Protocol

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This protocol describes the differentiation of human neural progenitor cells (hNPCs) in a microfluidic device containing a thin 3D matrix with two separate chambers, enabling a cleaner separation between axons and soma/bulk neurons. We have used this technique to study how mitochondria-associated ER membranes (MAMs) regulate the generation of somal and axonal amyloid β (Aβ) in FAD hNPCs, a cellular model of Alzheimer’s disease. This protocol also details the quantification of Aβ molecules and isolation of pure axons via axotomy.

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Highlights

3D microfluidic separation of axons from soma of AD neural cellular model, FAD hNPCs

Quantitation of axonal versus somal Aβ molecules released from FAD hNPCs

Isolation of pure axons from differentiated FAD hNPCs via axotomy

Application to show that MAM-modulation regulates axonal Aβ generation in AD

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Protocol

Microfluidic separation of axonal and somal compartments of neural progenitor cells differentiated in a 3D matrix

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SUMMARY

This protocol describes the differentiation of human neural progenitor cells (hNPCs) in a microfluidic device containing a thin 3D matrix with two separate chambers, enabling a cleaner separation between axons and soma/bulk neurons. We have used this technique to study how mitochondria-associated ER membranes (MAMs) regulate the generation of somal and axonal amyloid β (Aβ) in FAD hNPCs, a cellular model of Alzheimer’s disease. This protocol also details the quantification of Aβ molecules and isolation of pure axons via axotomy.

For complete details on the use and execution of this profile, please refer to Bhattacharyya et al. (2021).

BEFORE YOU BEGIN

This protocol provides a detailed workflow to carry out microfluidic isolation of the axons or axonal microenvironments from bulk neurons or somal environments using two-chamber microfluidic devices. We have extensively used human neural progenitor cells overexpressing the FAD-mutant of APP (APPswe/Lon), namely FAD hNPCs, in our studies because 10-day differentiated cells generated high levels of Aβ40 not only from bulk neurons in the somal compartments, but also from the axons in axonal compartments. Use of microfluidic chamber slides to separate axonal environments from somal to measure axonal and somal Aβ is not unique. Several studies have reported fluidic separation between somal and axonal compartments of microfluidic devices containing differentiated neurons (Niederst et al., 2015; Das et al., 2016). However, the fluidic separation does not prevent the flow of cells from somal to the axonal compartments, thus contaminating the axonal compartments with bulk neurons. Here, we have introduced a thin-layer 3D matrix in the groove towards the somal compartment of the microfluidic devices to prevent contamination of bulk neurons in axonal compartments. This technique allowed us to more precisely determine axonal Aβ generation in differentiated FAD hNPCs in these microfluidic devices.

The protocol described here utilizes both commercially available as well as in-house two-chamber microfluidic devices. The commercially available (Xona) devices are primarily used to measure the number of Aβ molecules per axon or per cell body upon modulation of axonal MAMs.
(mitochondria-associated ER membranes) via sigma-1 receptor activation and inactivation. Our in-house devices are designed and implemented to physically separate axons by axotomy to perform biochemical analysis to compare MAMs from axons versus soma/bulk neurons. The commercially available Xona devices are not ideal for biochemical assays because the devices are made with materials that are not appropriate for physically severing the axonal side from the somal side for axotomy. On the other hand, the in-house devices are not suitable for Aβ measurement because the somal and the axonal compartments only hold ~50 μL condition media as opposed to large somal and axonal compartments in Xona microfluidic devices that hold ~200 μL conditioned media. At least 100 μL axonal conditioned media is required to perform ELISA assays in duplicate for each sample. It is possible to design in-house devices containing large somal and axonal compartments. We are currently engaged in designing such devices.

**Validation (24 h)**

There are several experimental factors that need to be perfected before beginning this protocol. Below, we have provided these experimental considerations in detail.

**Figure 1. Measuring somal and axonal Aβ from FAD hNPCs differentiated in 3D microfluidic chambers**

(A) Schematic diagram of a commercial (Xona) microfluidic chamber. The somal groove on the somal compartment is attached with the axonal groove on the axonal compartment by 120 capillaries of 150 μm length. 3D matrix is formed by adding 30 μL Matrigel (1:10) in the somal groove to allow separation of the environments between the somal and the axonal compartments. In addition, use a phase separation by creating a 30 μL volume difference by adding 180 μL media each on the somal compartments and 150 μL media each on the axonal compartments.

(B) Representative Aβ ELISA assay validating that the phase separation effectively prevented any flow from the somal to the axonal side. Both Slide 1 and Slide 2 are microfluidic chamber slides containing 3D matrix (1:10 Matrigel) in the somal groove. Slide 1 contained 1 pM synthetic Aβ40 only in the somal compartments and no Aβ40 in the axonal compartments. Slide 2 was created by adding 1 pM Aβ40 only in the axonal compartments and no Aβ in the somal compartment. After 24 h, Aβ40 levels were measured in both compartments from Slide 1 and Slide 2. Slide 1 somal compartment retained nearly all Aβ40, while Slide 2 retained nearly all Aβ40 in its axonal compartments. (n=3).

(C) Representative image (20× objective) of GFP-expressing FAD hNPCs (Green fluorescence) seeded in 3D microfluidic chamber slide showing little or no contamination of the axonal compartment with bulk neurons (only 2 out of 30,000). Cells are also labeled with Hoechst (Blue fluorescence) to label nucleus.

(D) Representative image (60× objective) of Tau-labeled (red fluorescence) axons sprouting out of 6 capillaries (arrow heads) of a chamber slide containing 120 capillaries.

(E) Graphical representation of Table 3 showing number of Aβ molecules per cell body and per axon. N=6. p < 0.001.

(F and G) Quantitative analyses of number of Aβ molecules per cell body (F) or per axon (G) from untreated (veh) or NE-100 treated (NE) cells. N=3. p < 0.001.
Before measuring somal and axonal Aβ, we first validated that Aβ from the somal compartment did not flow to the axonal compartment in our 3D microfluidic chambers. We also validated that the 3D matrix prepared on the somal side prevented any flow of bulk neurons to the axonal side.

