Supporting Information for:

Spontaneous internalization of cell penetrating peptide-modified nanowires into primary neurons

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This file contains:

Materials and Methods

Figures S1-S3

Supporting References

Materials and Methods

Synthesis of silicon nanowires (Si NWs)

Si NWs were grown by a gold nanoparticle-catalyzed vapor–liquid–solid growth method.[1] Briefly, the growth substrate (600 nm Thermal SiO_2/Si, Nova Electronic Materials) was cleaned with an oxygen plasma (100 W, 3 min), treated with poly-L-lysine solution (0.1%, Ted Pella) for 5 min, and then rinsed thoroughly with deionized water. 1 mL of 80 nm gold nanoparticle solution (25 μg/mL, Ted Pella) was then deposited on the growth substrates (5 cm × 1 cm), maintained at room temperature for 5 min and then rinsed with deionized water. NW growth was carried out at 450 °C and 40 Torr with 1 standard cubic centimeters per minute (SCCM) SiH_4 (Ultra High Purity grade, Voltaix LLC.), 4 SCCM PH_3 (1000 ppm in H_2, Voltaix LLC), and 60 SCCM H_2 (99.9999%, Matheson Tri-gas) as reactant, dopant, and carrier gases, respectively. Growth was carried out for 15 min, and yielded NWs with lengths of *ca.* 10 μm; NW growth substrates were used within three weeks.

Surface modification of Si NWs

1. APTES modification: 95% APTES-ethanol solution was made with 9.5 mL of ethanol, 0.5 mL of deionized water, and 100 μL of (3-aminopropyl)-triethoxysilane (APTES, Sigma-Aldrich). The mixture was kept at room temperature for 20 min to promote activation of the APTES, and then a NW growth substrate was incubated in the solution for 20 min. The substrate was washed with ethanol 3 times and dried in a vacuum desiccator for at least one hour at room temperature. APTES coated NW substrates were immersed in deionized water for an hour prior to further modification to yield *ca.* monolayer APTES coating as reported previously.[2]

2. Streptavidin conjugation: A streptavidin solution was made by dissolving 0.2 mg of streptavidin labeled with Alexa Fluor 555 (STV, Invitrogen) and 4 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Sigma-Aldrich) in 200 μL of PBS. After washing the APTES-modified NW substrate three times with 10 mM PBS (pH 7.4), the STV and EDC solution was deposited onto the NW substrate and allowed to react for 2 hours at room temperature, and then rinsed 3 times with 10 mM PBS. To avoid evaporation during the modification step the reaction was done in a sealed petri dish with a 10 mM PBS-saturated sponge to maintain constant humidity. NWs were detached from the substrate by brief sonication in 10 mM PBS (~ 1 sec; 40 kHz, 120 W). The resulting STV conjugated NWs (STV-NWs) were used as is for control experiments.

3. TAT conjugation: 10 μL of biotin-TAT (47 – 57) (Anaspec Inc., 0.1mg/mL in PBS) was dropped on the STV-NW substrate and kept overnight at 4 °C in a humid environment. The substrate was then rinsed three times with 10 mM PBS and sonicated in 10 mM PBS (~ 1 sec; 40 kHz, 120 W) to detach NWs from the substrate. The resulting TAT-modified NWs were lyophilized or centrifuged and dried for preservation. The TAT-modified NWs were re-suspended in 10 mM PBS at a concentration of *ca.* 0.1 mg/mL immediately prior to use.

Culture of mouse hippocampal neurons

E18 Mouse combined cortex, hippocampus and ventricular zone tissue (BrainBits, LLC.) was digested and plated following manufacturer's protocol.[3,4] Briefly, the tissue was digested in 2 mg/mL of papain for 10 min at 30 °C, and triturated for 1 min with fire polished glass Pasteur pipettes. The cells were collected using centrifugation and plated onto poly-D-lysine (MW 70,000 – 150,000, Sigma-Aldrich) coated coverslips with the cell density of 1×10^6 in NbActiv4

media (BrainBits LLC). The cells were cultured for 7 – 14 days before use and the media was replenished every 3 days.

