Probing enzymatic activity inside living cells using a nanowire-cell “sandwich”assay

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Experimental methods

Preparation of Si NWs by Etching

SiNWs were formed by dry-etching silicon wafers coated with thermally grown silicon oxide (200 nm) as previously described (ref shalek et al, PNAS, 2010). Briefly, a resist, prepared by suspending 100-nm-diameter colloidal gold nanoparticles (Ted Pella) in a solution of 3% polymethyl-methacrylate (PMMA) in chlorobenzene, was spun-coat on top of each silicon wafer. The locations of the gold nanoparticles in the resulting 100 nm thick PMMA-nanoparticle film were then transferred to the wafer's thermal oxide using a CF4 plasma in an electron cyclotron resonance (ECR) reactive ion etch (RIE) system (NEXX Systems). After removing the gold nanopaticles with TFA gold etchant (Transene), the wafers were etched using a Bosch Process (a cycled SF6:C4F8 plasma) in an inductively coupled plasma (ICP) RIE (STS MPX/LPX) so as to generate high aspect ratio SiNWs at the sites of the patterned silicon oxide dots. Finally, the silicon oxide etch mask was removed by briefly dipping the wafers in 49% hydrofluoric acid.

Surface modification of SiNWs

Prior to functionalization, SiNWs were exposed to an oxygen plasma for 10 min, 25 mW. The treated SiNWs were subsequently incubated in 1.5% (v/v) 3-aminopropyltrimethoxysilane (APTMS, Sigma-Aldrich) in methanol in the presence of acetic acid (5% v/v) as a catalyst for 30 min at room temperature. The NW plates were then rinsed with methanol and water, and then dried. To covalently attach enzymatic substrate peptides to the SiNWs, the APTMS-treated SiNWs were further reacted with succinimidyl-6-[(β-maleimido-propionamido)hexanoate] (SMPH 10 mg/mL, Thermo Scientific) for an hour in sodium bicarbonate buffer (0.1M, pH 8.0). After rinsing with water, the SiNWs were incubated with the desired peptides (10 μM) for an hour in the same buffer. Simple adsorption of the fluorescent peptide on SiNWs was achieved by dropping a small aliquot of the fluorescent peptide solution (~ 5 μL) onto the SiNWs, followed by evaporation.

Cell culture and sandwich assay

HeLa and PC3 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) and RPMI 1640, respectively, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. For enzyme activity assays, cells were plated in 12-well plates

bearing fibronectin (25 μg/mL, Sigma-aldrich) coated SiNWs (5 mm x 5 mm) at a final density of ~ $5x10^4$ cells/cm^2, and incubated overnight. Before sandwiching, cell-seed NW plates were further treated with TrypLE Express (Life Technologies) to remove any excess cells that were not firmly immobilized on the SiNWs. Enzyme activity was measured by assembling a sandwich using two different SiNW samples as following: cells that had been immobilized on one SiNW plate were softly placed on top of a peptide-treated SiNW substrate that was immersed in an appropriate medium. When relevant, an apoptosis-inducer, staurosporin (500 nM, Sigma-Aldrich), or a PKA inducer, forskolin (10 μg/mL, Sigma-Aldrich), was added directly into the medium just prior to sandwich formation. Assembled SiNW sandwiches were disassembled after set periods of time for further analysis. For PTP inhibition, HeLa cells on SiNW were pre-incubated with PTP inhibitors, α-bromo-4-hydroxyacetophenone (Calbiochem, 80 μM) and sodium orthovanadate (Sigma, 200 μM) for 30 min before sandwich assembly and then incubated for an hour in the presence of the inhibitor.

Cell immobilization

The selection of 3-5 μm long SiNW arrays was beneficial in preventing the transfer of cells from the top SiNW sample to the bottom one while in a sandwich assembly. SiNWs of these lengths proved long enough to firmly anchor cells; shorter SiNWs (1-2 μm in length) work better than flat samples, but do not fully prevent cells from transferring to the peptide-attached SiNW sample (data not shown). We similarly found that it was critical to maintain an evenly distributed monolayer of cells on SiNWs: this allows the cells to be uniformly penetrated without any of them migrating to the peptide-attached NWs where they could affect subsequent analyses. To achieve this, we seeded an excess number of HeLa cells on the SiNWs (final density ~$5x10^4$ cells/cm^2) and treated the samples with a TrypLE Express solution supplied as 1X for 5 min before forming our sandwich assembly to gently remove any superficially adhered cells that were not firmly penetrated by the SiNWs. To examine whether cells were stably immobilized on the SiNW arrays, HeLa cells immobilized on one SiNW plate were sandwiched and incubated overnight. The following day, the cell distribution on both plates was visualized by staining with 1 μM CFDA. As shown in the left column of Figure 1b, in the absence of a TrypLE Express pretreatment, some cells were observed to transfer to the bottom SiNW plate; when these weakly bound, multilayered cells

were removed with a TrypLE Express pretreatment, no cells transferred (right column of Figure 1b).