**Phase separation of somal and axonal compartments**

To prevent flow of Aβ from the somal compartments to the axonal compartments, in addition to generating a 3D matrix inside the somal groove, we also created a 30 μL phase separation by adding 180 μL media to each somal compartment, and 150 μL media to each axonal compartment (Figure 1A). To validate that Aβ did not flow from the somal compartments to the axonal compartments or vice versa, we made two types of chamber slides:

**Slide 1:** Here, we prepared a 3D matrix into the grooves on the somal side and added 180 μL media containing 1 μM synthetic Aβ40 to each somal compartments. We added 150 μL blank media in the axonal compartments.

**Slide 2:** In a separate microfluidic chamber, after making the 3D matrix in the groove on the somal side, we added 180 μL blank media to the somal compartment and 150 μL media containing 1 μM synthetic Aβ40 to the axonal compartments.

After 24 h, we collected somal and axonal media to measure the Aβ40 levels in each compartment using Wako Aβ ELISA assay kit based on the standard values (Table 1, Raw data of standard, and the slope and intercept values). We measured 918.12 ± 49.96 pM Aβ40 in the somal compartment and undetectable amounts of Aβ in the axonal compartment from the Slide 1 chamber slides (Table 1 and Figure 1B). On the other hand, the somal compartment of the Slide 2 chamber slides contained no detectable Aβ40, while the axonal compartment contained 929.65 ± 69.10 pM Aβ40 (Figure 1B). This validated that our 3D chamber slides containing a 30 μL volume phase-separation between the somal and the axonal compartments effectively separated somal environment from the axonal environment.

**Note:** Synthetic Aβ40 peptides were prepared and purified by J. I. Elliott at Yale University (New Haven, CT) using solid-phase peptide synthesis. Bulk powdered Aβ peptides were initially dissolved and incubated (18 h) at room temperature (RT; 24°C) in 30% trifluoroethanol (1 mg/mL) before lyophilization and storage (−200°C) under nitrogen for 2–3 months. Before experimentation, dried peptide films were solubilized in 10 mM NaOH as described by Kumar et al. (2016).

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**Table 1. 3D matrix separated Aβ in somal compartments from Aβ in axonal compartments**

| Std (pM) | A450 | A450 | Average (A450) | Linear regression |
|---------|------|------|----------------|------------------|
| Standards: |      |      |                |                  |
| 0.0 (Blank) | 0.036 | 0.039 | 0.037          | Slope: 0.0148   |
| 1.50  | 0.077 | 0.104 | 0.090          | Intercept: 0.0949 |
| 3.12  | 0.148 | 0.154 | 0.151          |                  |
| 6.25  | 0.195 | 0.191 | 0.193          |                  |
| 12.50 | 0.265 | 0.35  | 0.307          |                  |
| 25.00 | 0.519 | 0.497 | 0.508          |                  |
| 50.00 | 0.877 | 0.887 | 0.887          |                  |
| 100.00 | 1.542 | 1.538 | 1.540          |                  |

| A450 | Aβ (pM) | Av. Aβ (pM) |
|------|---------|-------------|
| Somal (Slide 1) | 0.807 | 0.749 | 0.811 | 0.742 | 958.49 | 0.039 | 0.037 | 0.037 | 0.037 |
| Axonal (Slide 1) | 0.048 | 0.051 | 0.062 | 0.047 | −71.63 | −71.63 | −71.63 | −71.63 | −71.63 |
| Somal (Slide 2) | 0.061 | 0.04 | 0.049 | 0.05 | −53.99 | −53.99 | −53.99 | −53.99 | −53.99 |
| Axonal (Slide 2) | 0.901 | 0.714 | 0.794 | 0.834 | 950.35 | 832.27 | 940.85 | 995.14 | 929.65 |

Before measuring somal and axonal Aβ, we first validated that Aβ from the somal compartment did not flow to the axonal compartment in our 3D microfluidic chambers. We also validated that the 3D matrix prepared on the somal side prevented any flow of bulk neurons to the axonal side.
## KEY RESOURCES TABLE

