use. Quantum yields (QYs) for hydrophobic QDs (λEm =563 nm, QY= 68 %) and the pQDs (λEm =563 nm, QY= 56%) were determined by standard procedures using coumarin 314 in ethanol (λEm =477 nm, QY=68%) as a reference as used by CAN Gmbh**.**

Preparation of the ligands:

CPPs were prepared as maleimide compounds by N-[g-maleimidobutyryloxy]succinimide ester (GMBS) addition at a molar ratio of 1:10 and stored dessicated after HPLC purification. For use, they were dissolved in HBS**.**

CaRubies PEGylation: CaRubies have been PEGylated using NH_2-PEG-alkyne of variable length (10 kDa, 5 kDa, 3 kDa, Iris Biotech and synthesised 0.3 kDa PEG) taking advantage of their side chain for click chemistry. Details of the Cu-catalyzed azide/alkyne cycloaddition (CuAAC) click conjugation has been already reported.[2] Briefly, alkyne-PEG-NH_2 and CaRuby were added in a 1(2 µmol): 2 molar ratio in 2 ml of methanol. A heterogeneous solution of $CuSO_4 \cdot 5H_2O$ (20 eq.) and of sodium ascorbate (25 eq.) in 500 µl of water was then added. The mixture was stirred overnight in the dark. The product was extracted with dichloromethane (DCM) and washed with a 0.1M solution of EDTA. The organic phase was evaporated and the crude purified through a LH-60 column (DCM/MeOH : 1/1). The first colored fraction was evaporated, taken up in distilled water and lyophilized to give the clicked product as a purple solid (yield ~80% for PEG10-CaRuby). Ca^{2+} sensitivity of CaRubies was unaltered by the click reaction.

0.3 kDa PEG synthesis:



1 was synthesized following a published protocol [3] To a solution of **1** (1.00 g, 3.125 mmol, 1 eq) in DCM (15 mL) were added 4-pentynoic acid (0.367 g, 3.75 mmol, 1.2 eq), HOBt (0.506 g, 3.750 mmol, 1.2 eq) DIEA (1.620 mL, 9.375 mmol, 3 eq) and EDC (0.900 g, 4.687 mmol, 1.5 eq). The solution was allowed to stir overnight. The solution was washed with HCl (1M) neutralized with a saturated $NaHCO_3$ solution before being dried over $MgSO_4$, filtered and evaporated. The crude product was purified by column chromatography on silica gel (7/3 DCM/Acetone) to obtain 1.00 g of the desired product (80%) as a yellowish syrup. Rf = 0.45 (7/3 DCM/Acetone). This product was solubilized in DCM (8 mL) and TFA was added (3 mL). The reaction was sonicated for 1 hour. The solvents were then evaporated and the product was washed by addition of ether, sonication and removal of the supernatant (repeated 2 times). The remaining ether was evaporated and the product was dried under vacuum to give **2** in a quantitative manner as a yellowish oil. ^{1}H-NMR (300 MHz, D2O): δ 3.57 (s, 10H), 3.48 (t, *J* = 6.2 Hz, 2H), 3.17 (t, *J* = 6.5 Hz, 2H), 3.00 (t, *J* = 6.8 Hz, 2H), 2.38-2.26 (m, 5H), 1.84 (t, *J* = 6.3 Hz, 2H), 1.69 (t, *J* = 6.5 Hz, 2H).

To a solution of **CaRuby-Me-N_3** (10 mg, 0.01 mmol, 1 eq) and the **2** (15 mg, 0.037 mmol, 3.7 eq) in methanol (4 mL) was added a heterogeneous solution of $CuSO_4 \cdot 5H_2O$ (10 mg, 0.04 mmol, 4 eq) and sodium ascorbate (10 mg, 0.05 mmol, 5 eq) in water (500 µL). The mixture was stirred at room temperature for 3 days in the dark before being evaporated, the crude was purified by reverse phase chromatography on a C-18 column (ACN (0.1% TFA)/Water (0.1% TFA), 20/80 to 100/0) to obtain 10 mg of **CaRuby-small PEG-NH_2** (75%) as a purple solid after lyophilization.

