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In vivo imaging of acetylcholine release in the peripheral nervous system with a fluorescent nanosensor

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2 *Works for me* dx.doi.org/10.17504/protocols.io.bmmxk47n

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ABSTRACT

This protocol provides a detailed decription of the experimental procedure, including nanosensor farbication and characterization, *in vivo* microinjection, and *in vivo* imaging of acetylcholine release in the submandibular ganglion of mice.

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KEYWORDS

Nanosensor, Actylcholine, neurotransmission, optical sensor, fluorescent sensor, submandibular ganglion

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MATERIALS TEXT

MATERIALS

⊠ α-Bungarotoxin Alexa Fluor 647 conjugated **Thermo Fisher Scientific Catalog #B35450**

⊠ Anesthetic (Isoflurane) **Fisher Scientific Catalog #NC9259743** Step 3

⊠ Vesamicol **Sigma Aldrich Catalog #V104** Step 5

⊠ Acetylcholinesterase from Electrophorus electricus (electric eel) **Sigma Aldrich Catalog #C2888**

⊠ pHAb Reactive Dyes **Promega Catalog #G984A**

⊠ Tetrazine-PEG5-NHS ester **Contributed by users Catalog #BP-22681**

⊠ SM(PEG)12 (PEGylated long-chain SMCC crosslinker) **Thermo Fisher Scientific Catalog #22112**

⊠ TCO-PEG4-NHS ester **Click Chemistry Tools Catalog #A137**

⊠ Acetylcholine chloride **Sigma Aldrich Catalog #A6625**

SAFETY WARNINGS

α-bungarotoxin is a snake venom and is toxic. It should be properly disposed of.

Nanosensor fabrication

1 The structure of the DNA scaffold was designed to accommodate four AChE enzymes (electrophorus electricus, Millipore-Sigma), four pHAb fluorophores (Amine-reactive, Promega) and two BTX molecules (AF 647 conjugated, Thermo fisher) while minimizing the overall size. Preliminary sequences were obtained by a andom sequence generator to anneal into a dendritic secondary structure with four branches and a focal point as confirmed by RNAfold minimum free energy calculations and Nanoengineer-1 Program (Molecular Dynamics Studio).Single-stranded DNA oligonucleotides with amine or thiol functional groups were purchased from Sigma. pHAb dyes were conjugated to the DNA strands (L2, L3, L4) by the vendor.

1. Conjugation of Tetrazine groups to single-stranded DNA oligoneucleotides.

This is for further conjugation with BTX via Click Chemistry.

Reactions were conducted in 0.1 M $NaHCO_3$ solution (pH=~8.1-8.2) at a DNA concentration of 100-150 µM. Modified single strands were firstly separated from excess pHAb dyes and tetrazine cross linkers by 2 rounds of ethanol extraction, and further purified by illustra NAP-10 columns (GE Health) via gel filtration, followed by overnight lyophilization (Labconco).

2. Assembly of the DNA scaffold.

The DNA scaffold was self-assembled by incubating 5 DNA oligonucleotides following a temperature gradient from 95 °C to 4 °C for 6 h in a thermocycler (Mastercycler® nexus X2, Eppendorf) in Tris-EDTA buffer (TE buffer, 10mM tris and 1mM EDTA). Misfolded DNA scaffolds were removed by a size-exclusion column (Superdex 200 increase 10/300 GL column, GE Health) via HPLC (Infinity 1260, Agilent) using phosphate buffer (500 mM NaCl) as the eluent. The correct fraction was collected and resuspended in TE buffer via buffer exchange. The fully assembled DNA scaffold can be

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stored in a -20 °C freezer for up to 6 months.

3. Purification of AChE

We employed size-exclusion HPLC to purify AChE since the commercially-available AChE (from eel extract) is not of 100% purity. The correct fraction was collected for further modification with maleimide functional groups.

4. Conjugation of AChE and BTX to the DNA scaffold

For protein functionalization, 30 to 50 µM of AChE was swirled with maleimide linkers (maleimide-PEG_{12}-NHS ester, Pierce SM$(PEG)_{12}$ Thermo Scientific) for 2 h before being purified by 100 k Amicon centrifugal filter 5 times. BTX was maintained at ~500 µM when reacting with TCO linker (TCO-PEG4-NHS ester, 377 Click Chemistry Tools). In the aforementioned protein-linker reactions, either AChE and BTX solution was incubated with corresponding cross linkers at a molar ratio of 1:20 to keep the balance between linkage efficiency and protein activity. Assembled DNA scaffolds were pretreated with TCEP for 1.5 h at RT to cleave the disulfide bonds and then removed from excess TCEP via Amicon centrifugal filters (30k cut-off) prior to enzyme conjugation. Maleimide- functionalized AChE was later vortexed with the DNA scaffold at a molar ratio of 5:1 for 2 h. Afterwards, TCO-functionalized BTX was added to the reaction mixture at a BTX to DNA molar ratio of 20:1, allowing a TCO-tetrazine ligation reaction between BTX and the DNA scaffold for another 2 h. The unreacted thiol groups were later capped by ethylmaleimide (Fisher Chemical). The final reaction mixture was vortexed for 30 min before transferring to a 4 °C fridge for overnight incubation.