| REAGENT/RESOURCE                        | SOURCE                              | IDENTIFIER                  |
|-----------------------------------------|-------------------------------------|-----------------------------|
| **Antibodies**                          |                                     |                             |
| C66 (Anti-APP C-terminus (1:1000)       | In-house                            |                             |
| 22C11 (anti-APP N-terminus) (1:5000)    | MilliporeSigma                      | Cat#: MAB348                |
| Anti-Neurofilament heavy polypeptide antibody (1:250) | Abcam                              | Cat#: ab8135                |
| anti-Tau (1:500)                        | Cell Signaling Technologies         | Cat#: A0024                  |
| Anti-VDAC1 / Porin antibody [EPR10852(B)] (Rabbit) (1:1000) | Abcam                              | Cat#: ab154856              |
| anti-LaminB1 (1:1000)                   | Abcam                              | Cat#: ab65986                |
| Alexa Fluor 488 and 568 secondary antibody (1:250) | Life Technologies               | Cat#: A32723, A32731, A-11011, A-11004 |
| HRP conjugate secondary antibody (1:2000) | Life Technologies               | Cat#: G-21040, G-21234       |
| anti-SOAT-1/ACAT-1 Polyclonal (Rabbit) (1:1000) | Cayman Chemical                | Cat#: 100028                |
| **Chemicals, peptides, and recombinant proteins** |                                     |                             |
| DMEM/F12 with L-glutamine               | Gibco/Thermo Fisher Scientific      | Cat#: 11320-033              |
| B-27 Supplement (50x), serum free       | Gibco/Thermo Fisher Scientific      | Cat#: 17504044               |
| bFGF                                    | R&D System                          | Cat#: 233-FB                 |
| EGF                                     | Sigma-Aldrich                       | Cat#: 92090408               |
| Penicillin/Streptomycin/Amphotericin B  | Lonza                               | Cat#: 17-745E                |
| StemPro Accutase                        | Gibco                               | Cat#: 2023-01-30             |
| DMEM 4.5 g/L Glucose w/o L-Gln w/Phenol Red | Lonza                         | Cat#: BE12-614F              |
| NE-100                                  | Sigma-Aldrich                       | Cat#: SML0631                |
| Hoechst 3342                            | Thermo Fisher Scientific            | Cat#: H3570                  |
| Triton X-100                            | Sigma-Aldrich                       | Cat#: T8787                  |
| n-Octylglucoside                        | Sigma-Aldrich                       | Cat#: 10634425001            |
| NuPAGE 4–12% Bis-Tris gel               | Invitrogen                          | Cat#: NP0321BOX              |
| Matrigel Basement Membrane Matrix       | Corning                             | Cat#: 356234                 |
| NaCl                                    | Fisher Scientific                   | Cat#: 7647145                |
| Tris-HCL, pH 7.6                        | Boston BioProducts                  | Cat#: 42000000               |
| EDTA                                    | Life Technologies                   | Cat#: 41116134               |
| PBS–CM                                  | Thermo Fisher Scientific            | Cat#: 10010023               |
| Tween 20                                | Fisher Scientific                   | Cat#: 501657287              |
| BSA                                     | Fisher Scientific                   | Cat#: 501781532              |
| Gelatin                                 | WWR International                   | Cat#: 9000-70-8              |
| Paraformaldehyde                        | Sigma-Aldrich                       | Cat#: 8082590100             |
| Trifluoroethanol                        | Sigma-Aldrich                       | Cat#: H0200000               |
| Heparin                                 | Sigma-Aldrich                       | Cat#: A1075                  |
| Synthetic (monomeric) Aβ                 | Sigma-Aldrich                       | Cat#: EP0030710118-192EA     |
| T-25 Flask                              | MilliporeSigma                      | Cat#: 41122107               |
| Falcon 6 Well Plates                    | WWR International                   | Cat#: 294-62501              |
| Human/Rat beta amyloid (40) ELISA Kit   | Wako                                | Cat#: 290-62601              |
| **Critical commercial assays**          |                                     |                             |
| **Software and algorithms**             |                                     |                             |
| ImageJ Software                         | ImageJ 1.53a                        | N/A                         |
| Photoshop                               | Adobe Photoshop CC 20.0.10          | N/A                         |
| Graphpad Prism                          | Prism 9, version 9.0.2              | N/A                         |
| MS Excel                                | Microsoft Excel, version 16.30      | N/A                         |
| AutoCAD                                 | Autodesk, version 2019              | N/A                         |
| **Other**                               |                                     |                             |
| Microfluidic Slides (Commercial)        | Xona Microfluidics                  | Cat#: XC150                  |
| XonaChip 150 µm                          | Xona Microfluidics                  | Cat#: C150                   |
| *SU8-100 photore sist                   | Kayaku Advanced Materials           | Cat#: SU-8 100              |

(Continued on next page)
## MATERIAL AND EQUIPMENT

### Expansion media for hNPCs

| Reagent                      | Final concentration | Amount   |
|------------------------------|---------------------|----------|
| DMEM with L-glutamine        | N/A                 | 500 mL   |
| Heparin                      | 2 ug/mL             | 5 mL     |
| B27                          | 1x                  | 10 mL    |
| bFGF                         | 20 ng/mL            | 4 mL     |
| EGF                          | 20 ng/mL            | 5 mL     |
| Penicillin/Streptomycin      | 100 units/mL        | 5 mL     |
| Total                        | N/A                 | 516.4 mL |

[Be sure to filter media before adding penicillin/streptomycin. Store at +2 to +8 degrees Celsius for up to a month.]

### Differentiation media for hNPCs

| Reagent                      | Final concentration | Amount   |
|------------------------------|---------------------|----------|
| DMEM with L-glutamine        | N/A                 | 500 mL   |
| Heparin                      | 2 ug/mL             | 5 mL     |
| B27                          | 1x                  | 10 mL    |
| Penicillin/Streptomycin      | 100 units/mL        | 5 mL     |
| Total                        | N/A                 | 515.5 mL |

[Be sure to filter media before adding penicillin/streptomycin. Store at 4°C for up to a month.]

### Protein extraction buffer

| Reagent          | Final concentration | Amount |
|------------------|---------------------|--------|
| NaCl             | 1.5 M               | 43.8 g |
| Tris-HCl pH 7.6  | 100 mM              | 25 mL  |
| EDTA             | 20 mM               | 20 mL  |
| MQ-H2O           | N/A                 | 411.2 mL|
| Total            | N/A                 | 500 mL |

[Store at 4°C. This reagent can be stored for up to 3 months.]
**Fixing solution for immunostaining**

| Reagent       | Final concentration | Amount |
|---------------|---------------------|--------|
| Paraformaldehyde | 3.3%                | 2 mL   |
| DPBS          | 96.7%               | 8 mL   |
| Total         | N/A                 | 10 mL  |

[Store at room temp. This reagent should be made fresh.]

**Wash buffer**

| Reagent       | Final concentration | Amount |
|---------------|---------------------|--------|
| PBS-CM        | N/A                 | 500 mL |
| Triton X-100  | .1%                 | 500 µL |
| Tween 20      | .05%                | 250 µL |
| Total         | N/A                 | 506.25 mL |

[Stored at 4°C for up to a year.]

**Blocking solution for immunostaining**

| Reagent       | Final concentration | Amount |
|---------------|---------------------|--------|
| PBS-CM        | N/A                 | 500 mL |
| Triton X-100  | .1%                 | 500 µL |
| Tween 20      | .05%                | 250 µL |
| BSA           | 1%                  | 5 g    |
| Gelatin       | .1%                 | 5 g    |
| Total         | N/A                 | 506.25 mL |

[Make a fresh solution the day of staining. A master mix of PBS-CM, Tritonx100, and Tween 20 can be made and stored at 4°C for up to a year, but BSA and Gelatin must be added day of.]

**STEP-BY-STEP METHOD DETAILS**

**Plating 3D microfluidic chambers to measure somal and axonal Aβ**

In these steps, we have used cell culture and cellular differentiation methods to seed and differentiate FAD hNPCs in microfluidic chamber slides to isolate somal and axonal Aβ. For this, we have used commercially available microfluidic chambers from Xona Microfluidics. We used the microfluidic chambers containing 150 µm capillaries connecting the somal and the axonal compartments. We have seeded FAD hNPCs in 3D into the groove on the somal side prior to differentiating the cells to allow axons extending along the capillaries into the grooves towards the axonal compartments. The method allowed prevention of cells remaining concentrated in the somal compartments without contaminating the axonal compartments with bulk neurons. As an additional step, we created a small volume change between the somal and axonal compartments by adding 180 µL media in each of the somal compartments, while adding 150 µL media in the axonal compartment 24 h before collecting the conditioned media for Aβ analyses.

