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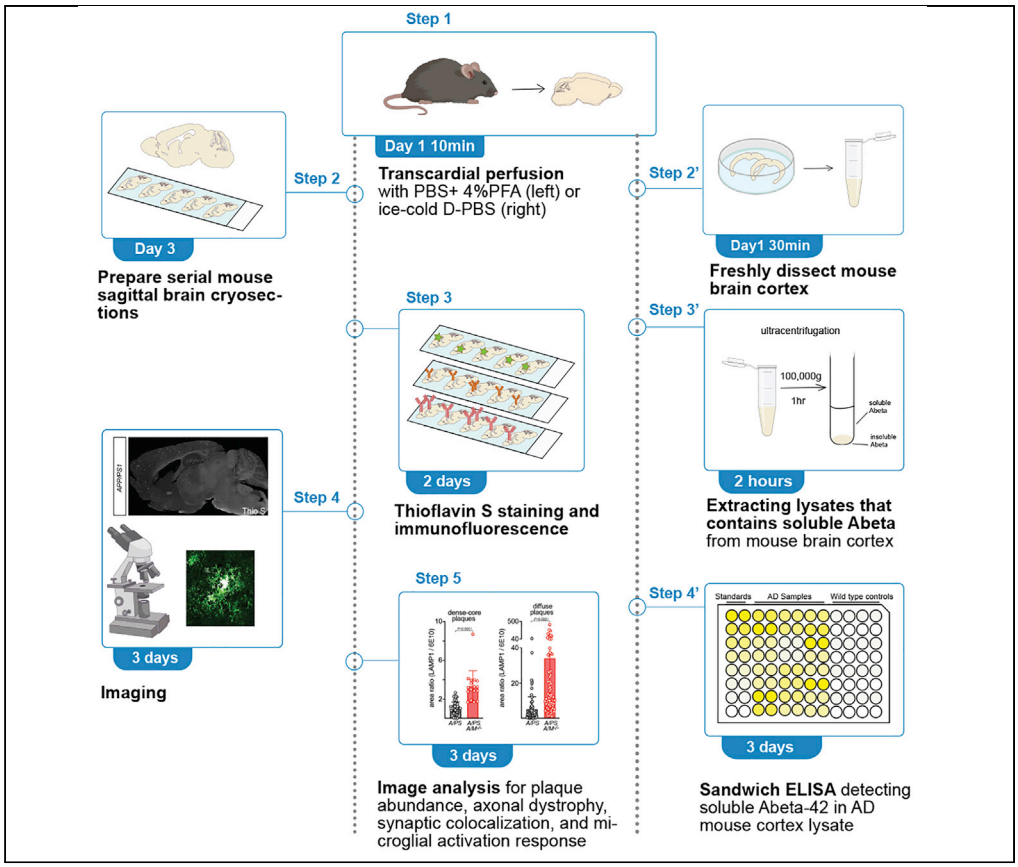
Adaptable toolbox to characterize Alzheimer's disease pathology in mouse models



Here, we describe a highly adaptable toolbox for characterizing and analyzing molecular and histopathological changes in Alzheimer's disease (AD) mouse models. We detail optimized and streamlined approaches from sample preparation to image analysis to facilitate reproducible analyses. We also describe the extraction and measurement of the soluble Aβ level by sandwich ELISA in the cortex and hippocampus of AD mouse models before and after plaque deposition. Finally, we outline the steps for image quantification and analysis using Imaris and ImageJ.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Streamlined steps to prepare fresh mouse brains for histological and biochemical assays

Details for sagittal cryosectioning of mouse brains

Toolbox for assessing molecular and pathological changes in AD mouse models

Highly adaptable techniques to extract and analyze soluble Abeta in AD murine brains

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Adaptable toolbox to characterize Alzheimer's disease pathology in mouse models

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SUMMARY

Here, we describe a highly adaptable toolbox for characterizing and analyzing molecular and histopathological changes in Alzheimer's disease (AD) mouse models. We detail optimized and streamlined approaches from sample preparation to image analysis to facilitate reproducible analyses. We also describe the extraction and measurement of the soluble Aβ level by sandwich ELISA in the cortex and hippocampus of AD mouse models before and after plaque deposition. Finally, we outline the steps for image quantification and analysis using Imaris and ImageJ.

For complete details on the use and execution of this protocol, please refer to Huang et al. (2021).[1]

BEFORE YOU BEGIN

The protocol below describes the specific steps for harvesting and processing mouse brain for detecting and analyzing molecular and histopathological hallmarks in AD mouse models. However, it can also be utilized for other studies using immunohistochemical (IHC) /immunofluorescence (IF) and light microscopy for murinecentral nervous system (CNS) tissues.

Mice

Mouse strains utilized in this protocol are dependent on the experiments. All lines have been maintained on a C57BL/6 background.

For AD mouse models, we have extensively characterized the course of microglial and pathological changes from juvenile mice at P30 to 18-month-old aged mice in two amyloidogenic AD mouse models. The first one is *B6.Cg-Tg(APPSwePSEN1dE9)* hemizygous mice[2,3] (*APP/PS1*) (JAX number: 005864), which carry a pathogenic, amyloidogenic 'Swedish mutation' of the human amyloid precursor protein (*APP*) gene and an activating (exon 9 deletion) mutation of the human presenilin 1 (*PSEN1*) gene,[2,3] both as transgenes driven by the mouse prion protein promoter. The second one is *APP41* mice, which express a 'Swedish' + 'London' (V717I) mutant human APP under the Thy-1 promoter,[4–6] were a kind gift of Drs. Kuo-Fen Lee and Jiqing Xu. Mice of both genders were randomly allocated to experimental groups unless otherwise noted. For more details, see Huang et al.,[1] for reference.

Institutional permissions

All animal procedures were conducted according to protocols approved by the Salk Institute Animal Care and Use Committee.

Animal studies must be approved by an Institutional Animal Care and Use Committee (IACUC) and performed in accordance with IACUC guidelines.

Setup procedure

Timing: 0.5–1 h

1. Set up for mouse transcardial perfusion.
   a. Checklist for perfusion setup in a ventilated hood:
      i. Anesthesia drug loaded in syringes (final concentration: 100 mg/kg Ketamine and 10 mg/kg Xylazine in accordance with IACUC guidelines).
      ii. Perfusion pump (Master Flex L/S, standard drive, model no.7520-10) with a two-part stop line that draws from PBS/Heparin and 4% PFA in 1×PBS, plugged in and flow rate at about 0.3 mL/s.

