Direct tracking of amyloid and Tau dynamics in neuroblastoma cells using nanoplasmonic fiber tip probes

Feng Liang, Yiying Zhang, Wooyoung Hong, Yuanlin Dong, Zhongcong Xie, and Qimin Quan

Nano Lett., Just Accepted Manuscript • DOI: 10.1021/acs.nanolett.6b00320 • Publication Date (Web): 06 Jun 2016

Downloaded from http://pubs.acs.org on June 9, 2016

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a free service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are accessible to all readers and citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.
Direct tracking of amyloid and Tau dynamics in neuroblastoma cells using nanoplasmonic fiber tip probes

Feng Liang 1, Yiyeng Zhang 2, Wooyoung Hong 1,3, Yuanlin Dong 2, Zhongcong Xie 2* and Qimin Quan 1*

1 Rowland Institute at Harvard University, Cambridge, Massachusetts, 02142, USA.
2 Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts, 02129, USA.
3 Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts, 02138, USA.

ABSTRACT: Amyloid plaques and neurofibrillary tangles are the pathological hallmarks of Alzheimer's disease. However, there has been a long standing debate on the dynamic relations between Aβ and Tau proteins, partially due to the lack of a tool to track protein dynamics in individual live neurons at the early stage of Aβ generation and Tau phosphorylation. Here we developed nanoplasmonic fiber tip probe (nFTP) technology to simultaneously monitor Aβ42 generation and Tau phosphorylation (at serine 262) in living, single neuroblastoma cells over 12 hours. We observed that Aβ42 generation, under clinically relevant anesthetic treatment,
preceded Tau phosphorylation, which then facilitated Aβ42 generation. This observation is also supported by measuring proteins in cell lysates using the ultrasensitive label-free photonic crystal nanosensors. nFTP therefore provides an advanced method to investigate protein expression and post-translational modification in live cells and determine outcomes of intervention of Alzheimer’s disease and other neurodegenerative disorders.

TOC GRAPHIC:

![Graph showing protein levels over time](image)

KEYWORDS: Nanoplasmonic fiber tip probe (nFTP), protein dynamics, Aβ, Tau, Alzheimer's disease, photonic crystal nanosensor

Aβ hypothesis posits that Aβ generation triggers a cascade of neurotoxic events, including Tau phosphorylation, which leads to neurofibrillary tangles ¹. However, Tau hypothesis suggests that Tau phosphorylation leads to Aβ accumulation and eventually the formation of amyloid plaques ², ³. Debates still remain on the precedence and causal relations between Aβ generation and Tau phosphorylation ⁴, partially due to the lack of a technique to simultaneously measure the
dynamics of Aβ and phosphorylated Tau proteins in the same individual neuron over time at
their native conditions. Current techniques that measure intracellular proteins either are
destructive to cells (e.g., enzyme-linked immunoassay, western blot) or require transfected cell
lines or transgenic animals (e.g., fluorescent imaging)\(^5,\,6\). Recent development in nanosensors
demonstrated measurement of action potentials using nanowire field-effect transistors\(^7\), optical
endoscopy with nanofibers\(^8,\,9,\,10\), carbon nanotubes\(^11,\,12\), nanowires\(^13\) and nanobeam cavities\(^14\).
Gold nanoparticles have been used to visualize localization of proteins and RNAs in live cells\(^15,\,16\). However, these approaches do not analytically quantify the sensitive changes of protein levels.
These limitations impose a major hurdle in studying protein dynamics in living cells and
clinically obtained primary samples. Here we demonstrate direct observation of dynamic change
of Aβ42 and phosphorylated Tau (at serine 262) proteins in single living neuroblastoma cells
using the nanoplasmonic fiber tip probe (nFTP) technology. Our measurements suggest a
hierarchical and circular pattern between Aβ generation and Tau phosphorylation: Aβ generation
precedes and leads to Tau phosphorylation, and phosphorylated Tau also promotes Aβ
generation. This conclusion is also supported by measuring protein concentrations from lysed
cells using photonic crystal label-free nanosensors, thus establishing nFTP as a reliable method
for live cell research.