Assembling functionalized QDs and purification: Prior to conjugation, the QDs were diluted to ~ 1 µM. All products to be conjugated at the desired molar ratio were prepared separately in a x5 GMBS (Thermo Scientific, USA) solution and left to react 1 hour at room temperature. Then, they were purified on G-10

Macro SpinColumn (Harvard Apparatus, USA) to remove excess of GMBS product. Finally, maleimide activated products and QDs were allowed to react overnight**.**
Dialysis was applied to remove excess of unbound molecules of PEGX-CaRuby. The samples were dialyzed in tubes with MWCO 50 kDa (Spectra/Por) against HBS buffer for 48 hours. In order to find optimal conditions for dialysis and to check its efficiency we used agarose gel electrophoresis. Gel electrophoresis was performed in 0.5% low electroendosmosis agarose (Ambion, USA) gels in 0.5X TAE buffer applying a 10 V/cm electric field for 20 minutes. The fluorescence of the complexes was detected and imaged directly on gels using ultraviolet excitation and a digital camera.

Electron microscopy:
Estimation of QDs diameter: For observation of QD at electronic level, formwar coated 400 mesh nickel grids are floated with the support film side facing up inside a few drops of the different specimens solutions for 30 mimutes. After washing in distilled water, grids are treated with 2 % of uranyl acetate in water, drained off after 15 minutes and dried before observation (Philips Tecnaï 12 at 80 kV).
The core/shell diameter was extracted from EM micrographs. Using MATLAB® we obtain radial profiles of the QD. Higher SNR (Signal to Noise Ratio) was achieved by subtracting background noise. Estimate radius corresponding to half width half maximum (HWHM) after denoising was (in nm): 1.8 ± 0.25 (mean ± SD), 1.8 ± 0.3 and 1.86 ± 0.22 for QD-KC, QD-PEG5CaRuby and QD-PEG10CaRuby respectively.

Cell EM: After loading with QDots-CPPs-CaRuby at the concentration of 150 nM, cells culture were prepared on ACLAR film (0,2 mm thick , Agar scientific UK), and fixed with 4 % paraformaldehyde and 0.1 % glutaraldehyde in PBS for 30 minutes at room temperature. They were then rinsed 3 times 10 minutes in the same buffer and postfixed in 1 % OsO4 in PBS for 15 minutes at 4 °C. After wash in water, cells were dehydrated in ethanol graded series of concentrations to 90 %, then in hydroxy propyl methacrylate (90, 95, 97 %) and finally embedded in Epon. Ultrathin sections (70 nm) were contrasted as previously, or for better visualization of the membranes they were stained with 5 % uranyl acetate prepared in 70% methanol.

Fluorimetry: Methods used have been published previously.[2,4] Briefly, CaRuby dynamic range for Ca2+ sensing was estimated from peak PL of CaRuby measured in a solution containing (in mM): 100 KCl , 30 MOPS, and pH was adjusted at 7.2 by KOH addition. KD was evaluated similarly adjusting the Ca2+ concentration as previously described using NTA for adjusting the Ca2+ concentration in the low micromolar range.[5] FRET fluorescence spectra (500-700 nm) were obtained by excitation light at 350 nm, and direct emission spectra (545-700 nm) were obtained by excitation at 535 nm. All values for FRET pairs were calculated, after spectral linear unmixing, by fit to the QD and CaRuby spectra (MatLab curve fitting tool)**.**
Kinetics of the Ca^{2+} binding reaction to free CaRuby-Me and to QD-CPP-PEG5CaRubyMe was performed with a stopped flow apparatus (SFM-3, Bio-Logic, Claix, France). The reaction was carried out in 100 mM KCl, 30 mM MOPS and 10 mM NTA to adjust free Ca^{2+} concentration in the µmolar range around CaRuby-Me KD in the µmolar range, pH 7.4, 20°C. For koff evaluation, no NTA was added. The excitation and emission wavelengths were 450 and 514 nm (Melles-Griot interferential filter) respectively. Data acquisition and processing were done with the Biokine software from the instrument manufacturer.