Culture of dorsal root ganglia (DRG) cells

Dissociated DRG cells were prepared as described previously.[5] P14-6 Long Evans rats (Charles River Lab Inc.) were anesthetized by respiratory inhalation of isoflurane. After deep anesthetization, the rat was decapitated, the spinal column was removed and the ganglia were detached and bisected. The ganglia were digested with papain (20 U/mL, Worthington Biochem Corp.) solution in 37 °C for 20 min and then transferred to collagenase (3 mg/mL, Worthington Biochem Corp.) / dispase (4 mg/mL, Roche Holding AG) solution for another 20 min. After digestion, the DRG cells were transferred to L-15 (Life Technology) and DMEM (Life Technology) media and triturated with fire polished glass Pasteur pipette. Nerve growth factor (NGF, 100 ng/mL, Sigma-Aldrich) was added to the media and 100 μL of cell solution was plated on poly-D-lysine/laminin-coated coverslips (12 mm, NeuVitro) for 30 min. After plating, 3 mL of Neurobasal media (Life Technology) with NGF was added and stored in 4 °C or cultured inside the incubator. All procedures performed on the vertebrate animal subjects were approved by the Animal Care and Use Committee of Harvard University. The animal care and use programs at Harvard University meet the requirements of the Federal Law (89-544 and 91-579) and NIH regulations and are also accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

Neuron culture with TAT- and STV-NWs

TAT- or STV- conjugated NWs were dispersed in NbActiv4 media at a concentration of 5 μg/100 μL, and then 100 μL of NW solution was drop-cast onto the mouse hippocampus neuron

culture and incubated for 30 min in the cell culture incubator. After rinsing with warm culture media, the NW-treated cell culture was kept in the incubator for 6, 20, and 32 hours; samples were analyzed (see below) for each of the specific incubation periods. For DRG cells, the NWs were dispersed in Neurobasal instead of NbActiv4 media with all other procedures remaining the same. Following incubation, WGA – Alexa Fluor 594 (Image-iT Live, Molecular Probe) diluted 1000x with culture media was added to the culture and incubated for 10 min at room temperature for staining the cell membrane. The cytoplasm was labeled with Calcein-AM (included in Neurite outgrowth staining kit as the cell viability indicator, Molecular Probe, diluted 1000x) for 20 min at 37 °C. Cells were then fixed with 5% of glutaraldehyde (Sigma-Aldrich) for 15 min at 37 °C and rinsed twice with 10 mM PBS buffer for 3 mins.

Fluorescence labeling for live cell imaging

5 μg of TAT-NWs or STV-NWs and WGA – Alexa Fluor 594 dye (diluted 1000x) was mixed together with 100 μL of the culture media and applied to the cells. The cells were incubated at 37 °C for 30 min and placed inside the live cell imaging chamber that was filled with warm culture media. Calcein-AM dye (diluted 1000x) was added for 20 min at 37 °C when labeling the cytoplasm was necessary for cell viability checks.

Live cell imaging chamber

The chamber shown in Figure 4a was made from a 50 mm diameter polystyrene petri dish, with. an ~1 cm hole was drilled in the lid for insertion of the objective lens for imaging. In addition, a 2 cm × 2 cm silicon substrate with gold markers attached to the bottom part of the petri dish to allow for registration of cultured cells (Figs. 4a,b). The gold markers were defined using standard methods[6] by electron beam lithography and thermal vacuum deposition. The chamber

was placed on a temperature controller TC344b (Warner Instrument) to monitor and control temperature at 37 °C.

Confocal Microscope Imaging

An Olympus FV1000 upright confocal microscope was used for imaging fixed and live cells. 405, 559, and 635 nm laser sources were used with water immersion objective lenses. Minimum pinhole size (150 nm) was used for Z-stack imaging with slice thickness of 500 nm. Differential interference contrast (DIC) was used for observation of cell morphology with mercury light source illuminated from the top of the microscope. All images were acquired by Olympus Fluoview software v2.1.

Image Processing.