The nFTP was comprised of a nanoscale optical fiber with a single gold nanorod
biosensor on its tip (Figure 1a). A broadband white light source (Tungsten-halogen lamp)
excited the localized surface plasmon resonance (LSPR) of the gold nanorod through the optical
fiber (Supporting Figure S1). The LSPR spectrum displayed a wavelength shift when proteins
bound to the gold nanorod (Figure 1b), which was detected from the inverted microscope
(Olympus IX73) and analyzed with a spectrometer (Princeton Instrument). The method and
fabrication of nFTP was specified in our previous work \(^{17}\). In the current research, our primary focus is to establish nFTP as a reliable method to study the Aβ-Tau problem. First, blocking non-specific binding is proved to be an essential step for intracellular measurements (Supporting Figure S2). Prior to the single cell measurements, each functionalized nFTP was tested in the cell culture medium containing pre-mixed solution of proteins. A positive spectrum shift, if observed, would verify the successful functionalization of the antibodies on the sensor surface (Supporting Figure S3). To dynamically measure protein expressions in the same cell, we used glycine solution (pH 2.5 tuned with hydrogen chloride) to unbind proteins from the antibodies while preserving the antibodies on the sensor (Supplementary Fig. S4). Figure 1c illustrates one measurement cycle: nFTP was sequentially incubated in the regeneration and blocking solution. It was then brought into proximity to the cell, controlled by the motorized stages. The baseline spectra were first taken outside of the cell. The nFTP was then inserted into the cell, incubated for two minutes and retracted out of the cell. The signal spectra were taken at the same focal position as the baseline spectra. The LSPR shift between the baseline and the signal spectra, induced by the captured proteins (e.g., Tau-PS262), quantified their intracellular concentrations. This regenerate-block-baseline-insert-incubate-retract-signal completed one measurement cycle, and could be repeatedly performed in the same single live neuron or cell.

We measured both Tau-PS262 and β-Actin levels in the wild-type neuroblastoma SH-SY5Y cell, Tau over-expressed SH-SY5Y cell (SH-SY5Y-Tau cell), mouse cortex neuron and Tau knock-out mouse cortex neuron (Neuron-Tau\(^{−/−}\)). All these cells were first treated with isoflurane to trigger Tau-PS262 generation. Results summarized in Figure 2a showed clear difference in Tau-PS262 expressions between different cell types, while similar levels of β-Actin levels were observed as control. LSPR shifts can be converted to protein concentrations by
calibrating nFTP using known concentrations of Tau-PS262 solutions. As shown in Figure 2b, the sensor has a detection range from 0.5 nM to 0.5 µM, equivalent to approximately 300 to 300,000 copies of proteins in the volume of a typical cell (~10 µm size). This dynamic range of proteins falls in the biologically relevant concentration of intracellular proteins.\(^{18}\)

We assessed the cell membrane integrity, enzymatic and metabolic activity after the cell was punctured by nFTP once every hour, consecutively for 12 hours. First, we used the cell viability/cytotoxicity assay (L3224, Invitrogen): 2 µM calcein-AM and 4 µM ethidium homodimer-1 (ethD-1) were applied to the cell. After incubating for 40 minutes, cell medium was replaced by 1 mL Dulbecco's phosphate-buffered saline (DPBS) and cells were imaged under fluorescent microscope. The calcein-AM is a green fluorescent dye that indicates intracellular esterase activity. EthD-1 is a red fluorescent dye that stains nucleic acid, indicating a compromised cell membrane. As shown in Figure 3a, the dash-circled cell was punctured by the nFTP and showed green fluorescence and no red fluorescence. The solid-circled cell, punctured by a microscale fiber showed red fluorescent color and no green fluorescence. These results indicated that puncturing by the nFTP (but not microscale probe) preserved esterase activity and membrane integrity.

Next, we performed cell vitality assay (L34951, Invitrogen): 500 nM C12-resazurin and 10 nM SYTOX DNA binding dye were applied to the cell and incubated for 15 minutes. Reduction of C12-resazurin to red-fluorescent C12-resorufin indicates active metabolic condition. SYTOX is a cell-impermeant, green-fluorescent nucleic acid stain that indicates compromised cell membrane. In Figure 3b, the dash-circled cell was punctured by the nFTP and the solid-circled cell was punctured by a microscale probe. The presence of red fluorescent color and
absence of green color in the dash-circled cell indicated that the nFTP (but not microscale probe) puncturing preserves cell metabolic activity and membrane integrity.