5. HPLC purification of the nanosensor

After protein-DNA conjugation, ACh nanosensors that contain DNA scaffold, AChE and BTX were separated from other impurities by a size-exclusion column (Superdex 200 increase 10/300 GL column, GE Health) via HPLC (Infinity 1260, Agilent). The elution solution was 0.15 M phosphate buffer with NaCl concentration of 500 mM. Three UV signals (i.e., 260 nm and 280 nm for DNA and protein absorbance, 652 nm for AF 647) and one fluorescence signal (i.e., Ex=532 nm, Em=560 nm for pHAb) were monitored during elution. After purification, ACh nanosensors were concentrated by 100k Amicon® ultra centrifugal filters, and then aliquoted for long-term storage at -20 °C.

Nanosensor calibration

2 Anesthetized mice were sacrificed by cervical dislocation immediately after the microinjection of anosensors/control nanosensors. The SMG along with the salivary duct and the gland were quickly excised under a dissection scope. The dissected tissue was positioned within an open diamond bath imaging chamber (RC-26, Warner Instruments). The salivary duct was carefully stretched with an insect pin as a metal harp (Warner Instruments) was placed on top to suppress and stabilize the SMG (Fig. 2). After placing the chamber onto the microscope stage, 200 µL of freshly prepared Ringer's buffer (pH=7.3-7.4) was added under the 63x water-immersion objective, followed by 500 µL injections of ACh solutions ranging in concentration from 0.001 µM to 10 mM. The final concentration of ACh was adjusted based on a dilution factor of 1.4. The entire experiment was performed using an Olympus BX61WI fluorescence microscope. After each measurement, the ACh solution was carefully removed using Kimwipes, followed by three rounds of washing with Ringer's buffer. The sample was then immersed and incubated in the buffer for 10 min to enable the nanosensor to recover to baseline fluorescence signals. For the calibration of control nanosensors, we repeated the same procedure, with the exception of using citrate-phosphate buffers for pH<6 and phosphate buffers for pH≥7. PBS (pH=7.3-7.4) was used for intermittent washings in between measurements of the control nanosensors.

In vivo staining of submandibular ganglion with nanosensor by microinjection 1h

3 1. Anesthetize the mouse by inhalation of 1.5-2%

Anesthetic (Isoflurane) **Fisher Scientific Catalog #NC9259743**

and place it on a rodent heating pad set at **37.7 °C**

2. Place the mouse on its dorsal side under the dissection scope.

3. Apply depilatory cream to the neck region for **00:01:00** and remove the hair.

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4. Make a small incision on the neck region and expose the salivary glands.
5. Seperate salivary ducts from the surrounding connective tissues.
6. Transfer the anethetized mouse to the stage of an upright fluoresence microscope (BX61W, Olympus)

Fixed stage microscope
Microscope
Olympus **N/A**

7. Lift the salivary ducts by using a support platform controlled by a micromanipulator for stabilization.
8. Pull glass needle using a P-97 micropippette puller and borosilicate glass tube with 1.0 452 mm/0.78 mm OD/ID.

P-97 micropipette puller
Flaming/Brown Micropipette Puller
Sutter instrument **N/A**

9. Load glass needle with **4 µl** of nanosensor solution.
10. Apply vacuum grease around the incision area to contain an aqueous solution without leakage.
12. Fill the cavity created by grease with Ringer's solution and switching to 63x objective.
13. Secure the needle to the injection arm controlled by Eppendorf Inject Man 4.

InjectMan 4
micromanipulation
Epperndorf **N/A**

14. Approach the needle towards the submandibular ganglion from the proximal end at an angle of 25 degree.
15. Under 63x objective, pierce the needle into the SMG for injection.
16. Incubate nanosensor with SMG for **00:05:00** .
17. Washing the injeciton area with Ringer's buffer for **00:10:00** .

In vivo imaging of ACh release evoked by electricical stimulation

4 1. After microinejction step is done, connect a parallel bipolar electrode (FHC Inc.) to the micromanipulator arm.

parallele bipolar electrode
microelectrode
FHC **30210**

2. Place the electrodes directly on the salivary ducts, sitting above the SMG (closer to the lingual nerve).
3. Produce electrical pulses using a MyoPacer filed stimulator triggered by a pulse generator. Use bipolar pulses with an amplitute between 3-10V, frequency of 10Hz, pulse duration of 10ms, and pulse number between 10-100.

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MyoPacer Cell Stimulation
Stimulator

Ion Optix **N/A**

PulserPlus
Pulse generator

Prizmatix **N/A**

In vivo imaging of ACh release blocked by ACh inhibitor

5 1. After microinjection is done, apply [M]**100 Micromolar (µM)**

Vesamicol **Sigma**

Aldrich Catalog #V104

to the neck region for **00:10:00** .

2. Image ACh release by electrical stimulation.
3. Wash away drug solution with Ringer's buffer for **00:10:00** .
4. Image ACh release by elecrical stimulation to study recovery in response.

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