   Alternatives: manual perfusion using with 30 mL syringes filled with corresponding solutions connected to a 25G butterfly needle.

      iii. PBS/Heparin (working concentration 20 U/mL) – prepare for at least 30 mL per animal.
      iv. 4% PFA in 1×PBS – Prepare 100 mL of paraformaldehyde (PFA) per adult animal.
      v. 70% ethanol for sanitization and preparation of the mouse's abdominal/chest area.
      vi. 22-G needle for heart puncture (covered with a short piece of silicone tubing (inner diameter 1/32 inch) so that only the tip of the needle and its beveled edge remains uncovered to prevent over-puncture during transcardial perfusion).
      vii. Sanitized tools for mouse brain dissection (from Fine Science Tools: ToughCut standard scissors #14054-13; Taylor forceps #11010-17; CeramaCut small scissors #149 958-11; Micro-Adson rat tooth forceps #11019-12; Hartman hemostats straight #13002-10; Graefe forceps small forceps curved #11051-10; Dissector scissors bone scissors #14082-09; double-ended curved spatulas and micro spring scissors for fresh cortical and hippocampus dissection).
      viii. Styrofoam support, pins (25G needles), and glass tray for drainage collection.
      ix. Acrylic coronal mouse brain matrix with a midline sagittal cut to facilitate separation of the left and right hemisphere and better results in PFA post-fixation.
      x. Animal carcass bag.
      xi. If needed, tubes/plate for collecting tail for genotyping before perfusion.

   ⚠ CRITICAL: Make sure to handle PFA and PFA-related steps under chemical hood with proper suction and ventilation.

   Note: Clean the area and tools thoroughly with 70% ethanol before the setup.

2. Prepare the PBS/Heparin and 4% PFA buffers for perfusion if needed. Make sure to prepare 4% PFA as fresh as possible (preferably the day before mouse perfusion).
3. Preparation for mouse brain collection for PBS/PFA perfusion, pre-label and pre-fill 10 mL of PFA in a glass vial per mouse brain. Keep at 4°C until brain dissection.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-Axl, polyclonal goat (for IF, 1:50)	R&D Systems	Cat# AF854, Lot CTC0214101, RRID: AB_355663
anti-Mer, monoclonal rat, clone: DS5MMER (eBioscience) (For IF, 1:100–200)	Invitrogen	Cat # 12575182 (lot 4285684) RRID: AB_2572623
anti-Mer, polyclonal goat (For IF, 1:50)	R&D Systems	Cat# AF591, Lot DGS0517061, and Lot DGS0213111, RRID: AB_2098565

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
anti-mouse Gas6, polyclonal goat (For IF, 1:50–100)	R&D Systems	Cat# AF986, lot: EFU0314121, RRID: AB_2263130
anti-human-b-amyloid, 1–16 antibody, clone 6E10 (For IF, 1:1000)	Biolegend	Cat# 803001, RRID: AB_2051358
anti-Iba1, polyclonal rabbit (For IF, 1:200)	Wako	Cat# 019-19741, RRID: AB_839504
anti-Iba1, polyclonal goat (For IF, 1:50)	Novus	Cat# NB100-1028, RRID: AB_521594
anti-cleaved Casp3, polyclonal rabbit (For IF, 1:200)	Cell Signaling	Cat# 9661, lot 45, RRID: AB_2341188
anti-Tmem119, monoclonal rabbit (For IF, 1:200)	Abcam	Cat# AB209064, lot GR320057-1, RRID: AB_2800343
anti-RTN3, polyclonal rabbit (For IF, 1:200)	EMD Millipore	Cat# ABN1723, lot 3109186
anti-LAMP1, monoclonal rabbit, clone 1D4B (For IF, 1:200)	BD Biosciences	Cat# 553792, RRID: AB_2134499
anti-Trem2, polyclonal sheep (For IF, 1:200)	R&D Systems	Cat# AF1729, RRID: AB_354956
anti-Trem2, polyclonal sheep, biotin-conjugated (For IF, 1:50)	R&D Systems	Cat# BAF 1729, RRID: AB_356109
anti-CD31, polyclonal goat (For IF, 1:100)	R&D Systems	Cat# AF3628, RRID: AB_2161028
anti-laminin, polyclonal rabbit (For IF, 1:500)	Sigma	Cat# L-9393, RRID: AB_477163
anti-vGlut1, polyclonal guinea pig (For IF, 1:1000–2000)	Millipore	Cat# AB5905, RRID: AB_2238022
anti-PSD95, polyclonal rabbit (For IF, 1:400)	Life Technologies	Cat# 51-6900, RRID: AB_2533914
JRF AB042/26 for Aβ42 capture antibody (final conc. 1.5 μg/mL) and detection antibody JRF/AbN/25, unlabeled	made in-house from Janssen Pharmaceuticals	N/A
Donkey anti-rat, AF488 or Cy3 conjugated, 1:500	Jackson ImmunoResearch	712-545-153 (RRID: AB_2340684) or 712-165-153 (RRID: AB_2340667)
Donkey anti-goat, AF546 conjugated, 1:500	Life Technologies Jackson	A-11056, (RRID: AB_2534103)
Donkey anti-rabbit, AF488 conjugated, 1:500	Life Technologies	A-21206, (RRID: AB_2535792)
Donkey anti-sheep, AF546 conjugated, 1:500	Thermo Fisher Scientific	A-21098, (RRID: AB_2535752)
Donkey anti-mouse, AF488, Cy3, or Cy5 conjugated, 1:500	Jackson ImmunoResearch	715-545-150 (RRID: AB_2340846), 715-166-150 (RRID: AB_2340816) or 715-175-150 (RRID: AB_2340819)
Donkey anti-guinea pig, AF488, Cy3, or Cy5 conjugated, 1:500	Molecular Probe Jackson ImmunoResearch	A-11073 (RRID: AB_2534117) 706-165-148 (RRID: AB_2340460) 706-175-148 (RRID: AB_2340462)
Chemicals, peptides, and recombinant proteins		
Paraformaldehyde (PFA)	Sigma	P6148
Bovine serum A (BSA), IgG-free	American Bioanalytical	AB01240-00100
Citric acid	Sigma	C0759
Trisodium citrate dihydrate (sodium citrate tribasic dihydrate)	Sigma	C7254
Methoxy-X04	Tocris	4920
Tissue-Tek O.C.T. compound	Sakura Finetek	4583
Tissue freezing medium (TFM), clear	Electron Microscopy Sciences	72592
TrueBlack® Lipofuscin Autofluorescence Quencher	Biotium	23007
Thioflavin S (ThioS)	Acros Organics	213150250
bisbenzimide (for Hoechst 33258)	Sigma-Aldrich	B-2883
Synthetic human $A\beta_{1-42}$ peptides for ELISA standards	Anaspec	20276
NaN3	Sigma	S2002
Fluoromount-G mounting medium	Southern Biotech	0100-01
Vectashield mounting medium for fluorescence	Vector Laboratories	H-1000
Sucrose	SIGMA	S7903
Heparin (10,000 U/mL)	Sigma-Aldrich	H3393-10KU
Propylene glycol	Fisher Scientific	P355-1
Tris-HCl	OmniPur	9310