Now to study Aβ42 and Tau-PS262 dynamics simultaneously in the same cell, we built the mechanically revolving dual-nFTP ([Supporting Figure S6]) and functionalized the gold nanorod surfaces with Aβ42 antibodies and Tau-PS262 antibodies, respectively. Inhalation anesthetic isoflurane has been shown to induce Aβ accumulation \(^{19}\) and Tau phosphorylation (increase in Tau-PS262 levels without significant changes in total Tau levels) \(^{20}\), which provides a clinically relevant tool to simultaneously induce both Aβ generation and Tau phosphorylation.

We first performed a control experiment in the SH-SY5Y-Tau cells without the treatment of isoflurane. As shown in **Figure 4a**, neither Aβ42 nor Tau-PS262 levels increased under this control condition. Each data point in all of the plots represents the averaged value of three consecutive measurements in the same cell. Next, we treated SH-SY5Y-Tau cells with 2% isoflurane for two hours, and then measured Aβ42 and Tau-PS262 levels simultaneously every two hours up to 12 hours. In contrast to the control condition, isoflurane significantly increased the Aβ42 and Tau-PS262 levels (**Figure 4b**). Moreover, the time to reach a 50% increase in Aβ42 level (approximately 4 hours) was less than that needed for a 50% increase in Tau-PS262 level (approximately 7 hours). These data suggest that Aβ42 generation precedes Tau phosphorylation (at serine 262).

Next, we set out to study whether Aβ generation and Tau phosphorylation could interfere with each other. We treated the SH-SY5Y-Tau cells with 0.5 μM γ-secretase inhibitor (L-685,458, Sigma L1790) one hour before the isoflurane treatment, which has been demonstrated to suppress Aβ accumulation \(^{19}\). In the same cell, we observed simultaneous decrease in the Tau-PS262 level (**Figure 4c**). We also used 3 μM Tau phosphorylation inhibitor
(K252a, Abcam ab120419) to treat SH-SY5Y-Tau cells for one hour before the isoflurane treatment, which has been shown to suppress Tau-PS262 generation\textsuperscript{21, 22}. Western blot was also carried out to verify K252a efficacy (Supporting Figure S7). In the same cell, we observed Aβ42 level first increased but finally reduced after 10 hours (Figure 4d). These data indicate that inhibition of Tau phosphorylation led to a partial reduction in Aβ42 levels. Taken together, these results suggest a hierarchical and circular pattern between Aβ generation and Tau phosphorylation: Aβ generation precedes and leads to Tau phosphorylation, and phosphorylated Tau also promotes Aβ generation.

Our studies are the first direct measurements of intracellular Aβ generation and Tau phosphorylation in living cells. To further verify our major conclusion, i.e. Aβ precedes Tau phosphorylation, we prepared multiple cell cultures, treated them with isoflurane, lysed them and harvested their intracellular proteins at different times after the isoflurane treatment. As proteins were significantly diluted during this process, we used the ultra-sensitive photonic crystal label-free nanosensors to measure the Aβ and Tau-PS262 levels in each sample.

The photonic crystal nanosensor is a silicon waveguide with periodic structures deterministically designed and patterned along the waveguide (Figure 5a)\textsuperscript{23, 24}. These structures provide constructive interference and create a high-\textit{Q} optical resonance, which is sensitive to any proteins that bind on the sensor surface\textsuperscript{25, 26}. Therefore, protein concentrations in the cell lysates can be quantified by measuring the optical resonance shift. Precise fabrication of the designed structures was achieved using electron beam lithography and reactive ion etching processes (see Supporting Information). A Polydimethylsiloxane (PDMS) microfluidic channel was bonded on the chip for fluid delivery\textsuperscript{27}. We functionalized the surface of the photonic crystal nanosensor with Aβ42 antibody (ab10148, Abcam) and phospho-specific Tau antibody (anti-Tau-PS262, sc-
32828, Santa Cruz) respectively (see Supporting Information). Cell lysates taken at different times after the isoflurane treatment were flowed into the microfluidic channel (Figure 5b). The resonance shifts observed from the photonic crystal nanosensors are highly consistent with the live-cell measurement by nFTP: as shown in Figure 5c, the time to reach a 50% increase in Aβ42 level (approximately 5 hours) was less than that needed for a 50% increase in Tau-PS262 level (approximately 7 hours). The nFTP approach, therefore, is proved to be a reliable method that has the unique capability of revealing protein dynamics in single live cells. The limitation of nFTP includes low throughput to interrogate large amounts of cells, which would be improved by multiplexing single probes into probe arrays. For the same reason, we did not perform statistical analysis for the data obtained; however, the findings were confirmed in repeated experiments.