Fluorescent nanowire labeling and confocal imaging

For fluorescent SiNW labeling experiments (figure 1a), samples were coated with 3 μL of 1 mg/mL Alexa Fluor succinimidyl ester (SE) (Invitrogen) in DMSO and incubated at 37C. After 30 minutes, samples were washed thrice in distilled, sterile water, and blown dry. The day after plating, cells were incubated with 1 μg/mL Hoechst (Invitrogen) for 30 minutes and then subsequently dipped into a solution containing a 1:200 dilution of Vybrant DiI cell labeling solution (Invitrogen) in PBS. After 5 minutes, these samples were rinsed through PBS and then imaged using an upright confocal microscope (Olympus). Confocal reconstructions were performed using Imaris (Bitplane).

Cell viability and fluorescence microscopy

Cell viability and membrane integrity were examined by staining cells with carboxyfluorescein diacetate (CFDA, Sigma-Aldrich) and propidium iodide (PI, Sigma-Aldrich). After sandwich disassembly, SiNW-plated cells were incubated in full media supplemented with 1 μM of each solution for 5 min. Samples were then washed with Dulbecco's Phosphate Buffered Saline (DPBS) twice before imaging. Fluorescence images were acquired using an upright fluorescence microscope (Zeiss Axioskop2 FS plus with AxioCam MRm camera). Fluorescence images for measuring peptide stability and caspase-3 activity were obtained using either an upright fluorescence microscope or an inverted fluorescence microscope (Deltavision, Applied Precision).

In our caspase-3 experiments, imaging of the peptide substrate-containing SiNW sample showed spot-like fluorescence coming from the SiNWs even in staurosporine's presence (data not shown), suggesting that most TAMRA molecules on peptides remained on the SiNWs.

MALDI measurements

After disassembly of the SiNW sandwiches, the degree of enzyme-mediated peptide

modification was quantified by measuring mass changes to our peptide probes. Since all of the peptides used in this study were designed to have a TEV protease recognition site (ENLYFQ), our covalently attached peptides could be released from the SiNWs by treating them with TEV protease. To achieve this, peptide-coated SiNWs were immersed in TEV buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM DTT) containing ~50 unit AcTEV protease (Invitrogen), and incubated at 30 ℃ for 4 hours. The collected peptide solution was then loaded on a $ZipTip_{c18}$ (Millipore) to remove excess salts and proteins and extensively washed with 0.1% trifluoroacetic acid in water. Next, the washed samples were eluted from the tip with α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) in 50% acetonitrile/50% water containing 0.1% trifluoroacetic acid and transferred to a MALDI sample plate. MALDI-TOF measurements were performed on a Voyager analyzer (Applied Biosystems).

Peptide substrate preparation

All of the substrate peptides used in this study were prepared by Peptron (South Korea). Peptide purity (>95%) was doubly confirmed by HPLC and MALDI spectra. The exact sequence of each peptide is described in SI table 1.

cAMP measurements

$5x10^4$ of PC3 cells were seeded on 24-well plate and incubated overnight. Cells were treated with or without forskolin and incubated appropriated time (15, 30, and 60 minutes). Then, 1 mL of 0.1M HCl was added to the cells and left for about 10 min for cell lysis. After verifying cell lysis by inspecting cells under a microscope, supernatants were collected and centrifuged at >600 g for 10 min. Proteins in the supernatants were quantified by Bradford assay, and the same amounts of cell lysates were applied for cAMP measurements (cAMP enzyme immunoassay kit, Sigma-Aldrich). Detailed experimental procedures were followed according to the protocol provided by the supplier.