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Halt protease and phosphatase inhibitor	Thermo Fisher scientific	78440
1% casein blocking buffer	Bio-Rad	1610783
Triton X-100	Bio-Rad	X100
Tween-20	Corning	170-6531
Normal donkey serum	Jackson	017-000-121
DPBS 1×	Gibco	14040-133
Isopentane (2-methylbutane)	Electron Microscopy Sciences	18550
Critical commercial assays		
EZ link Sulfo-NHS-LC Biotin	Thermo Fisher	Cat #21217
Slide-A-lyzer dialysis cassette	Pierce 0728	prod # 66415
BCA assay	Thermo Scientific Pierce	23225
Experimental models: Organisms/strains		
Mouse: C57BL/6J, (age 2–18 months, both sexes)	The Jackson Laboratory	JAX #000664
Transgenic mouse: *B6.Cg-Tg(APPSwePSEN1dE9)* hemizygous (APP/PS1), (age 2–18 months, both sexes)	The Jackson Laboratory	JAX number: 005864[2,3]
Transgenic mouse: APP41 (age 2–18 months, both sexes)	Gift from Dr. Kuo-fen Lee lab (Salk)	Rockenstein et al.[5]
Software and algorithms		
Fiji	Schindelin et al.[7]	https://imagej.net; RRID: SCR_003070
Imaris (version 9.1.2)	BitPlane	RRID: SCR_007370
FlowJo	TreeStar	RRID: SCR_008520
Zen black / blue	Zeiss	RRID: SCR_013672
Virtual Slide Imager (VS-desktop)	Olympus	N/A
GraphPad Prism	Prism	RRID: SCR_002798
Other		
Perfusion pump for transcardial perfusion	Cole-Parmer Instrument co. Master Flex L/S	Model no: 7520-10 Standard drive 3 AMP, 115 VAC, 50/60 Hz
Coverslips 22 mm × 50 mm; #1 $^{1}/_{2}$	Fisher Scientific VWR	12-544-D Cat. No: 48311-703
Cryostat	Hacker Industries	Bright OTF5000
0.22-μm filter	Sigma-Aldrich	SLMP025SS
Superfrost plus micro slides	VWR	48311-703
Confocal and super-resolution microscope	Zeiss	LSM 780, LSM 880 Rear Port Laser Scanning Confocal and Airyscan FAST microscope
96-well plate reader	TECAN	TECAN Infinite® 200 PRO reader
Virtual Slidescanning microscope	Olympus	VS-120
Polycarbonate centrifuge tubes	Beckman Coulter	343778
Whatman 1 filter paper (90 mm diameter)	Cytiva	1001-090
Kontes pellet pestle motor (with 1.5 mL pestle)	Kimble	749540-0000 749521-1500
96-well V Bottom plate	Thermo Fisher	277143
Ultracentrifuge with rotor (TLA 120.2)	Beckman	TLA 120.2

MATERIALS AND EQUIPMENT

4% PFA/PBS

Reagent	Final concentration	Amount
Paraformaldehyde (PFA)	4%	40 g
PBS	1×	1,000 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
1 M NaOH	N/A	4 drops
Total	**N/A**	**1,000 mL**

Closely monitor the temperature when heating until PFA powder is fully dissolved and the solution turns clear. DO NOT exceed over 70°C. Cooldown before filtering through 0.22 μm filter and store at 4°C for same week use or store at −20°C for up to 6 months.

30% sucrose/PBS		
Reagent	Final concentration	Amount
Sucrose	30%	30 g
PBS	1×	100 mL
Total	**N/A**	**100 mL**

30% sucrose/PBS is used for cryoprotection for CNS tissues after overnight PFA fixation.

When sucrose is fully dissolved, filter with 0.22 μm filter and store at 4°C for up to 1 month.

Citrate buffer for antigen retrieval	
Reagent	Amount
Stock Solution A: citric acid (C_6H_8O7)	1.83 g in 100 mL H_2O
Stock Solution B: trisodium citrate ($C_6H_5O_7Na_3.2H_2O$)	15.66 g in 500 mL H_2O

Stock Solution A and B (10×) can be stored at 4°C for up to 1 year.

For making 1,000 mL working citrate buffer solution for epitope retrieval = 18 mL solution A + 82 mL solution B +900 mL H_2O, solution can be stored at room temperature for up to 6 months.

For the antigen retrieval step, preheat the working citrate buffer solution in a Coplin glass jar to 65°C–70°C (see protocol below for more details).

Blocking buffer for IHC and IF		
Reagent	Final concentration	Amount
Triton-X100	0.3%	150 μL
Tween-20	0.1%	50 μL
Normal Donkey serum	5%	2.5 mL
IgG-free BSA	2%	1 g
1×PBS	1×	45 mL
Total	**N/A**	**50 mL**

Shake vigorously on vortex until thoroughly mixed. Filter through 0.22 μm filter. Aliquoted immediately and keep frozen at −20°C until use or store at 4°C for up to 1 week.

PBS-T Wash buffer for IHC and IF		
Reagent	Final concentration	Amount
1×PBS	N/A	1,000 mL
Tween-20	0.1%	1 mL

To accurately measure Tween-20, use a 1cc syringe to draw and expulse Tween-20. Mix well in the bottle. Store at room temperature up to 6 months.

RIPA buffer for tissue homogenization and fractionation	
Reagent	Final concentration
Tris-HCl	50 mM
NaCl	150 mM
SDS	0.1%
Triton-X100	1%
Sodium Deoxycholate	0.5%
Total	**N/A**

Make in H_2O. pH=8.0. Use together with Halt protease and phosphatase inhibitor and Na orthovanadate for best phosphatase inhibition. Use at ~4:1 RIPA volume/brain wet weight. See the protocol below for detailed steps. Store at 4°C.

Thioflavin S (ThioS) staining solution		
Reagent	Final concentration	Amount
ThioS	0.1%	0.1 g
EtOH	80%	80 mL
dH_2O	20%	20 mL
Total	**N/A**	**100 mL**

Filter with 0.22 μm filter. Make freshly right before ThioS staining procedure and use within 15 min.

Methoxy-X04 staining solution		
Reagent	Final concentration	Amount
Methoxy X-04 (MX04)	10 mg/kg body weight of mice	3 mg (enough for 10 mice)
DMSO	10%	100 μL
Propylene glycol	45%	450 μL
1×PBS	45%	450 μL
Total	**N/A**	**1 mL**

Aliquot immediately upon fully mixing and store in −20°C

This can be used for both immunofluorescence and for *in vivo* imaging. For the latter, stock MX04 was first dissolved in 100% DMSO then injected i.p. at a final concentration of 10 mg/kg in 10% DMSO, 45% propylene glycol, 45% PBS, pH 7.5) 24 h prior to imaging.

D-PBS supplemented with BSA		
Reagent	Final concentration	Amount
BSA	0.5%	0.5 mg
D-PBS w/Ca^{2+} Mg^{2+}		100 mL
Total	**N/A**	**100 mL**

Filter with 0.22 μm filter. Store at 4°C up to 1 month.

ELISA Coating buffer		
Reagent	Final concentration	Amount
Tris HCl (Stock 2 M)	10 mM	2.5 mL
NaCl (Stock 5 M)	10 mM	1 mL
NaN_3 (Stock 0.5 M)	10 mM	10 mL
Distilled H_2O	N/A	500 mL
Total	**N/A**	**500 mL**

Bring volume to 500 mL with distilled H_2O; Adjust to pH 8.5; Filter on a 0.22 μm filter. Store at 4°C for up to 6 months.