In conclusion, nFTP technique brings traditional immunoassay to the nanoscale dimension and nano-molar sensitivity by integrating a nanoplasmonic sensor on a nanoscale optical fiber tip. This technology, at the same time, enables the detection of post-translational modifications of proteins in live cells, which traditionally have been measured by first separating the proteins via electrophoresis, chromatography or immunoprecipitation, and detecting them with mass spectrometry or fluorescent-labeling methods. The nFTP approach is particularly powerful to study protein dynamics and post-translational modifications in live cells, as well as to determine the outcomes of interventions of neurodegenerative diseases. Its high-sensitivity, free-of-labels and single cell capability make it a unique tool to diagnose clinically obtained, limited, primary cells.
Materials and Methods

Nanoplasmonic fiber tip probe (nFTP) fabrication. The nFTP was fabricated by wet-etching an optical fiber (SM28, Thorlabs Inc.) with hydrogen fluoride chemistry by precisely monitoring and controlling the etch time. Gold nanorods were dispersed on the cover slip and then picked up onto the nano-size tip of the nFTP by using a micro-manipulator under the dark field of an inverted microscope. A UV-curing optical adhesive (Norland NOA 128) was used to adhere a nanorod to the tip. The nFTP had a tip size of approximately 50 nm; the gold nanorod was 86 nm long, and had a 25 nm diameter cross-section (Nanopartz Inc.).

Surface functionalization. 1 mL of cetrimonium bromide (CTAB) capped gold nanorods (Nanopartz Inc.) was mixed with 100 µL of a 20 mM solution of 11-mercaptoundecanoic acid (11-MUA, Sigma-Aldrich) prepared in ethanol. The mixture was sonicated for 90 minutes at 55°C and kept at room temperature overnight. These nanorods were centrifuged at 5,500 rcf for 10 minutes and re-dispersed in water to remove the excess 11-MUA. A single nanorod was immobilized on the tip of the nFTP. The assembled nFTP was incubated in 100 mM 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Sigma-Aldrich) with 100 nM of antibodies to the target proteins. In this work, we used anti-Ab42 (ab10148, Abcam), anti-Tau-PS262 (sc-32828, Santa Cruz) and anti-β-Actin (ab13822, Abcam). To block the non-specific binding, the FTP was incubated in the cell culture medium in which fetal bovine serum (FBS) was replaced by 1% bovine serum albumin (BSA).

Cell culture. The wild-type human neuroblastoma SH-SY5Y cells [CRL-2266, American Type Culture Collection (ATCC)] and Tau overexpressed SH-SY5Y-Tau cells (generous gift of Dr. Luc Buee from Inserm UMR1172, France) were cultured at 37 °C in a humidified incubator with
5% CO₂ in a DMEM F-12 medium (ATCC, 30-206) supplemented with 10% fetal bovine serum (ATCC, 30-2020), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma, P4333), 1% (v/v) nonessential amino acids (Sigma, M7145) and 2 mM L-Glutamine (Sigma, 59202C).