Quantitative real-time PCR analysis

HeLa cells, cultured on SiNWs for 24 hours were sandwiched with Probe NWs for 4 hours. Total cellular RNA from both sandwiched cells and non-sandwiched cells culture on SiNWs as a control was isolated using QIAGEN RNeasy Mini Kit (QIAGEN), according to the

manufacturer´s instructions. Quantitative mRNA expression analysis of five housekeeping genes (hypoxanthine phosphoribosyltransferase 1 (HPRT1), β-actin (ACTB), β-glucuronidase(GUSB), β-2-microglobulin(B2M), glyceraldehydes 3-phophate dehydrogenase(GAPDH)) were performed as described previously (ref Shalek et al, PNAS, 2010). The mRNA expression for each gene was normalized to the 18SrRNA control and compared to the 2-ΔΔCT method.

Figure S1. Cell viability tests after sandwich incubation and disassembly. Cell viability and membrane integrity were confirmed using two different staining methods. Live cells were stained with CFDA, while the nuclei of dead cells were stained using PI. HeLa cells on SiNWs (Untreated) showed very bright CFDA fluorescence and no PI staining. Disruption of the cell membrane with the detergent Triton X-100, on the other hand, induced cell death, as indicated by low levels of CFDA fluorescence and high nuclear PI fluorescence. Meanwhile, HeLa cells on SiNWs after sandwich disassembly showed positive CFDA staining and were negative for PI fluorescence (Sandwiched), indicating that sandwiching did not adversely affect cell viability or membrane integrity. Scale bar, 50 μm.

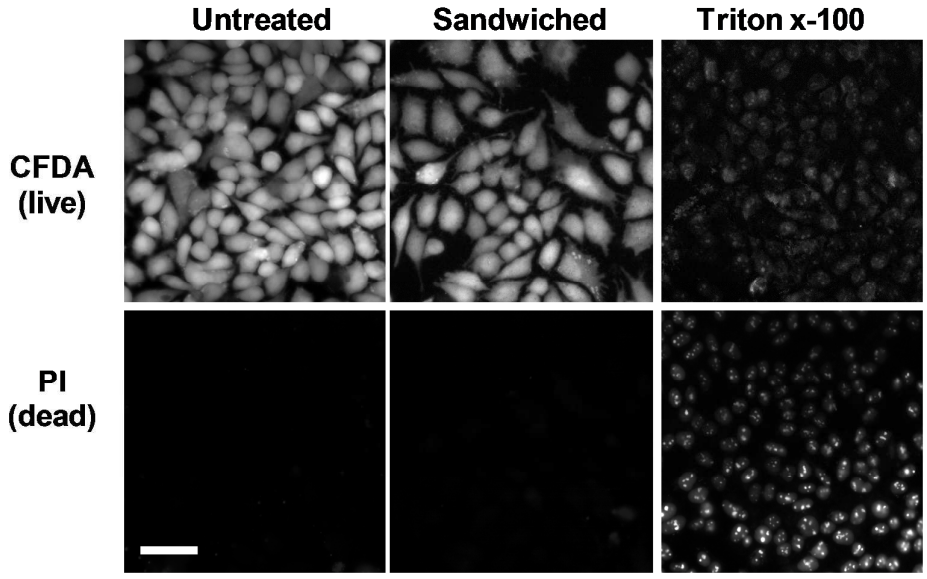

Figure S2. Relative mRNA expression levels were determined by quantitative real-time PCR for cells on NWs and sandwiched cells with NWs for 4 hours. The expression levels of five housekeeping genes were minimally affected by the sandwich assay.