⚠ CRITICAL: Change differentiation media every 3 days. For media changes, do not aspirate all media. Leave 20% of the media and replace the rest with fresh media to avoid cell death. For drug treatments, prepare the reagents at a concentration 1.25-fold than the required concentrations.

**Preparation of somal and axonal Aβ from FAD hNPCs differentiated in 3D microfluidic chambers**

The steps to prepare differentiated FAD hNPCs in microfluidic chambers to separate axons from bulk neurons are as follows:

© Timing: 5 h
1. Precoat the chambers as described in the manufacturer’s (Xona) protocol (https://xonamicrofluidics.com/protocols/) as follows (Refer to Figure 3): [2 h]
   a. Pipette 100 μL of precoat (provided by the manufacturer) into the top left well of the microfluidics chamber (facing away from the hole leading to the middle chamber to avoid bubbles) to allow it to flow through the middle chamber.
   b. Wait 1 min then add another 100 μL into the bottom left chamber.
   c. Wait 5 min then repeat steps a and b with the right side of the chamber.
   d. Aspirate off.
   e. Repeat steps a-d 2× only this time instead of using precoat use PBS to wash the chambers.
   f. Repeat steps a and b. This time use Matrigel solution (1:100 Matrigel to DMEM/F12) to coat the chambers.
   g. Incubate the slides at 37°C for 1 h for effective Matrigel-coating of the microfluidic chambers.
   h. Gently aspirate the media using a pipette.

   Note: To avoid aspirating off Matrigel, do not use vacuum suction to aspirate.

   i. If not immediately used, store the Matrigel-coated chambers inside a sealed container at 4°C. The chambers can be stored for maximum 1 month.

2. Prepare cells for plating in the microfluidic chambers by following these steps: [3 h]
   a. Aspirate media off a healthy plate of FAD hNPCs. (Note: we use FAD hNPCs. We plate cells (passage number 12 or less) in expansion media inside T-25 flasks and wait until the confluency reaches to 70%–80%.)
   b. Wash cells with PBS as to remove any unwanted dead cells.
   c. Aspirate off PBS and apply enough accutase to cover all the cells and place in 37°C CO2 (5%) incubator until cells are fully detached from the plate (around 2–3 min).

   Note: A T-25 flask only requires around 300 μL of accutase to detach cells. If cells stick you can lightly tap plates to help them fully detach.

   d. Dislodge the cells from the bottom of the dishes by gentle taps and resuspend the cells in 3 mL expansion media.
   e. Take 10 μL of the suspension and count the number of cells using a cell counter.
   f. Take appropriate number of cells (~30,000 for each microfluidic device) and centrifuge at 500×g for 3 min.
   g. Discard supernatant and collect the cell pellet.
   h. Resuspend cell pellets in 30 μL prechilled differentiation media containing Matrigel at 1:10 ratio of media to Matrigel. Resuspend cells on ice and gently pipette 15 μL and add it in the top left well of microfluidics plate being sure to pipette it into the hole that leads to the middle chamber.

   Note: During step g, keep all solution on ice and use pre-chilled pipette tips during cell resuspension. Reference troubleshooting for what to do if a bubble is visualized in between steps.

   i. Repeat steps g and h with the rest of 15 μL cell suspension on the lower left well of the somal compartment of the microfluidics plate again making sure the pipette is aimed towards the hole that leads to the somal grooves.
   j. Wait 5 for min then place 150 μL of differentiation media into the top left corner of the microfluidics plate.
   k. Wait 1 min then place 150 μL of differentiation media into the bottom left corner of the microfluidics plate.
   l. After waiting another 5 min repeat steps i and j for the right side (axonal compartments) of the microfluidics plate and place it in the 37°C CO2 (5%) incubator for 18 h.
m. Next day exchange the media with fresh differentiation media and allow differentiation for 10 days. Add fresh differentiation media every 2–3 days with 150 µL media for each of the four chambers.

△ CRITICAL: While exchanging the media, do not replace all of the media. Leave 20% of the old media and exchange 80% of the old media with fresh media.

n. Differentiate the cells for 10 days by exchanging the media every 2-days.

The steps to collect conditioned media from axonal and somal compartment after 10-day differentiation of FAD hNPCs in microfluidic chambers:

 grades Timing: 25 h

3. 24 h before collecting the somal and axonal conditioned media (CM) for Aβ ELISA analysis, take the microfluidic chamber slides containing 10-day differentiated cells (step 2n) and add 180 µL media to the somal compartments and 150 µL media to the axonal compartments containing desired reagents (vehicle or 10 µM NE-100).

4. Collect the CM to perform Aβ ELISA to measure Aβ40 level. (Note: we did not measure Aβ32 because the level of Aβ32 was undetectable in the axonal compartments.).

△ CRITICAL: After collecting the CM, immediately flash freeze the samples and store at −80°C freezer. Avoid freezing and thawing the samples to prevent Aβ degradation.

5. Use 5 µL somal CM diluted in 50 µL dilution buffer (provided by the manufacturer) in for each well of the 96-well plate to measure somal Aβ40 level. To measure axonal Aβ level, use 50 µL of undiluted CM from the axonal compartments for each well of the Aβ ELISA plate.

6. Perform ELISA according to the manufacturer’s (Wako) protocol (https://labchem-wako.fujifilm.com/us/product/detail/W01W0129-6470.html) and (Takeda et al., 2010)).