CPP labeling with Cy5: The $Had_{UF1.11}$-C peptide was labeled with Cy5 according to the manufacturer's protocol (GE Healthcare). The peptide was dissolved at a concentration of 200 µg/ml in 1 M $NaHCO_3$ buffer, pH 9.3. 500 µl of the solubilized peptide was added to a Cy5-maleimide containing tube. The mixture was incubated during 2 hours at room temperature and then purified by HPLC using an analytical Vydac C18 column. Elution of the Cy5-labeled peptide was performed using a 10-60% acetonitrile linear gradient containing 0.1% trifluoroacetic acid. The pure peak fraction was lyophilized and the peptide quantified by UV spectrophotometer at 650 nm.

Cell culture and transfection for experiments on cells suspension: BHK-21 (clone 13) (baby hamster kidney) cell line was obtained from European Collection of Cell Cultures. Cells were cultured in a flask in DMEM medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS, Sigma), Pen Strep (100 g/mL), pyruvate (110 mg/L) and glutamate (100 mg/L) and incubated in 5% CO_2 at 37°C. The cells were collected from 90% confluent cell culture flasks by aspirating off the media and incubating with trypsin-EDTA (Gibco) for 2 min. DMEM medium, containing 10% FCS was added to stop trypsinization. Then, this solution was separated from the medium by centrifugation at 800 rpm for 3 min. The cells pellet was suspended in DMEM to obtain a homogeneous cell suspension at the desired final concentration for the toxicity assay or for investigation of QD-CaRuby-CPP penetration. For the intracellular Ca^{2+} measurements, HEK293 cells, transfected with NR1/NR2B NMDA receptors, were used. Cells were cultured in a flask in the same way as BHK-21 cells. Transfection was carried out on 80% confluent cells and nucleic acid-jet PRIME® complexes, containing 5 µg NR1 and 5 µg NR2B DNA plasmid, were added. After 4 hours incubation, the medium was replaced for fresh medium containing 200 µM APV and cells were left for 48 hours in the incubator to allow the expression.

Cell culture of a BHK cell line stably expressing NR2-NMDAR: Baby Hamster Kidney (BHK) cells stably expressing the NR1 and NR2 subunits of the N-methyl-D-aspartate receptor (NMDAR)[6] were grown in presence of two selection antibiotics (geneticine and blasticidine for each plasmid respectively). Further cells were maintained in presence of the two competitive antagonists of the glycine and glutamate binding sites, 5,7 dichlorokynurate (DCKA, 200 µM) and amino- 5, phosphonovalerate (APV, 200 µM) respectively. To reduce intracellular autofluorescence cells were cultured in phenol-red free DMEM.
For imaging, cells were transferred onto the stage of a custom microscope[7] (see below) and were continuously perfused at 3 ml/min with extracellular HEPES buffered saline containing (in mM): 140 NaCl, 2 KCl, 1.3 CaCl2 and 1.1 MgCl2, 10 Hepes and pH was adjusted to 7.4 (NaOH). 'Zero Ca^{2+}' solution contained was nominally Ca^{2+} free and containted 1.1 mM $MgCl_2$. 1 day before imaging, cells were loaded by exposure for 2 hours to 100 nM QD-CaRuby-CPP, trypsinized and re-plated on uncoated 25-mm ∅ #1.5 quartz coverslips (Technical Glass Products, Painesville, OH) that had previously been cleaned by successive baths in acetone, ethanol, water and were finally exposed to UV light for one hour.

Toxicity assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT, assay): Cells were plated into 96-well microplates and grown until reaching 75% confluence. After 2 days of culture, the cells were incubated for 24 hours at 37 °C with CPPs at a concentration of 10 µM. Control wells containing cell culture medium alone or with cells, both without peptide addition, were included in each experiment. 2% SDS was used as a toxic agent for comparison. The cells were then incubated with MTT added at 1.5 mM for 30 min (stock at 15 mM (25 mg/5 ml in HBS; MW 335). Conversion of MTT into purple-colored MTT formazan by the living cells indicates the extent of cell viability. The crystals were dissolved with 100 µl DMSO, and the optical density was measured at 490 nm using a microplate reader (Biotek ELx-800, Mandel Scientific Inc.) for quantification of cell viability. All assays were run in triplicates**.**