Other solutions

- PBS/Heparin perfusion buffer (prior to PFA fixation): 20 U/mL heparin in 1× PBS. Make freshly before use.
- Hoechst 33258: 1% solution of bisbenzimide in water. Store at room temperature.
- ELISA Wash buffer: 1×PBS + 0.05% Tween. Store at room temperature.
- Permeablization buffer for IHC/IF: 0.3% Triton-X100 in 1×PBS. Store at room temperature.
- Autofluorescence treatment buffer for aged brain tissue and/or human postmortem samples: 1× TrueBlack in 70% Ethanol (store in dark, according to manufacturer's instruction). Make freshly before use.

STEP-BY-STEP METHOD DETAILS

Mouse perfusion, brain harvest and cryopreservation

Timing: 3 days

This part of the protocol will guide you through harvesting mouse brains through transcardial perfusion to prepare for a variety of downstream assays such as cryosection and imaging analysis or fresh microglia isolation.

Day 1

1. Mouse anesthesia.
   a. Using a 1 cc syringe, anesthetize the mouse with IP injection of 100 mg/kg Ketamine and 1 mg/kg Xylazine in accordance with IACUC guidelines.

   Note: check for motor reflex by pinching the hind paws before proceeding onto dissection.

   Optional: meanwhile, heat up the PBS and PFA solutions by microwave to 37°C for an optimal fixation result; use ice-cold D-PBS only if proceeding to fresh brain/cortex collection for downstream cell isolation or molecular assays.

2. Transcardial perfusion.
   a. Once the mouse shows no sign of pain and motor reflex, pin the four limbs onto a Styrofoam board with four 25 G needles.
   b. Sanitize and prepare the chest and abdomen area with 70% ethanol.
   c. Using the rat tooth forceps to pinch the abdominal skin and make a small incision upward through the skin and peritoneum, and then cut parallel through the diaphragm, then parallel to the body through the ribs to just above the heart.

   Note: Please be careful not to nick the liver and lung (avoid by gently push down the liver with the side of the scissors while cutting) because the efficiency of the perfusion will be compromised. Be sure to also cut away the connective tissue around the heart as well for a full and unobstructed display of the heart.

   d. Clamp the sternum/rib with a pair of hemostats to open the rib cage.
   e. Gently nick the right atrium, then inject the needle tip laterally into the left ventricle in the lower left quadrant of the heart at an upward 20-degree angle toward the midline.

   Note: a nick in the right atrium should result in blood oozing out; the injection needle is covered by a short piece of Tegon tubing 'sleeve' so that only the tip and the beveled edge of the needle is exposed in order to prevent over-puncturing the heart.

f. Perfuse for 30 s- 1 min with PBS/Heparin at speed of 0.3 mL/s at 37°C if followed by PFA fixation. Alternatively, if collecting fresh brains, perfuse with a total of 2 min by ice-cold D-PBS.

Note: the first sign of a good perfusion at this point is that the liver should switch from dark red to pale mustard color[8] (See Methods video S1: real time transcardial perfusion, related to step 2f).

g. If collecting fresh brains, jump to step 35 for "Isolation of soluble Aβ from transgenic AD mouse cortex and hippocampus and its measurement by sandwich ELISA", otherwise switch over to perfusing with 4% PFA/PBS for 5 min at 37°C or room temperature, add 30 s- 1 min each step for aged/larger mice.

Note: a good fixation should result in stiff body and organs, and the brain should be devoid of red patches and visible vasculature pattern.

3. Brain dissection and preparation.
   a. Decapitate the fixed mouse, then make an incision down the midline to tease apart the skin of the head.
   b. Then from the temple-posterior side inward and forward, gently nick and peel away the skull bone using bone scissors starting from the cerebellum and brainstem.

Note: be careful to cut away from the brain to not damage the brain parenchyma and delicate parts such as the olfactory bulb and cerebellum. For a detailed step-by-step protocol for the dissection of mouse brain, please refer to Meyerhoff et al.[9]

   c. Place the dissected whole brain into the acrylic mouse brain matrix, align with symmetry and slice down the midline with a clean razor blade to separate the two hemispheres.
   d. For fresh brain collection, place two hemispheres on a petridish on ice lined with a filter paper pre-wetted with PBS or continue to protocol "Isolation of soluble Aβ from transgenic AD mouse cortex and hippocampus and its measurement by sandwich ELISA" (step 35) below for using fresh frozen tissue for downstream assays.
   e. For fixed brain collection, immerse the dissected brain hemispheres into a glass vial filled with at least 10 mL of 4% PFA in PBS for fixation overnight at 4°C.

Day 2

4. Switch to 30% sucrose/PBS (at least 10 mL per brain) to infiltrate overnight at 4°C.

Note: A full infiltration of 30% sucrose as cryoprotectant for CNS tissues would result in the brains sinking into the bottom of the vials, so do not proceed if the brains have not sunken. Using 30% sucrose/PBS is crucial for preventing the formation of ice crystals and other freezing artifacts when processing CNS tissues.

Pause point: the brain hemispheres can be stored in 30% sucrose/PBS at 4°C for up to a week if not immediately prepared for frozen tissue block for cryosection; after that, 0.1%–0.2% of PFA will need to be added so as not to reverse the paraformaldehyde crosslink.

Day 3

5. Fill the foil mold to 90% fullness with TFM (Tissue Freezing Medium).

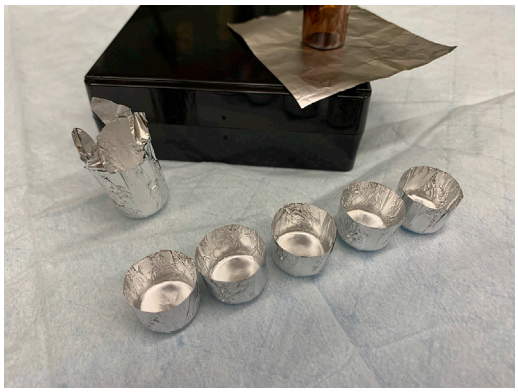

Figure 1. Homemade brain tissue freezing foil molds that are optimized for rapid freezing for downstream sagittal cryosections (image courtesy of Patrick Burrola)

Note: TFM, which cuts more smoothly than OCT at colder temperature (e.g., at −25°C), is used to freeze PFA-fixed tissues in general; OCT is used for freezing fresh frozen tissues (and cut best at −20°C to −15°C).

We customize our tissue freezing molds using foil of tissue-fitted sizes to ensure fast conduction to the center of the tissue block (Figure 1).

Avoid bubbles. Use tweezer tips or pipette tips to fish out bubbles if any.

6. Fill a glass petri dish with 20–30 mL of isopentane and set on dry ice to cool down.

   Note: Keep this system under a chemical hood or suction system to remove any fume from the isopentane.

Drop a chuck of dry ice into isopentane to speed up the cooling. When the liquid reaches the proper temperature, it will stop boiling and is ready for freezing tissue blocks.

7. Use curved tweezers, gently pick up the brain hemispheres from sucrose and thoroughly blot dry their surfaces using Kimwipe tissues.

   Note: This step is crucial because residual sucrose on the surface will result in edges of the tissue section curling and detaching from the mounting media when cutting.