Calculation of axonal and somal Aβ from 3D microfluidic chambers

Timing: 4 h

The key to measure Aβ from cell bodies and from axons is to calculate the number of axons generated from each capillary after 10-day differentiation of FAD hNPCs in the 3D-2D microfluidic chambers. We counted ~300 axons per chambers from 30,000 cells seeded in the somal compartments (Figures 1C–1G). The steps to calculate the amount and the number of Aβ40 molecules per axon or per cell body is as follows:

7. First calculate Aβ concentration (in pM) in the condition media from somal (bulk) and from axonal (axons) compartments employing the Wako Aβ ELISA assay (Table 2, Raw data) following the manufacturer’s protocol as described in step 6.
8. To calculate Aβ from only cell bodies follow this formula:

Concentration of Aβ (in pM) from cell bodies alone = the bulk Aβ concentration (from 30,000 bulk neurons) in pM minus the pM concentration of the axonal Aβ multiplied by 30,000 (total cells plated) /333 (average number of axons counted).

This will provide the concentration of Aβ (in pM) from cell bodies alone. In our case we obtained approx. 380 pM Aβ from the bulk neuron containing 30,000 cells (somal compartment) and approx. 3.5 pM Aβ from the axonal compartment containing 333 axons. Based on this we measured that the axons in the somal compartments would contribute to 3.5 x 30,000/300 pM amount of Aβ, which
was subtracted from the bulk neuron Aβ (~380 pM). This gave us 30–140 pM Aβ from cell bodies alone (Table 3).

9. Calculate the total axonal Aβ amount (in picogram) following this formula: Total axonal Aβ (in picogram) = the concentration (pM) Aβ × molecular weight (4330 Dalton) × total volume (150 μL) divided by 10^6. Aβ amount can be presented in picogram. To present it as number of Aβ molecule, multiply the total pg amount with Avogadro’s number (6.02 × 10^23) divided by 10^12. This will give total number of Aβ molecules per axon. Multiply the data was by 10 to match the 10-times more axonal conditioned media compared to that of somal in the experiments (Table 2).

10. Calculate total Aβ amount (in picogram) from bulk neurons in the somal compartment by following this formula: Total bulk neuronal Aβ (in pg) = the concentration (pM) Aβ from somal compartment × molecular weight (4330 Dalton) × total volume (180 μL) divided by 10^6.

11. To calculate total number of Aβ molecule per cell body, use the total pg of cell body Aβ amount obtained from step 9 and use the method from step 10.

12. Perform statistical analyses by Student’s t-test using Prism 9 software.

**Note:** NE-100 treatment results in a severe loss of Aβ40 from axons. Therefore, Aβ40 released from cell bodies is nearly identical to Aβ40 released from bulk neurons.

### Immunostaining of differentiated FAD hNPCs in microfluidic chambers

**© Timing:** 36 h

FAD-APP (APP<sub>Swe/Ion</sub>) expressing hNPCs (FAD hNPCs) are generated by transfecting naïve hNPCs with IRES-mediated polycistronic lentiviral vectors encoding human APP<sub>Swe/Ion</sub> with GFP as a reporter for viral infection. The fluorophore GFP in FAD hNPCs can be imaged directly because the fluorophore has provided a sufficiently strong signal (Figure 1C). Immunocytochemistry is performed to detect endogenous or exogenously expressing cellular proteins. This is standard immunocytochemistry workflow for detecting fluorescent-labeled proteins (specifically Tau as a marker for axons) by labeling fixed cells with primary antibodies followed by secondary antibodies conjugated with fluorophores.

### Table 2. Normalized raw data of somal and axonal Aβ from 30,000 FAD hNPCs after 10-day differentiation (6 independent experiments)

| Somal (450), raw data | Av. (450) | Axonal (450), raw data | Av. (450) |
|-----------------------|----------|------------------------|----------|
| 0.415                 | 0.35     | 0.128                  | 0.115    |
| 0.316                 | 0.406    | 0.126                  | 0.111    |
| 0.305                 | 0.319    | 0.119                  | 0.112    |
| 0.315                 | 0.325    | 0.131                  | 0.124    |
| 0.316                 | 0.306    | 0.116                  | 0.141    |
| 0.405                 | 0.319    | 0.109                  | 0.122    |

### Table 3. Number of somal and axonal Aβ molecules per 24 h from 30,000 FAD hNPCs after 10-day differentiation

| Bulk Aβ (pM) | Axonal Aβ (pM) | Cell body Aβ (pM) | Aβ molecules/cell body | Aβ molecules/axon (average axon: 333) |
|--------------|----------------|-------------------|------------------------|---------------------------------------|
| 382.36       | 2.68           | 141.24            | 1.8E+09                | 3.1E+08                               |
| 353.17       | 2.40           | 136.52            | 2E+09                  | 2.8E+08                               |
| 296.17       | 2.00           | 116.19            | 1.5E+09                | 2.3E+08                               |
| 297.53       | 3.63           | 29.893            | 3.9E+08                | 4.3E+08                               |
| 285.31       | 3.76           | 44.823            | 5.8E+08                | 4.4E+08                               |
| 296.17       | 2.00           | 96.397            | 1.3E+09                | 2.3E+08                               |
13. Fix the cells with 3% paraformaldehyde (PFA) for 20 min at room temperature. 3% PFA is made in PBS containing calcium and magnesium.
14. Wash cells three-times with PBS.
15. Add Blocking Solution and incubate the cells for 1 h at room temperature.
16. Add primary antibody solutions and incubate the cells with primary antibodies for 16 h at 4°C. Use anti-Tau antibody at 1:1000 dilution to label axons for counting.
17. Wash the cells 3-times 5 min each with Washing Buffer.
18. Add secondary antibody at 1:250 dilution and incubate for 18 h at 4°C.
19. Wash the cover slips 3-times 5 min each with Washing Buffer.
20. If necessary, add 1:10,000 diluted (made in dH2O) solution of Hoechst to label nucleus.
21. Immediately proceed to microscopy.
22. We use Nikon C2 Eclipse Ti2 inverted confocal microscope to capture fluorescent images using NIS Element AR software.
23. Convert the images to photoshop images (Figure 1D).

⚠️ CRITICAL: Counting the number of axons by eye may result in errors. Because the axons are growing inside 3D matrix, while counting adjust the focus using fine adjustment to look at different planes. While counting axonal Aβ, we followed the methods described by Niederst et al. (2015) with one exception. We only counted the number of axons sprouting from the tip of the capillaries. Use model cells similar to FAD hNPCs that produce significant level of Aβ so that would be sufficient to calculate axonal Aβ even by counting only the axons extending out of the capillaries separating the somal and the axonal compartments. In our case, we obtain ~333 axons per microfluidic chamber.