Confocal microscopy: BHK-21 cells were plated into 8-well Lab-Tek plates (at 10% confluence). Before incubation with quantum dots, cells were washed twice with DMEM/F-12 medium without serum and antibiotics (assuming that the serum could prevent the CPP penetration into the cell). The complexes at the concentration of 100 nM QD were added to a final volume of 150 µl per each well and were incubated for 2 hours at 37° C and 5% CO_2. Then, nuclei were stained by adding Hoechst 34580 (Invitrogen) at a final concentration 1 µl/ml and cells were further incubated for 10 minutes. Afterwards

cells were washed twice with DPB to remove free Hoechst and free QDs. Finally, after addition of 150 µl of DMEM/F-12 (without serum and pH indicator), plates were left in the incubator.
Just before imaging, plasma membranes were labeled by adding Concanavalin A-Alexa Fluor 647 (Invitrogen) to a final concentration of 25 µg/ml (150 µl final volume) and incubating for a 1 minute at room temperature. Cells were then washed 2 times with DPBS and finally kept in 250 µl of DMEM/F-12 (without serum and pH indicator). Live cells were immediately visualized by confocal microscope.

For analysis of the cell entry of Cy5-labeled-$Had_{UF1\text{-}11}$-C peptide in living cells, cell cultures were incubated with the fluorescent peptide (in DMEM/F-12 nutrient medium only) for 2 hours, and then washed twice with phosphate-buffered saline (PBS) alone. For analysis of cell entry of a dye, just before imaging, the plasma membrane was labeled with 5 (25) µg/ml rhodamine-conjugated concanavalin A (Invitrogen) for 5 (1, respectively) min at room temperature. Cells were washed twice DPBS and finally kept in DMEM/F-12 (without serum and pH indicator). Live cells were then immediately analyzed by confocal laser scanning microscopy using a Zeiss LSM operating system. Rhodamine (580 nm) and Cy5 (670 nm) were sequentially excited and emission fluorescence was collected.

Fluorescence imaging was carried out with a confocal microscope (LSM 710, Zeiss, Germany). The QDs-CaRuby-H11 were excited with 561 nm laser line and detected with 550-610 nm band-pass filter, Hoechst 34580 was excited with 405 nm laser line and detected with 389-500 nm band-pass filter and Concanavalin A-Alexa Fluor 647 was excited with 633 nm laser line and detected with 620-740 nm band-pass filter. The total acquisition time was 3min. Images were collected by the microscope software (ZEN 2011).

Flow cytometry analysis: BHK cells were incubated with various concentrations of Cy5-labeled peptide in DMEM-F12 culture medium without serum at 37°C for 2 hours. The cells were then washed with PBS to remove excess extracellular peptide and treated with 1 mg/ml trypsin-EDTA (Invitrogen) for 5 min at 37°C to detach cells from the surface, and centrifuged at 200 g before suspension in PBS. Flow cytometry analyses were performed with live cells using an Accuri C6 flow cytometer (BD Biosciences). Data were obtained and analyzed using CFlow Sampler (BD Biosciences). Live cells were gated by forward/side scattering from a total of 10,000 events.

Intracellular Ca calibration experiments were performed using a BD LSR Fortessa (BD Biosciences) flow cytometer. After QDs loading (as above), BHK cells were resuspended in a 30 mM MOPS and 10 mM NTA- buffered solutions at variable free [Ca2+] concentrations Slight cell membranes permeabilization was achieved by 0.06% Tritonx100 addition just before flow cytometer analysis. Live cells were gated by forward/side scattering from a total of 10,000 events and mean fluorescence intensity (MFI; 561 nm and 610 ± 20 nm for excitation and emission respectively) was determined for every condition.

Imaging cytometry: For imaging flow cytometry analysis, the same procedure of plating and incubation with QDs as for confocal microscopy was applied with the difference that after incubation the cells were trypsinized and resuspended in DPBS. DAPI staining (at a final concentration of 2.5 µg/ml) was used to reject dead cells. Cell images were acquired using multispectral imaging flow cytometer (Image Stream 100 with 12 channels and 2 cameras, Amnis Corporation), collecting 500 events per sample at 60 x magnification. A 561 nm wavelength laser was used to excite QD-CaRuby complexes and the emission fluorescence was collected using the 595-660 spectral detection channel. Cell images were analyzed using IDEAS image-analysis software (Amnis). Three main criteria were used to select cells for analysis. Starting from the “Begin analysis” wizard, firstly cells within the focal plane were selected using a histogram of the root-mean-squared gradient of the bright field image. Secondly, a gating based on a two-dimensional plot of area versus aspect ratio was used to isolate a population of single cells. A third step was to eliminate dead cells using a histogram of the fluorescence intensity in the DAPI channel. After that, “Masks” feature was applied to determine the cytoplasmic part of the cell. For this purpose an "Object" mask was created on the bright field image and it was eroded by 8 pixels to separate cell