8. Drench the brain with TFM on a paper towel, then pick it up gently using curved scissors and guide the hemisphere all the way to the bottom of the foil molds with the flat midline side facing down until adherence.

   Note: Gently push the hemisphere down including more delicate parts such as the olfactory bulb and the cerebellum to ensure flat sagittal cryosections.

9. Remove the frozen block from the isopentane once the center of the block solidifies. Store the frozen blocks in –70°C for long term storage.

Cryosection of mouse brain

Timing: ~30 min/brain

10. TFM tissue blocks with frozen brain hemisphere are cryosectioned at −25°C.

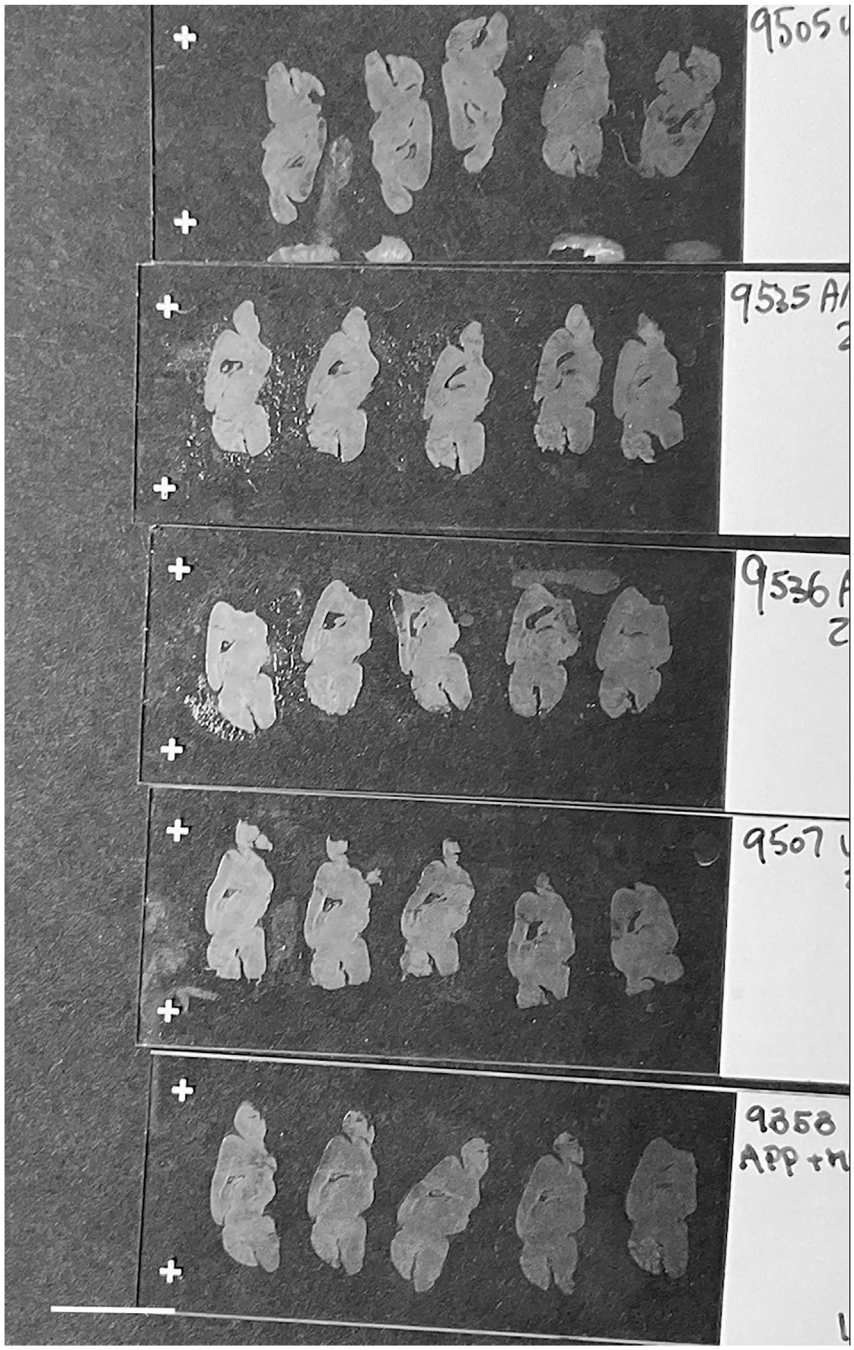

Figure 2. Serially mounted sagittal brain cryosections on glass microscopy slides
Scale bar, 20 mm.

11. Label microscopy slides with permanent markers or pencil.
12. To obtain sufficient technical replicates and consistent and representative cryosections for quantification purposes, we mount a serial set of 15 μm sagittal sections (5 sections/slide and 5 or 10 slides per hemisphere, see Figure 2).

Note: we used hippocampus and lateral ventricles as our anatomical landmarks and a mouse brain atlas as a reference map (ISPN: 9780123742445).

a. Starting trimming from the midline, then start collecting from when the hippocampus begin to be fully shown (with DG, CA3 and CA1 shown on the same plane), from approximately 0.25 mm–0.85 mm from the midline, each spaced 0.15 mm apart.
b. Use a thin art brush to guide the TFM bottom edge of a section downward to obtain a flat and smooth surface, be careful to grab with the brush on the mounting media but not on the tissue section to prevent damaging the tissue.
c. Add one drop of water on the desired location on the slide, then use a pre-chilled fine tip tweezer to grab the medium part of the section, and in the cryostat, gently place the section flat on top of the water drop.
d. Then quickly use a small transfer pipette to suck around and away the residual water underneath the section until it adheres flat onto the slide.

Note: This technique is crucial and reliable in our hand for obtaining wrinkle-free and intact brain cryosections, which is most ideal for downstream immunolabeling and semi-automated quantification for plaque abundance.

13. Slides are air-dried at room temperature overnight and then are either used immediately for immunofluorescence experiments (see protocol "immunofluorescence" below) or stored at −80°C in a slotted slidebox with desiccant.
14. Unfinished frozen TFM tissue blocks can be re-sealed by applying a thin layer of TFM at the cut surface stored at −80°C in airtight containers for reuse anytime.

Note: Avoid storing cryo tissue blocks at −20°C as temperature changes (freeze-and-thaw cycles, etc) can cause ice crystal damage to the tissue blocks.

Pause point: TFM tissue blocks can be kept in −80°C and glass slides mounted with cryosections can be saved for long-term use/reuse with proper storage conditions.

For common issues with cryosections and preparations for downstream immunofluorescence experiments, please refer to troubleshooting problem 1 and 2.

Immunofluorescence

Timing: 2–3 days

This protocol describes the key standard steps of conducting immunofluorescence experiments on transgenic AD brain cryosections for analysis and quantification of Aβ plaque abundance, axonal dystrophy, synaptic colocalization, and microglial activity. This protocol is partly adapted from recent publications[10–13]). Note that steps such as antigen retrieval and autofluorescence quenching are optional and will need to be evaluated for the specific antigen/antibody and imaging setup case by case.