**Anesthetic isoflurane treatment.** Twenty-one percent oxygen, 5% carbon dioxide, balanced nitrogen, and 2% isoflurane were delivered from an anesthesia vaporizer to a sealed plastic box in a 37°C incubator containing plates seeded with the SH-SY5Y-Tau cells for two hours. Twenty-one percent oxygen, 5% carbon dioxide and balanced nitrogen without isoflurane were used in the control condition.
Figure 1. Nanoplasmonic fiber tip probe (nFTP). (a) Scanning electron micrograph (SEM) image of nFTP. Inset shows a single gold nanorod attached to the nFTP. (b) Localized surface plasmon resonance (LSPR) spectra measured before (green) and after (red) inserting the nFTP into the cell. LSPR spectrum was collected using an electron multiplying charge coupled device (EMCCD) spectrometer (Princeton Instrument) on an inverted microscope (Olympus IX73). (c) During each measurement cycle, nFTP was first regenerated in pH 2.5 glycine solution. 1% bovine serum albumin (BSA) mixed into the cell culture medium (without fetal bovine serum) was then used as the blocking solution. The baseline LSPR spectra were taken in close proximity to the cell. The nFTP was then inserted into the cytoplasm, incubated for two minutes, and retracted from the cell. The signal LSPR spectra were immediately taken at the same focus spot as the baseline spectra. The LSPR shift from the baseline spectra to the signal spectra \( \Delta \text{LSPR}=4.5 \text{ nm} \) in (b) quantified the intracellular Tau-PS262 level.
Figure 2. Specificity and sensitivity. (a) The nFTP sensors were functionalized with anti-Tau-PS262 (sc-32828, Santa Cruz) and anti-β-Actin (ab13822, Abcam), and were employed to measure Tau-PS262 and β-Actin proteins in different cell types after the cells were treated with isoflurane. The error bar comes from three measurements on the same cell. (b) LSPR shift at different concentrations of Tau-PS262 solutions. 0.5, 1, 5, 10, 50, 100, 500 nM Tau-PS262 (Santa Cruz, sc-32828p) in the cell culture medium (without FBS) were prepared, and sequentially tested. At each concentration, 1,000 spectra were measured. The nFTP was regenerated between two different concentrations.
Figure 3. Cell viability and vitality assay on SH-SY5Y-Tau cells. The dash-circled cell was punctured by nFTP once every hour for 12 hours. The solid-circled cell was punctured by a microscale fiber. (a) Cell viability/cytotoxicity (L3224, Invitrogen) assay. Green-fluorescence indicated active esterase condition. Red-fluorescence indicated loss of cell membrane integrity. (b) Cell vitality (L34951, Invitrogen) assay. Green-fluorescence indicated loss of cell membrane integrity. Red-fluorescence indicated active metabolic condition.
Figure 4. Aβ42 and Tau-PS262 dynamics. (a) Aβ42 and Tau-PS262 levels were monitored in the same SH-SY5Y-Tau cell when no isoflurane was applied (control condition). (b) Aβ42 and Tau-PS262 levels were monitored after the treatment of isoflurane in the SH-SY5Y-Tau cell up to 16 hours. (c, d) Dynamic Aβ42 and Tau-PS262 levels in SH-SY5Y-Tau cells, which were pre-conditioned with 0.5 µM γ-secretase inhibitor (c) or 3 µM phosphorylation kinase inhibitor K252a (d) prior to the isoflurane treatment.
**Figure 5.** Aβ42 and Tau-PS262 detection in the SH-SY5Y-Tau cell lysates using photonic crystal nanosensors. (a) Scanning electron microscope (SEM) image of the photonic crystal nanobeam cavity. (b) Schematics of the sensor chip, where light paths are denoted in blue and fluid paths are denoted in grey. The sensor chip is fabricated in silicon-on-insulator (SOI) platform. Telecom lasers were coupled to the edge of the chip and collected from the opposite side. Microfluidic channels were bonded on the chip for sample delivery. (c) Resonance shifts of the photonic crystal nanosensors measured in different cell lysates collected at different times after the isoflurane was applied.
ASSOCIATED CONTENT

Supporting Information Available: The supporting information includes details of the surface functionalization, regeneration, calibration and mechanical design of the revolving dual-nFTP and western blot validation of Tau phosphorylation inhibitor K252a. It also includes the fabrication and surface functionalization of the photonic crystal nanosensors.

This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

* Email: quan@rowland.harvard.edu (Q.Q) and zxie@mgh.harvard.edu (Z.X).

Author Contributions

Q.Q., Z.X., Y.Z., F.L. designed the research. F.L. performed the experiments with contributions from Y.Z., W.H.. Y.D. prepared the mice primary neurons. Q.Q., Z.X., F.L., Y.Z. analyzed the experimental results and wrote the manuscript.

Funding Sources

The research was supported in part by the Rowland Junior Fellowship Award at Rowland Institute at Harvard University, Cambridge, Massachusetts (to Q.Q.), and in part by R01 GM088801 and R01 AG041274 from the National Institutes of Health, Bethesda, Maryland (to Z.X.).

Notes

The authors declare no competing financial interest.
ACKNOWLEDGMENT

The authors are thankful for helpful discussions with Diane Schaak at Rowland Institute at Harvard University. The authors thank Fang Fang and Lining Huang from Massachusetts General Hospital and Harvard Medical School for their help in harvesting cortex neurons of mice. The cost of isoflurane was generously provided by the Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital and Harvard Medical School.

REFERENCES

Measure protein levels in live cells

TOC graphic
391x157mm (300 x 300 DPI)