To obtain reconstructed cell surfaces, confocal Z-stack images were processed with Volume Viewer v.2.01 plugin in ImageJ, NIH.[7] Confocal images were converted to RGB format and reconstructed with a built-in function and standard parameters.

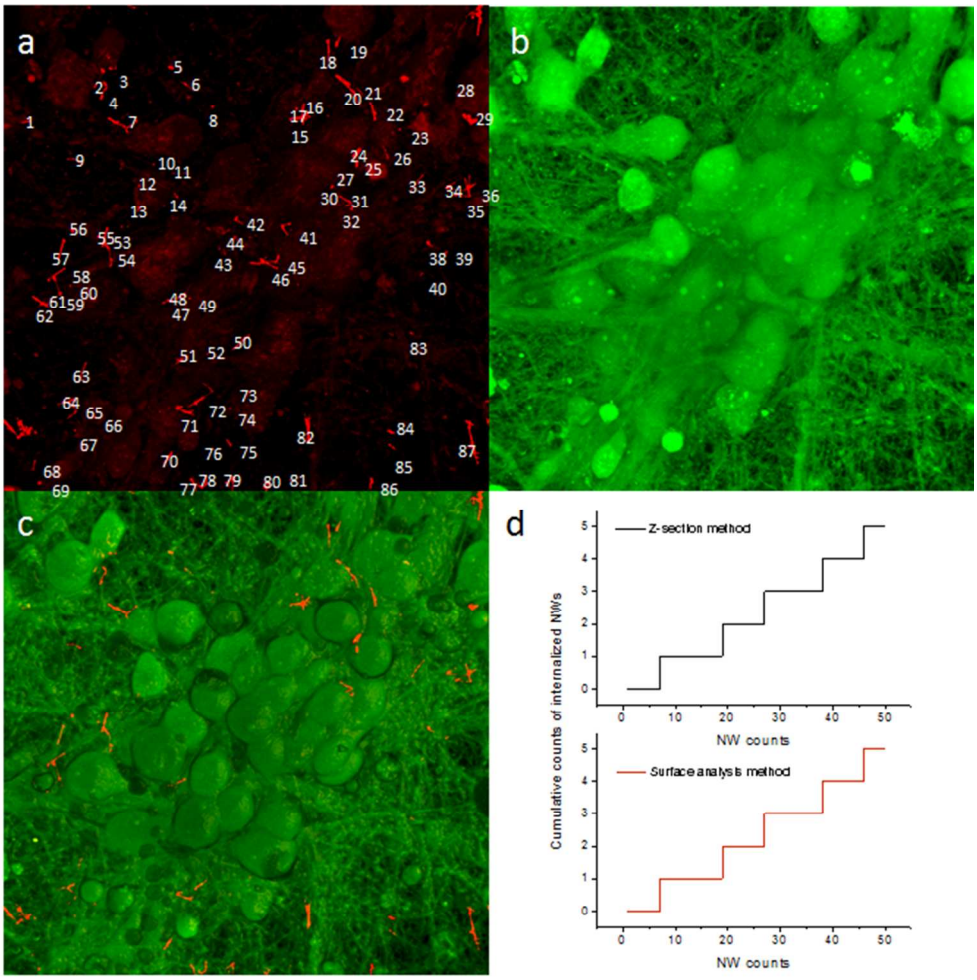

Figure S1. Large area analysis of TAT-NW internalization. (a) Confocal Z-stack image of the NW channel showing TAT-NWs labeled with Alexa-555. All TAT-NWs visible in this channel were counted; 20x objective lens. (b) Confocal microscope image of the neuron channel labeled with Calcein-AM dye. (c) Surface-reconstructed image of (a) and (b) processed by *Volume Viewer* in *ImageJ, NIH*. Panel (a) and (c) were compared and NWs visible in (a) but not in (c) were considered internalized. (d) Performance comparison of the surface analysis method and the standard confocal Z-section. The plots show cumulative counts of internalized NW assessed by confocal Z-section (Top) and surface reconstruction (Bottom) methods. 50 NWs were randomly selected and their internalization was assessed with both methods on the same NW. The two methods show the same cumulative plots, indicating that the surface reconstruction has the same performance with confocal Z-section method.