Day 1

15. Thaw slides (stored at −80°C before use) in room temp for 10 min.
    a. Make sure the cryosections are completely dry on the slides and do not have extra mounting medium underneath, so they adhere well to the slides. This is especially important if performing ThioS staining.

16. **Optional:** ThioS staining can be done first in combination followed by standard immunofluorescence protocol.

a. Prepare solutions (the volume below is intended for glassing histology staining tubs that hold 20 slides back-to-back, increase or decrease volume accordingly based on the size of the choice of staining container.
   i. 70% Ethanol 400 mL.
   ii. 80% Ethanol 600 mL.
b. Freshly make 0.1% ThioS in 200 mL 80% ethanol solution.

Note: ThioS solution must be made fresh and be filtered through 0.22 μm filter right before each use.

c. Wash slides with 70% Ethanol for 1 min.
d. Wash slides with 80% Ethanol for 1 min.
e. Incubate slides in filtered thioflavin S solution for 15 min in dark.

Note: protect thioflavin S from light and protect stained slides from light.

f. Wash slides with 80% Ethanol for 1 min in dark.
g. Wash slides with 70% Ethanol for 1 min in dark.
h. Wash with distilled water twice before mounting directly or continue to immunofluorescence.

Note: ThioS can be used for visualizing β-pleated sheets in pathology AD brain sections, including the dense core in neuritic amyloid plaques as well as Tau pathology and therefore can be useful to be simultaneously with antibodies that recognize various forms of amyloid-β (e.g., 6E10, 3D6, 4G8) or phosphorylated Tau such as AT8. To achieve high consistency in batch analysis, ThioS staining (and IF experiments in general) should be done in cohort batches when possible to ensure comparable background and staining intensity whenever possible and as little experimental variability as possible. Highly variable immunosignals can be normalized to background or to the expression level in wild type/control samples for relative fold change quantification.

17. Wash slides with 1×PBS for 5 min.

Optional: In the meantime, prepare citrate-based antigen retrieval (AR) buffer and fill 50 mL of AR per coplin glass jar that holds 10 slides back-to-back; if more slides, increase antigen retrieval solution correspondingly.

AR recipe:

A solution: 1.83 g citric acid ($C_6H_8O_7$) in 100 mL H_2O.

B solution: 15.66 g trisodium citrate ($C_6H_5O_7Na_3.2H_2O$) in 500 mL H_2O.

Working citrate buffer solution =1,000 mL = 18 mL solution A + 82 mL solution B +900 mL H_2O.

Preheat to ~60°C–70°C: Microwave for 30 s on power 1 (10% of total power) and again for 15 s on power 10.

18. Transfer the slides from the PBS buffer and immerse into the pre-warmed AR buffer.
19. Microwave the AR jar for 2 min on power 10 and the solution reaches a temperature of 80°C–90°C.

20. Once the slides are cool, rinse and wash the slides once with PBST for 5 min.
    a. Recipe for PBS-T (Wash buffer): 1 mL of Tween-20 in 1,000 mL PBS.

Table 1. Primary antibody in use and working conditions

Purpose	Antibody	Company, Cat #	Working dilution	Notes & tips
Labeling Different forms of amyloid β	Mouse anti-human β amyloid, clone 6E10	Covance SIG-39320	1:1000	Labels diffuse and dense-core plaques. Can be used in conjunction with ThioS or Methoxy-X04.
	Mouse anti- Aβ amyloid 17–24, clone 4G8	Covance catalog# SIG-39200	1:1000	4G8 ß-amyloid antibody reacts to abnormally processed isoforms, as well as precursor forms. Recognizes both rodent and human ß-amyloid.
For neuro-degenerative phenotypes	Rabbit anti-cleaved Caspase 3, Asp175	Cell signaling 9661	1:200	
	Rat anti-LAMP-1	BD Biosciences, Cat# 553792, clone 1D4B	1:200	Works better for labeling axonal dystrophy without antigen retrieval
	Rabbit anti-Reticulon 3 (RTN3)	EMD Millipore ABN1723	1:200	
For studying microglial morphology and response during AD pathogenesis	Rabbit anti-Iba1 Goat anti-Iba1	Wako 019-19741 Novus NB100-1028	1:200 1:50–500	
	Rabbit anti-Tmem119	Abcam AB209064	1:200	Labels resident or resting state microglia
	Rat anti-CD68	BioRad MCA1957	1:200	
	Rat anti-mouse Mer Goat anti-mouse Mer	eBioscience DS5MMER or R&D AF591	1:100–200 1:50	Works better with citrate antigen retrieval
	Goat anti-mouse Axl	R&D AF854	1:50	
	Sheep anti-Trem2	R&D systems, AF1729 and BAF 1729	1:200 and 1:50, respectively	Works better with citrate antigen retrieval
For studying excitatory synapses in hippocampus CA1	Guinea pig anti-vGlut1	Millipore, AB5905	1:1000–2000	See protocol below for synaptic imaging and analysis
	Rabbit anti-PSD95	Life Technologies, 51-6900	1:400	

Note: due to the high viscosity of Tween-20, use a 1cc syringe or go by weight for an accurate addition of Tween-20.

b. In the meantime, prepare a humidified chamber ("immunobox") by wetting tissue paper with sufficient H_2O to prevent evaporation during incubation.

21. Then quickly use kimwipe to suck off the residue liquid on the edges.
22. Use a hydro-barrier PAP pen to draw edges on the glass slides. Wait one minute before adding blocking buffer (BB).
    a. BB recipe: 2% BSA, 5% natural donkey serum, 0.1% Tween-20, 0.3% Triton X-100.
    b. BB can be frozen into small aliquots and store in −20°C for up to 6 months. If stored in fridge, use within 1 week.
23. Add ~100 μL BB to each sagittal brain section. Incubate slides in immunobox at room temperature for 1 h.
24. Make primary antibody mixture in BB. Again, estimate ~100 μL antibody solution for each brain section. Make sure antibody solution should sufficiently cover the sections to avoid drying overnight. Mix well before adding on brain sections.
    a. Follow respective manufacturer's instruction to prepare and store the stock antibodies. Example primary antibodies for characterizing various aspects of AD-related pathological changes are listed in Table 1 as a guideline.
25. Incubate in primary antibody mixture in the humidified chamber O/N in the dark at 4°C.

Note: Spin down the antibodies at max speed on a tabletop centrifuge for 30 s to remove any precipitated antibodies. Make sure the slides are placed level in the chamber and are not obstructed by anything atop.

Optional: Additional 100 mM L-lysine in BB to assist with synaptic labeling.

Day 2

26. Rinse and wash with wash buffer for 3 × 10 min.
27. In the meantime, make secondary antibody mixture (depending on the optic setup on the confocal microscope, I usually use a combination of Alexa Fluor 488 and 546 sometimes Alexa Fluor 647 on far red channel, with a 1:500 dilution in BB).

Note: Spin down the antibodies at max speed on a tabletop centrifuge and only withdraw from the top to avoid fluorescent antibody precipitates.