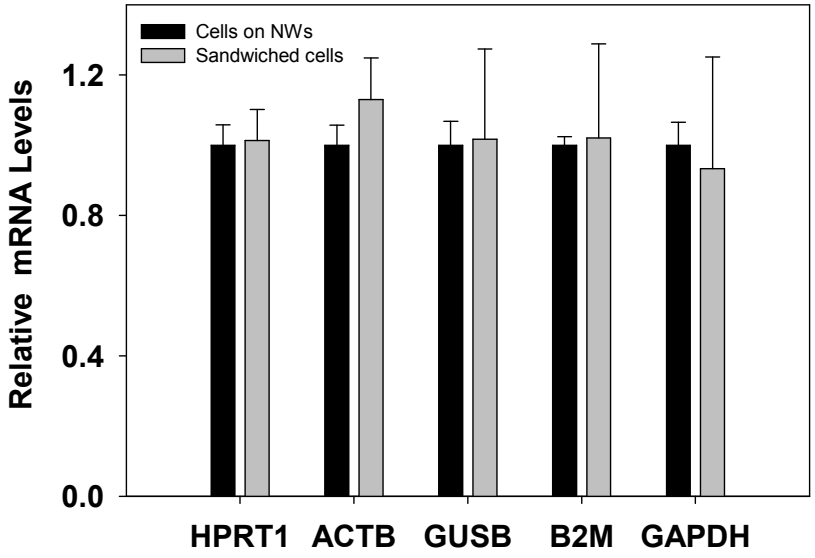

Figure S3. Representative SEM image of gold nanoparticle (AuNP)-attached NWs via biotin-straptavidin chemistry. APTMS-treated SiNWs were treated with NHS-biotin, followed by straptavidin-coated AuNPs (d ~30 nm). Reagents were used at lower concentrations than usual to clearly visualize the AuNP binding. The NW plate bound with AuNPs was then examined by SEM (Nova NANOSEM 200, FEI Company).

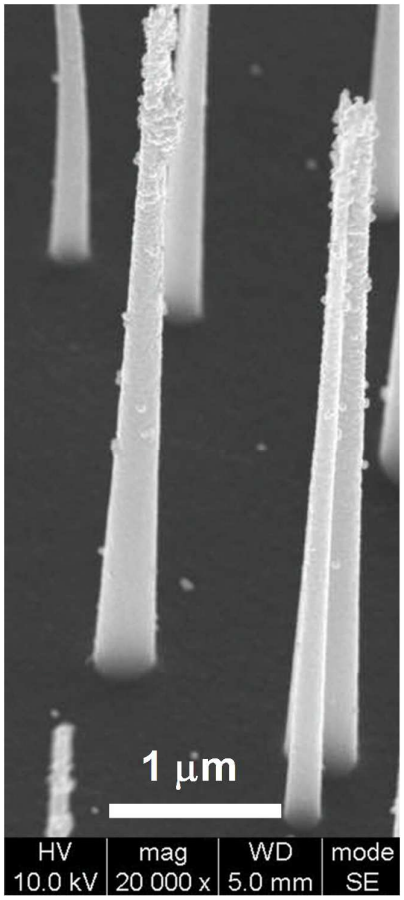

Figure S4. To evaluate the reproducibility of the assay, we repeated the PTP sandwich assay and mass spectrometric data were obtained from five independent experiments using independently prepared NWs and HeLa cells based on the same experimental procedure as in Figure 3.

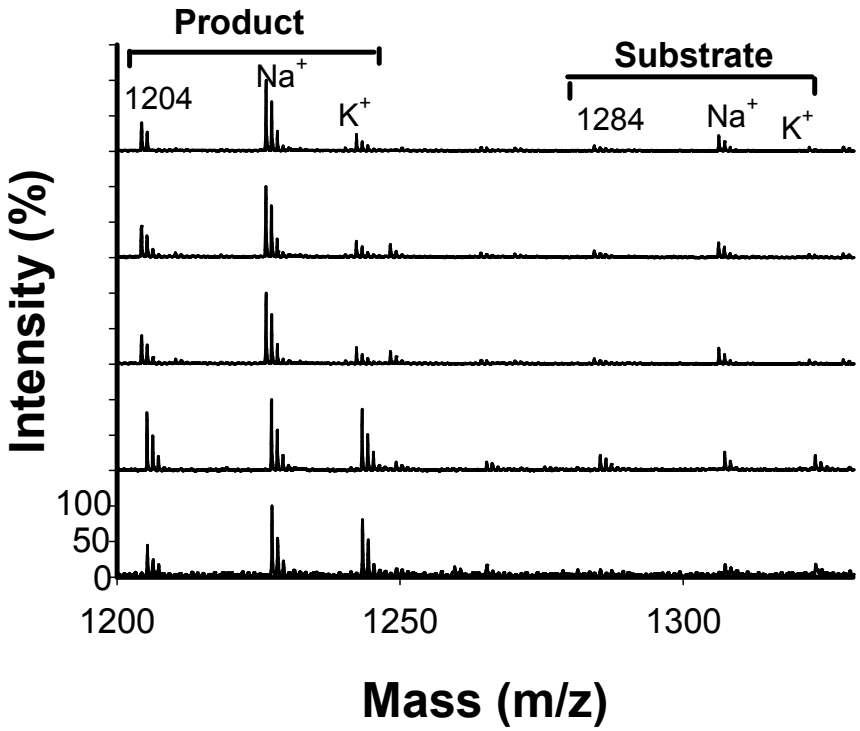

Figure S5. Endogenous PKCδ activity in HeLa cells. PKCδ substrate peptide molecules were covalently attached to the probe NWs, and then sandwiched with immobilized HeLa cells on the NW cell holder for 60 minutes. Bound peptides were subsequently released with TEV and analyzed by MALDI-TOF (Voyager analyzer). The peptide substrate sequence for PKC3 was KRREILSRRPSYRENLYFQGG-Ahx-C-NH_2 , where Ahx is 6-aminohexanoic acid.