membrane and cytoplasm of the cells. Finally, complex masking schemes as described in the “Spot Counting“ protocol (Amnis) were used to identify and calculate the number of particles inside the cells. More precisely, after a "Spot" mask was applied, an "Intensity" threshold was determined. Then a "Peak" mask was used to separate two close spots with one mask into two and a "Range" mask was applied to limit the mask to spots of a certain size in order to eliminate the noise. Final counting was carried out in the CaRuby channel.

Intracellular Ca^{2+} measurements on cells suspension: Plated 80% confluent HEK293 cells expressing NR1 and NR2B subunits of the NMDA receptor were exposed for 4 hours to 100 nM QDs in the medium without antibiotics nor foetal calf serum; after cells scraping, they were centrifuge to remove excess of QD, and then they were suspended in HEPES buffered extracellular medium, containing 1.3 mM Ca^{2+}.
Just before the measurement 10 mM Ca^{2+} final concentration was added to cells suspension and they were allowed to rest for 5 minutes. In order to reduce the basal fluorescence introduced by quantum dots attached to the external membrane and exposed to the high extracellular Ca^{2+} concentration 40 µM final concentration $CuSO_4$ was applied. The minimal concentration quenching both CaRuby emission and QDs was found experimentally.
Intracellular Ca^{2+} flux was measured using a JASCO FP-8300 spectrofluorometer (Japan) equipped with a CSP-829 sample compartment lid with syringe port for drug injection. A time-course measurement mode was performed with excitation at 545 nm (bandwith 5 nm) and a 606 nm (bandwith 5 nm) filter. A baseline fluorescence (F_{basal} signal) was recorded over 10 seconds, after which agonists of NMDA receptors (100 µM glutamate + 20 µM glycine final concentration) or antagonist of NMDA receptors (100 µM APV final concentration) were injected with a Hamilton micro syringe to the cuvette containing a 450 µl suspension of approximately 2 $x10^5$ cells and fluorescence (F) in response to the application was measured for 3 min.

The results are presented as $F- F_{basal}/ F_{basal}$. All measurements were performed using 2 replicates and in 3 independent experiments.

In cell imaging of single nanoparticules using TIRF microscopy: For single-nanobiosensor imaging we used a prism-type variable-angle total internal reflection fluorescence microscope (VA-TIRFM) described earlier[7] and modified for the experiments, Fig. S7. The beams of a 405-nm solid state laser (LasNova20, Lasos, Jena, Germany) and the 568-nm line of an Ar^+/Kr^+ mixed-gas laser (35 KAP 431, CVI MellesGriot, Carlsbad, CA) were combined (zt405RDC, AHF Analysentechnik, Tübingen, Germany), directed via all-mirror optics and a precision quartz prism (Bernhard Halle Nachf., Berlin, Germany) at a supercritical angle (q = 65.5 ± 0.5° [q_c=60.9°] to the cell/substrate interface. The calculated penetration depths of the evanescent fields set up by total internal reflection (TIR) were (1/e-intensity decay) ~75 nm (110 nm) at 405 (568) nm, respectively. Fluorescence was collected through a ×10/NA 0.3w (UMPlanFl) or a ×60/NA1.1w objective (LUMFl, both from Olympus-Europe, Hamburg, Germany), filtered with a HQ490DCXR primary dichroic and BA520IF long-pass filter, split up with a HQ590DCXR secondary dichroic into a green (BL562/40) and red (BP620/60) colour channel. Scattered laser light was rejected with two stacked 488/568-nm holographic rugate notch filters (Barr Precision Optics, Westford, MA) and the red and green component images projected via an achromatic telescope (f_1 = 50 mm, f_2 = 40 mm) side-by-side onto an electron-multiplying CCD detector (QuantEM512SC, Photometrics, Tucson, AZ). A wavelength-tunable Xe-arc monochromator (Polychrome II, TILL Photonics, Gräfelfing, Germany) provided whole-field epifluorescence (EPI) excitation. A green-light emitting diode (LED) mounted underneath the prism provided bright-field (BF) illumination. All shutters, light sources, acquisition and the piezo-focus drive (PIFOC, Physik Instrumente, Karlsruhe, Germany) were controlled with METAMORPH (MDS Analytical Technol., Sunnyvale, CA). Typical laser powers in the sample plane were ~1 mW for 405- and 300 µW for 568-nm excitation, with exposure times of 125 ms. Final magnifications were 508 ± 20 nm/px at ×10 with an extra magnification of ×2.