Optional: for aged or AD CNS tissues with lipofuscin, a step of autofluorescence quenching is necessary. Autofluorescence has broad spectrum and therefore can be observed and obscure immunofluorescent signals during imaging in all channels. To quench autofluorescence, add sufficient TrueBlack (20×) or Sudan Black D (0.1%) dissolved in 70% EtOH to cover sections, and incubate for 1 min before rinsing in PBS for 5 min before secondary antibody incubation.

Note: after autofluorescence quenching step, no detergent should be used in the following steps (i.e., substitute wash buffer with PBS, and non-detergent-containing blocking buffer).

28. Then incubate section in secondaries at RT in dark.
29. Make Hoechst33258 solution for nuclei staining:
    a. Stock Hoechst solution: 1% Bis Benzamide solution.
    b. For working dilution, use 1:1000 in PBS.
    c. Alternatively, use DAPI-containing mounting media at the end.
30. Suck off the secondaries with a vacuum suction system and incubate in Hoechst solution for 5 min at RT in the dark.
31. Rinse and wash 3 × 10 min with wash buffer.
32. Rinse and wash with PBS once. Then wash once with dH_2O.
33. Use mounting medium Fluoromount-G approx. ~20 µL/1 drop per section or ~50 µL for covering the whole slide. Gently lay down the coverslip of the fitted length (#1 $^1/_2$ that is optimized in optical index for subsequent confocal imaging).

Note: release the cover slip very slowly with fine point tweezers from one side to the other and carefully to avoid bubbles. If small bubbles inevitably formed, use a pipet tip to gently tap near the bubble on the cover glass to drive out any bubbles.

Optional: a good practice for efficient mounting and bubble formation is to do reverse mounting by applying mounting medium to coverslip instead of onto the sections (~60 µL per 50 mm long coverslip).

Alternatives: instead of doing a separate Hoechst staining, mount coverslips into Vectashield mounting medium with DAPI (Vector Laboratories Inc., Cat. No: H-1200) or ProLong Gold antifade mountant with DAPI onto glass slides.

34. The mounting media will solidify at RT at a dry and dark place overnight before imaging. Store slides at 4°C.

For common issues with immunofluorescence experiments, please see troubleshooting problem 3 and 4.

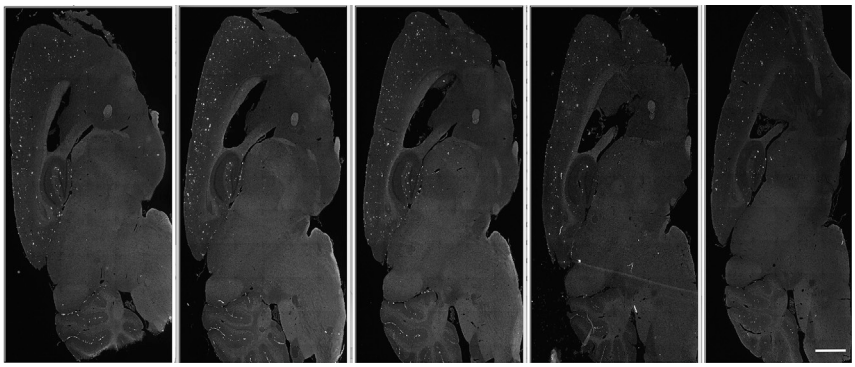

Figure 3. Representative images of Thioflavin S (ThioS)-staining for dense core plaque burden
Images are from serially cut 15 μm cryosections from a 12 mo. APP/PS1 left hemisphere. Scale bar, 1 mm.

Isolation of soluble Aβ from transgenic AD mouse cortex and hippocampus and its measurement by sandwich ELISA.

This protocol is partially adapted from Yang et al.[14] and Salas et al.[15] This protocol can be used for extracting and measuring the level of soluble Aβ in the cortex and hippocampus of AD transgenic mice both prior to plaque deposition (i.e., 2 mo and 4 mo in APP/PS1 mice) and at a plaque-laden stage (i.e., 12 mo. in APP/PS1 mice) in the disease course.

Day 1. Extraction soluble Aβ from AD transgenic mouse brains

Timing: 3–4 h

35. Fresh hippocampus and cortex dissociation.
    a. Following the 2 min transcardial perfusion with ice-cold D-PBS (step 2f).
    b. Transfer the perfused brain to a dry filter paper and quickly roll a full round to roughly remove the meningeal layer, then quickly place the brain onto a D-PBS-wetted filter paper on a petridish surface on ice (Figure 4A).
    c. Under a dissection microscope, first use a clean razor blade to slice down the midline of the brain. Then, use rounded edge of spatula to tease away brainstem, cerebellum, olfactory bulb, and other subcortical parenchyma.
    d. Place the two halves of cortex down, use iris micro-scissors and the tip of the curved spatula to gently tease outward the crescent-shaped hippocampus without protruding into the cortex (Figure 4B).
    e. Finally, use micro-scissors to trim as much white matter as possible to yield the cortex (Figure 4C).

Pause point: Snap freeze the dissected tissues in the corresponding cryo-safe tubes in liquid nitrogen and then either store at −80°C until further processing or transfer the dissected cortex and hippocampus to the corresponding homogenization tube on ice and proceed.

36. For soluble Aβ extraction from AD transgenic brains.
    a. Add 500 μL prepared ice-cold lysis buffer (RIPA + Halt protease and phosphatase inhibitor cocktail (Thermo Scientific)) to each hemisphere of cortical sample and add 250 μL lysis buffer to each pooled bilateral hippocampal sample.
    b. Tissue homogenization and fractionation by ultracentrifugation.
        i. Tissue is homogenized in lysis buffer with a motorized pestle (Kontes 749521-1500) for 30 s on ice and is set on ice for 20 min with agitation every 5 min by briefly vortexing and inverting the tubes.

Note: switch to a new pestle for each sample.

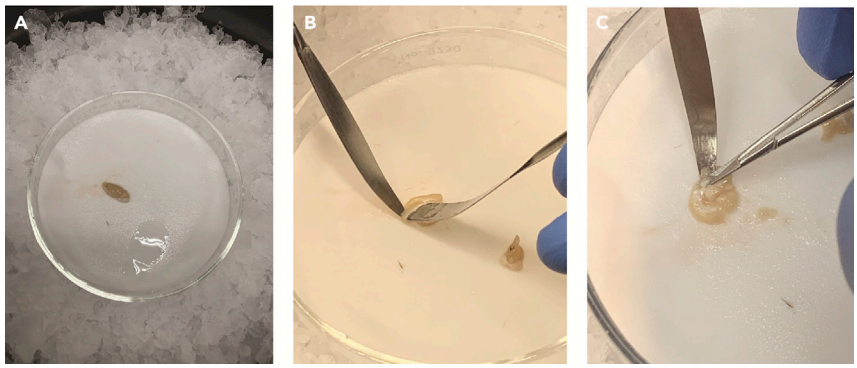

Figure 4. Overview of steps for fresh mouse brain dissection

(A–C) Microdissection of freshly perfused mouse brain on ice (A) for various brain regions, such as hippocampus (B) and cortex (C).

ii. In the meantime, pre-chill rotor (BeckmanTLA-120.2) to 4°C in ultracentrifuge; set up speed and program on the ultracentrifuge.
iii. After incubation, first spin down for 5 min at 16,000 g (12,000 rpm) at 4°C.
iv. Transfer 200 μL supernatant samples to ultracentrifuge tubes that are chilled on ice; discard debris.