In-house microfluidic chamber slides preparation
This section lists the major bioengineering steps to manufacture the microfluidic chamber slides used for collecting pure axons from bulk neurons after axotomy. Our laboratory is equipped with a contamination-free room that is essential to manufacture the devices with precision.

Fabricating microfluidic chambers
All designs can be generated using AutoCad software. To our knowledge, no reliable alternative software for designing a similar microfluidic chamber is commercially available. An overview of the fabrication process is shown in Figure 2.

24. Master Fabrication

่ม Timing: 4 h

a. Design the microfluidic chip using AutoCad software and print it onto the chrome photomask.
b. Dehydrate a silicon wafer in an oven at 250°C for ~20 min. Allow the 4-inch silicon wafer to cool down for ~2–3 min to room temperature.
c. Plasma-treat the silicon wafer using an oxygen plasma machine (March PX-2527 Plasma System) for 3 min at 100 watts.
d. Blow-dry the plasma-treated silicon wafer with nitrogen gas and place it onto the spinner chuck (Machine World 2 Step Spinner) and turn on the vacuum.

Note: Spinner chuck is commonly used for lithography. Here, Machine World 2 Step Spinner from Solitech Wafer Processing, Inc was used. However, different spinners with adjusting spin rate and time may be used. EMS 6000 photo resist spinner from Electronic Microsystems (http://electronicmicrosystems.co.uk/products/ems6000/) is a widely used spinner, which can be used as an alternative to Machine World 2 Step Spinner.
Figure 2. Manufacture of in-house microfluidic chamber slides for axotomy
(A and B) Schematic diagram of in-house microfluidic chamber preparation and axotomy.
(C) An image of a microfluidic chamber slide.
(D) Representative image of FAD hNPCs expressing GFP fluorophore after 10-day differentiation in the microfluidic chamber slide.
(E) Image of a severed slide prior to preparation for protein extraction from the somal and the axonal chambers.
(F) A representative Western blot image showing extraction of APP, MAM-protein VDAC1. Lamin b confirms the purity of the axonal preparation.
e. Add ~5 mL of SU8-2 on top of the silicon wafer and spin it at 500×g for 5 s, followed by spinning at 1000×g for 30 s. This spinning protocol produces a first layer photoresist thickness of 5 μm for microgrooves.

f. Place the coated silicon wafer onto a hot plate. Soft bake the wafer at 70°C for 2 min, followed by baking at 100°C for 5 min. Allow the coated silicon wafer to cool down to room temperature.

g. Place the coated silicon wafer onto the exposure stage of Neutronix-Quintel NXQ4006 Mask Aligner, facing the UV lamp. Place the first layer photomask (microgrooves) with the design onto the exposure stage. Open the shutter of the UV lamp and expose the photoresist for 8 s.

h. Place the exposed silicon wafer onto a hot plate, with the SU8-2 coating facing upwards. Post-bake the silicon wafer at 70°C for 1 min, followed by baking at 100°C for 3 min. Allow the coated silicon wafer to cool down to room temperature.

i. Develop the exposed silicon wafer using the developer solution for ~3 min, followed by developing in a fresh developer for another 1 min.

j. Rinse the silicon wafer with fresh developer and blow-dry using nitrogen gas.

k. Place the silicon wafer onto the spinner chuck (Machine World 2 Step Spinner) and turn on the vacuum to generate the second layer.

l. Add ~5 mL of SU8-100 on top of the silicon wafer and spin it at 500×g for 5 s, followed by spinning at 1200×g for 30 s. This spinning protocol produces a first layer photoresist thickness of 100 μm for channel compartments.

m. Place the coated silicon wafer onto a hot plate. Soft bake the wafer at 70°C for 30 min. Allow the coated silicon wafer to cool down to room temperature.

n. Place the coated silicon wafer onto the exposure stage of Neutronix-Quintel NXQ4006 Mask Aligner, facing the UV lamp. Place the first layer photomask (microgrooves) with the design onto the exposure stage. Open the shutter of the UV lamp and expose the photoresist for 40 s.

o. Place the exposed silicon wafer onto a hot plate, with the SU8-100 coating facing upwards. Post-bake the silicon wafer at 70°C for 10 min, followed by baking at 100°C for 30 min. Allow the coated silicon wafer to cool down to room temperature.

p. Develop the exposed silicon wafer using the developer solution for ~10 min, followed by developing in a fresh developer for another 2 min.

q. Rinse the silicon wafer with fresh developer and blow-dry using nitrogen gas and check under microscope that no SU8 leftover on the silicon wafer. If there is some, place it in developer for another 5 min, and rinse with fresh developer and blow-dry.

r. Place the silicon wafer onto the exposure stage with the SU8-coated surface facing the UV lamp. Place a clear photomask or glass on top of the silicon wafer. Post-expose the photoresist for another 65 s.

s. Place the exposed silicon wafer onto a hot plate with the coated layer on top. Post-bake the wafer at 70°C for 2 min, followed by baking at 100°C for 5 min. Allow the coated silicon wafer to cool down to room temperature and store for later use. [Can be stored indefinitely.]

25. PDMS Mold Fabrication

© Timing: 8 h

a. Prepare 10:1 PDMS by combining 40 g of Sylgard 184 base with 4 g of curing agent in a plastic dish. Mix thoroughly and degas the mixture for ~2 h inside a desiccator connected to an in-house vacuum to remove all air bubbles generated during mixing.

b. Gently pour the PDMS mixture over the silicon wafer mold.

c. Bake the PDMS-coated silicon wafer in an oven at 75°C for four hours.

d. Cut out the PDMS chip from the silicon wafer substrate using a sharp blade.
e. Punch the inlet and outlet of the microfluidic chip using 3 mm diameter puncher.

f. Place the PDMS spheroids array block on a clean tray inside the oxygen plasma machine (March PX-2527 Plasma System) with the design facing up. Place the glass bottom plate on the same tray and expose both surfaces to plasma for 70 s at 50 watts.

g. Invert the PDMS chip block and bond it to the glass bottom plates or dishes. Bake the bonded PDMS-glass slide in a 70°C hot plate for 20 min.