Small islets of flat, elongated BHK cells were imaged consecutively in bright field (BF) and upon 405-nm and 568-nm evanescent-wave (EW) excitation. Cells outside the excitation spot were not exposed to light, thereby minimizing photobleaching and allowing imaging several cells on the same coverslip. Glutamate, glycine and high Ca^{2+} (at 100 µM, 20 µM and 5 mM final concentration, respectively) were applied locally whilst the bath perfusion with standard extracellular saline was kept running to speed up solution exchange. 300 control and stimulation images were continuously acquired at 4Hz at 250-ms long exposure in the "stream" mode and processed using Metamorph (Molecular Devices).

We subtracted a dark image from each fluorescence image, extracted the average signal from 2-µm diameter circular regions of interest and corrected the resulting trace for photobleaching (fit of a single exponential). Ca^{2+} transients are shown as F/F_O, where F_O is the average basal fluorescence before NMDAR activation. Absolute signals emitted by a nanobiosensor were small and noisy as expected from the roughly 10 CaRuby molecules per QD. We therefore measured the mean peak F/F_O amplitude over 6 frames around the peak location determined from the 9-point box-filtered trace. Similarly, fluorescence risetimes were determined by a sigmoid fit to the rising phase of the F/F_O transient, again on the 9-points box-filtered trace.

ABBREVIATIONS

A, acceptor in a FRET pair; ACN, acetonitrile; APV, (2R)-amino-5-phosphonovaleric acid NMDAR antagonist; BAPTA,1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetra-acetic acid; BHK, baby hamster kidney cell line; Ca^{2+}, calcium (ion); CPP, cell penetrating peptide; CuAAC, Copper-Catalyzed Azide-Alkyne Cycloaddition; DCM, dichloro-methene; DMEM, Dulbecco's minimal essential medium; D, donor in a FRET pair; DHLA, dihydrolipoic acid; DIEA, N,N-Diisopropylethylamine; DPBS, Dulbecco's Phosphate-Buffered Saline ;E, FRET efficacy estimated on the donor; EDC, N-(3-Dimethylaminopropyl)-N'-ethylcarbonate; EDTA, Ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; F, fluorescence intensity; FP, fluorescent protein; FRET, Förster resonance energy transfer; GMBS, N-[γ-maleimidobutyryloxy]succinimide ester; H11, 11 aminoacids long peptide, $Had_{UF1\text{-}11}$, prepared from Hadrucalcin toxin; HEK: human kidney cells; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HWHM, Half Width at Half Maximum; MeOH, methanol; HOBt, hydroxybenzotriazole; MOPS, 3-(N-morpholino) propanesulfonic acid; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide ; NMDA, N-methyl-D-aspartate; NMDAR, NMDA-sensitive glutamate receptor type; NTA, nitrilotriacetic acid; pC, Ac-CGSESGGSESG(FCC)3F-amide peptide ; pK, NH_2-KGSESGGSESG(FCC)3F-amide peptide ; PEG, poly-ethylene glycol; PET, photoinduced electron transfer; PL, photoluminescence; QD, quantum dot, or fluorescent colloidal nanoparticle; SNR, signal to noise ratio; TAE, Tris-acetate-EDTA buffer; TEM, transmission electron microscopy; TFA, trifluoroacetic acid; TOP/TOP, Tri-n-octylphosphine/Tri-n-octylphosphine oxide.

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