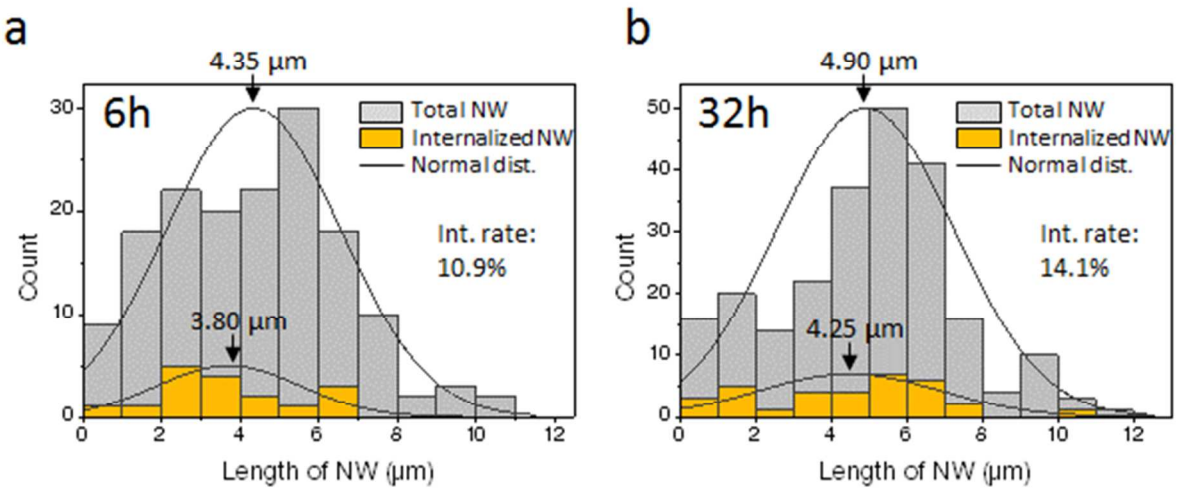

Figure S2. Histograms of TAT-NW internalization in mouse hippocampal neurons with incubation times of (a) 6 and (b) 32 hours at 37 °C. The gray and orange colored bars represent total TAT-NWs and internalized TAT-NWs, respectively. Overall internalization percentages were 10.9% and 14.1% for incubation times of 6 and 32 h, respectively. Mean values of TAT-NW lengths calculated from normal distribution fittings are shown on top of each curve. The surface analysis method was used for the analyses.

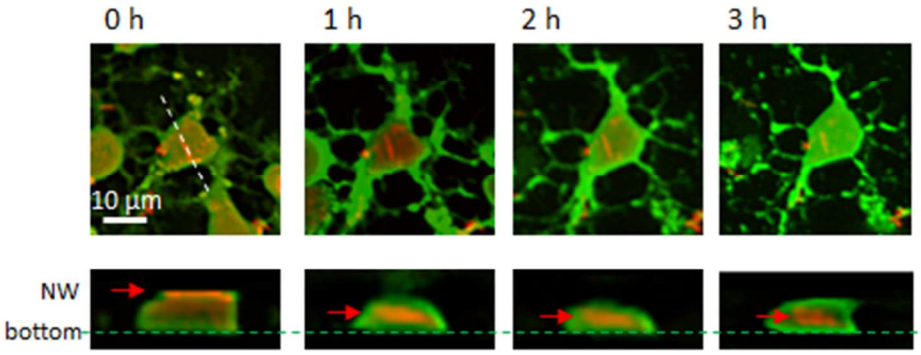

Figure S3. Live cell confocal microscope images of a neuron (green) treated with TAT-NWs (red). The cell was imaged with confocal Z-stacks every hour for three hours. Top row: top-down view of the cell with all Z-stack images overlaid at the maximum intensity; bottom row: Z cross-section view on NW along the white dashed line in the top left panel. The TAT-NW observed on top of the neuron at 0 h was later observed inside the cytoplasm after 1 h. Neuronal cytoplasm and TAT-NW were labeled with Calcein-AM and Alexa-555, respectively.

Supporting References

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