26. Cell plating and axotomy

**Timing: 6 h**

This is standard workflow to detect axonal MAMs from differentiated FAD hNPCs using axotomy.

a. Before seeding the cells, make Matrigel plates by adding 1 mL media containing 10 μL Matrigel (1:100 dilution) for a T-25 flask. Seed cells in expansion media until the flasks achieve approximately 80% cell confluency.

b. Prepare the in-house microfluidic chamber slides by adding Matrigel at 1:100 dilution, as before.

c. Next, take 0.5 × 10^6 cells in 1:10 Matrigel mixture in expansion media, add quickly add inside the groove on the somal side.

d. Wait for 5 min for 3D matrix to form and flood both chambers with expansion media for 24 h prior to adding differentiation media to differentiate the cells into neurons.

e. Allow differentiation for 10–14 days.

f. After 10–14 days of differentiation perform live cell confocal microscopy to confirm formation of axons along the capillaries.

g. Aspirate off the media and place the chamber in ice.

h. Take a clean glass plate and place it on ice to chill.

i. Take a diamond cutter and sever the strip containing the capillaries. Cut the strip into small pieces and store the pieces inside a pre-chilled 15 mL conical tube.

j. Add 100–200 μL of protein extraction buffer per strip, and vortex. Keep the tube on ice for 30 min with vortexing for 30 s every 5 min.

k. Repeat the same process for somal chambers.

l. Centrifuge the tubes at 500 g for 1 min and collect the suspension.

m. Next, centrifuge the suspension at 15,000 g for 20 min and collect the supernatant. The supernatant contains total axonal or somal protein extract.

n. Obtain appropriate amount of axonal and somal protein extracts to perform Western blot analysis to detect MAM-protein IP3R3 and APP in soma and in axons.

**EXPECTED OUTCOMES**

Using the 3D microfluidic chambers, we routinely obtain 10–12 × 10^8 molecules of Aβ from each cell bodies and about 2–3 × 10^9 Aβ from each axon from ~30,000 FAD hNPCs seeded at the somal groove in 3D microfluidic chambers and differentiated for 10–14 days (Table 3 and Figure 1D). The amount may vary depending on the detectable levels of Aβ. However, the key to the experiment is to observe little or no Aβ release in the axonal chambers when cells are treated with NE-100 that reduces axonal MAM levels ((Bhattacharyya et al., 2021) and Figures 1E–1G).

**QUANTITATIVE AND STATISTICAL ANALYSIS**

Perform statistical analyses using Microsoft Excel or GraphPad Prism v.6 software (Graphpad). Exact values for experimental numbers and p values are reported in the figures and corresponding figure legends. Bars and error bars on the graphs represent mean values and SEM for three independent experiments. Statistical significances are determined by unpaired Student’s t test for two groups or one-way ANOVA with Tukey’s multiple comparisons test for multiple groups. All statistical analyses
were performed using a two-tailed Student’s t test. Data in graphs are expressed as mean values SEM. p < 0.01 are considered significant.

**LIMITATIONS**

**Limitation 1**

Our protocol describes a detailed method to measure the number of Aβ molecules derived from axons or from cell bodies of a well-characterized neural cellular model of AD, namely FAD NPCs that were differentiated in 3D inside commercially (Xona) available microfluidic two-chamber devices.

Although our method was only applied to FAD hNPCs, we speculate that the protocol will be applicable for other neuronal cells such as naïve hNPCs or primary neurons. However, the major limitation of our method is that it can reliably detect axonal Aβ$_{40}$, but not Aβ$_{42}$, because the number of axons in the axonal chambers of the microfluidic devices was insufficient to obtain detectable level of Aβ$_{42}$. It is important to detect Aβ$_{42}$ level along with Aβ$_{40}$ because the degree of dementia in AD largely correlates with the Aβ$_{42}$/Aβ$_{40}$ ratio. In unpublished data we have been able to detect axonal Aβ$_{42}$ levels by employing a single-cell-derived clonal hNPCs overexpressing FAD mutants of APP (APP$_{Swe/Lon}$) and γ-secretase (PS1ΔE9), namely HRen-mGAP#A4H1 cells that generated high level of Aβ$_{42}$/Aβ$_{40}$ ratio when differentiated in 3D (Kwak et al., 2020). In addition to using HRen-mGAP#A4H1 cells, we also employed a more sensitive Aβ detection method namely MSD assay (Meso Scale Discovery, CA).

**Limitation 2**

We have also described a versatile protocol of axotomy to isolate pure axons from FAD hNPCs differentiated in our in-house microfluidic two-chamber devices.

We have used this method and successfully detected a series of MAM-associated proteins namely IP3R3, VDAC1, and ACAT1 in the purified axons of FAD hNPCs. We have demonstrated that inactivation of MAM-associated sigma-1 receptor (S1R) downregulated axonal MAM-proteins (Bhattacharyya et al., 2021). We speculate that our protocol will be able to isolate pure axons and axonal proteins from other neuronal cells including naïve hNPCs and primary neurons. However, our protocol is limited to the isolation of axonal proteins, but not that of somal proteins because the somal compartments of microfluidic chambers contain bulk neurons. Thus, to compare the effect of axonal versus somal proteins on axonal and/or somal Aβ production, we will require more sophisticated microfluidic chambers. A prototype of such chamber is under preparation in our laboratory.

**TROUBLESHOOTING**

**Problem 1**

The Matrigel solution (1:10) for making 3D matrix starts to polymerize too early, or while transferring the Matrigel-containing cell suspension into the somal grooves (step 2h).

**Potential solution**

Keeping every solution and the tubes and the pipet tips chilled at 4°C can solve the problem (Figures 3A–3C). If the gel solidifies, it creates bubbles inside the somal groove. Keeping the solutions and the materials cold and allowing a smooth flow of the cell suspension inside the grooves will prevent bubble formation. Even after taking all precautions bubbles may be formed (Figure 3D). We recommend discarding the microfluidic chamber and start afresh. This is the primary reason to use in-house microfluidic chambers over commercially available microfluidic chambers to keep the cost of the experiments reasonable.