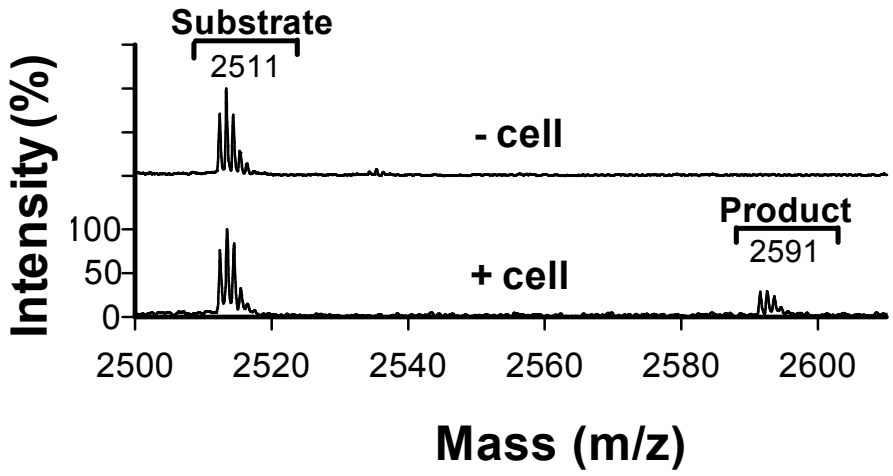

Figure S6. cAMP measurements in PC-3 cells. Cells seeded on 24-well plate were either non-treated or treated with forskolin and incubated for appropriated times. Same amounts of cell lysates produced by addition of 0.1M HCl were applied on the cAMP-specific antibody coated plate wells to quantify cAMP concentration using known standards. cAMP levels increased within 15 minutes, and remained high but not fell down to the basal level for 1 hour.

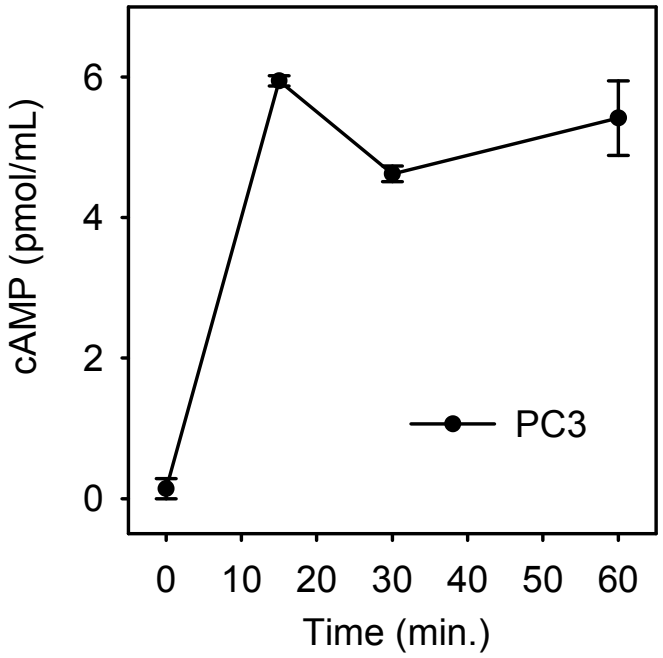

Table S1. Peptide substrate sequences used in this study. All of the peptide substrates have a cysteine residue either at their C- or N- terminus to enable covalent attachment to the SiNWs. The recognition site for each target enzyme is highlighted in red while the TEV enzyme cleavage site is highlighted in blue.

Target enzyme	Sequence
PTPs	phosphoYGGGGENLYGQGGGG-Ahx*-C-NH_2
Caspase-3	AADEVDGGASLGGENLYFQGGGGAhx*-C-NH_2
Caspase-3	TAMRA5**-AADEVDGGGGENLYFQGGGGAhx*-C-NH_2
PKA	C-Ahx*-GENLYFQGGLGGLRRSLG-NH_2
Phosphatase	LRRA-phosphoS-LGGENLYFQGGGG-Ahx*-C-NH_2

* Ahx: 6-aminohexanoic acid

** This peptide was modified with TAMRA for fluorescence imaging