**Problem 2**

The color of the differentiation media changes to yellow in 1–2 days in the somal chambers and the axons are not formed in the capillaries (step 2m).
Keeping the cell numbers ~30,000 per microfluidic somal chamber may solve the problem. However, media color also changes due to evaporation of the media. Because the volume of media in the somal compartment is small (~200 μL) evaporation of media is expected. Always keep the chambers inside a 100 mm culture dish with a lid. If the discoloration or drying of the media persists (Figure 4A), carefully add extra media around the chambers (this will preserve the media which is expensive) or flood the culture dish with media (Figure 4B). On the day of treatment, place the microfluidic chamber inside a fresh culture dish without flooding with media. Add exact volume of media to the somal and axonal chambers containing the appropriate concentration of drugs. For example, 180 μL 10 μM PRE-084/NE-100 in each somal chamber and 150 μL 10 μM PRE-084/NE-100 in each axonal chamber to test the effect of PRE-084/NE-100 on somal and axonal Aβ. Keep the treatment time no more than 24 h to avoid any evaporation or discoloration of the media.

Problem 3
Axonal chambers are contaminated with bulk neurons (step 2n).

Potential solution
This problem is more common with the commercial Xona microfluidic slides compared to the in-house devices because the capillaries of the Xona devices are thicker (accommodate ~3 axons per capillary) than the capillaries of our custom-made in-house devices (accommodate 1 axon per capillary) (Figure 5). To solve this problem, carefully monitor under a light microscope that no cell
enters the capillaries and the 3D matrix (1:10 Matrigel) is quickly formed inside the somal groove while adding the cell mixture to the microfluidic device (Figure 5B, c and d). It is also noteworthy that each lot of Matrigel has a different protein concentration, therefore every lot needs to be pre-tested. We use 1:10 dilution of Matrigel to make thin 3D matrix inside somal grooves. However, 1:15 or 1:20 dilutions may also be used as is described by Kim et al. (2015).

Problem 4

Increasing the number of cells in the somal chambers does not increase axonal protein amounts after axotomy (step 26m).

Potential solution

100,000 FAD hNPCs per microfluidic chamber is sufficient to isolate ~30 μg of axonal protein after 10-day differentiation. However, more amount of axonal protein is required to study subcellular distribution of proteins of interest such as axonal MAM-distribution of APP, IP3R3 or VDAC1. Increasing cell numbers does not increase axonal protein amount after axotomy because the number of axons in the microfluidic chambers is dependent on the number of capillaries connecting the somal and the axonal compartments, which remains fixed for each microfluidic chamber (450 capillaries per in-house microfluidic chamber slides). To obtain more than 30 μg axonal proteins, the number of microfluidic chamber slides will have to be increased. We have isolated ~200 μg axonal proteins from 5 in-house microfluidic chambers. Alternatively, more axonal proteins can be isolated by allowing longer differentiation time. We differentiate FAD hNPCs for 10 days prior to axotomy and extraction of axonal protein. More than 1 week differentiation is sufficient to study Aβ pathology in FAD hNPCs. However, to study tau-pathology, which is another hallmark of AD, FAD hNPCs has been differentiated for 3 weeks or longer without any adverse effect on cell viability (Kwak et al., 2020). Increasing the length of differentiation from 10 days to 3 weeks will generate ~2-fold increase in the amount of total axonal proteins.

Problem 5

Even when axonal protein amount is high, Western blot does not detect protein of interest.

Axonal chambers are contaminated with bulk neurons (step 26n).
Potential solution

Matrigel is made of extracellular matrix (ECM) proteins. The amount of protein from the Matrigel (1:10) in the somal chamber is negligible compared to significantly high level of proteins extracted from bulk neurons in the somal compartments. In contrast, the amount of axonal protein isolated from the capillaries and the axonal compartments is significantly low. If small amount of Matrigel enters the capillaries while adding cells (in 1:10 Matrigel) in the somal groove (Figure 1A) before differentiation, the proteins in the Matrigel may contaminate axonal proteins. Protein estimation will erroneously show higher amount due to Matrigel proteins, while the actual axonal protein level is low. To solve this problem, carefully monitor under a light microscope that the cell mixture (in 1:10 Matrigel) solidifies inside the somal groove and does not enter the capillaries of the microfluidic chamber slides.

RESOURCE AVAILABILITY

Lead contact
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Materials availability
All materials are available upon request. Contact: bhattacharyya.rraja@mgh.harvard.edu.

Figure 5. FAD hNPCs generates single axon per capillary inside in-house microfluidic chambers but more axons per capillary in commercial (Xona) microfluidic chambers

(A) Representative confocal image of GFP-expressing FAD hNPCs after 10-day differentiation inside the capillaries of in-house microfluidic chambers connecting somal (Left) and axonal (Right) compartments. (B) Representative images of two commercial (Xona) microfluidic chambers growing FAD hNPC axons along the capillaries (a and b). Arrow (white) indicates contamination of cell body inside a capillary (a). Image of a capillary showing no cell has contaminated the capillary (b). Panels c and d are representative light microscope images of cells seeded on the somal groove before differentiation. Representative image of Matrigel (1:10)-cell mixture not solidifying faster in c than in d, thus resulting in cells entering a capillary in c (arrows) but not in d.
Data and code availability
The protocol includes all raw datasets generated or analyzed during this study. If required, additional information can be obtained from the lead author (R.B.).

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AUTHOR CONTRIBUTIONS
R.B. and R.E.T. conceived the study, analyzed the data, supervised the study, and wrote the manuscript. R.B., M.S.L., and M.B.T. performed the experiments. M.S.L. and M.B.T. contributed equally. M.J. designed and microfabricated in-house microfluidic systems and contributed to manuscript figures and writing. D.M.K. provided valued insights and contributed to manuscript writing.

DECLARATION OF INTERESTS
R.E.T. and D.M.K. hold intellectual property and patents on the topic of acyl-CoA: cholesterol acyltransferase inhibitors (patent no. US20050118226A1 and methods and compositions relating to modulating amyloid precursor protein cleavage (patent no. US20050170437A1).

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