Note: the remaining supernatant lysate can be stored in a separate tube at −80°C for immunoblotting assays.

v. Centrifuge the samples at 4°C for 1 h at 100,000 g (or 55,000 rpm).

Note: make sure to weigh the tubes beforehand to balance for the ultracentrifugation.

vi. Meanwhile, set up a BCA assay for measuring protein concentration in the ultracentrifuged supernatant.
vii. Follow manufacturer's instruction and calculate respective concentration of each supernatant sample before and after ultracentrifugation, with a dilution factor of 10 and 2, respectively.
viii. Aliquot the supernatant to the prepared tubes and parafilm seal the pellet in the polycarbonate tubes for further extraction of insoluble Aβ.
ix. Freeze the supernatant and pellet tubes in −80°C or at −20°C for ELISA the next day.

Note: formic acid can be used to extract insoluble forms of Aβ associated with plaques from the pellet tubes after ultracentrifugation.[16]

Pause point: Samples can be stored in −80°C for long-term use.

37. If continue to ELISA measurement of soluble Aβ concentration from tissue supernatant:
    a. Prepare solution for the next day:
        i. Coating buffer.
        ii. Wash buffer.
        iii. Substrate solution.
38. Coat the plate:
    a. Prepare one 96-well transparent flat-bottom plate (costar transparent flat bottom).
    b. Dilute human Aβ42 capture antibodies (Aβ 42: JRF A042/26, Janssen Pharmaceutica) for a final concentration of capture antibody = 1.5 μg/mL.
    c. Load 50 μL of solution each well.

39. Incubate overnight on shake at 4°C, covered with plate seal to avoid evaporation.

Day 2. Sample incubation

Timing: 4 h

40. Wash plate 5× with 150 μL of ELISA wash buffer per well.
41. Add 50 μL of 1% casein blocking buffer to block and incubate in RT for 3 h.
42. Prepare a serial dilution for standards with Aβ42 peptides in diluent:
    a. Stock concentration of Aβ 42 is 1 μg/mL.
    b. Diluent is casein blocking buffer + 10% of RIPA for a 1:10 sample dilution (or the right % of RIPA depending on how samples are diluted).

Note: a pilot study with a gradient of dilution factors is crucial for determining the dilution factor of AD brain samples and the detection sensitivity. We recommend starting from a 1:5 dilution for brain lysate supernatant to avoid a strong matrix effect. For 12 mo. cortical samples from APP/PS1 mice, we used a 1:10 dilution and for hippocampal samples, we optimized our results for 1:4 or 1:10 dilution.

    c. Prepare the serial dilution for 1 plate with eight Aβ42 standards in duplicates (31.25–2,000 pg/mL).

Note: prepare this step within approx. 30 min prior to finishing blocking.

43. Prepare sample.
    a. Use a round bottom 96 well plate to mix samples; label plate if needed.
    b. Prepare sample dilutions.
        i. For each 12 mo. cortex sample from APP/PS1 mice, for a 1:10 dilution, dilute 6 μL of supernatant sample in 54 μL of casein blocking buffer).
        ii. For each 12 mo. hippocampus sample from APP/PS1 mice, for a 1:4 dilution, dilute 15 μL of supernatant sample in 45 μL of casein blocking buffer).

Note: prepare at least extra 10 μL per diluted sample to avoid bubbles/inaccuracy in duplicates.

⚠ CRITICAL: optimize sample dilution and readout accuracy by running a pilot study with a gradient of dilution factors to determine an optimal and reliable detectable range of the specific sample type and age. See troubleshoot problem 5 for details.

44. Wash 5× with PBS + 0.05% Tween-20.
45. Load 25 μL of standard dilution #1–8 and 25 μL of samples. Both in duplicates.
46. Prepare 1/2000 dilution of N25-biotinylated human antibody in casein blocking buffer for a final concentration = 1.07 μg/mL.

Note: the N25 biotinylation and purification can be performed by using EZ link Sulfo-NHS-LC Biotin (Thermo Fisher Cat #21217) and Slide-A-lyzer dialysis cassette (Pierce 0728 #66415) following manufacturers' instructions.

47. Add 25 μL of the dilution to each well on top of standards and samples.
48. Incubate overnight at 4°C.

Day 3. Development

Timing: 1–1.5 h

49. Wash 5× with ELISA wash buffer.
50. Prepare 1:2500 dilution of streptavidin-HRP for 50 μL/well.
    a. 2.5 μL diluted in 5 mL of BB.
    b. Incubate for 30 min.
51. Wash 5× with ELISA wash buffer.
52. Prepare substrate right before the last wash with a 1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine) (Cat # DY999).
    a. Immediately load 50 μL of substrate into the well.
53. Incubate in the dark for 15 min or until color fully develops then add 50 μL of stop solution (H_2SO_4).
54. Plates were immediately measured on a TECAN Infinite® 200 PRO reader at 450 nm.

Note: read plate within 30 min of adding the stop solution.

EXPECTED OUTCOMES

For cryosection technique for obtaining serially cryosectioned sagittal brain sections, see Figure 2. After air drying, one would expect a serially cut wrinkle-free sagittal brain sections that adhere well to the microscopy slides.

For representative image of Thioflavin S (ThioS)-stained for dense core plaque burden in serially cut cryosections, see Figure 3. A well-executed batch ThioS staining should result in with a uniformly applicable threshold on the ThioS channel with distinctive border marking the ThioS-labeled dense cores of amyloid β plaques.

For image analysis of LAMP1 immunofluorescence as a proxy for measurement of dystrophic neurites in transgenic AD mouse brains, see Figure 5.

QUANTIFICATION AND STATISTICAL ANALYSIS

Image analysis

Imaging and quantification of hippocampal CA1 excitatory synapses using Imaris

Note: image analysis should be done with the experimenter/analyst blind to genotype or experimental conditions.

This synaptic analysis protocol is adapted from several recent publications.[12,17,18]

1. Synaptic brain region of interest should be selected consistently among sections.
    a. For example, our synaptic abundance featuring the excitatory presynaptic marker vGlut1 and post-synaptic marker PSD95 focused on the CA1 apical dendrite area in aged hippocampal brain sections in CA1 brain sections approximately 500 μm–800 μm from lateral from the midline (CA1 of the hippocampus is a vulnerable brain region in aging and neurodegenerative diseases that is subjected to synaptic loss and long-term depression).
    b. Collect z-stack images of 3 μm thickness in designated brain region (optical slice 0.29 μm, 11 slices per 3 μm stack) using confocal or Airyscan imaging.
2. Analyze the co-localization of vGlut1 and PSD95 in the apical dendrite area of CA1 using Imaris software (version 9.1.2).
    a. To enhance and smoothen the signals of synaptic markers, a Gaussian filter can be added in the beginning of each image analysis.
    b. Built 'spots' for positive puncta of vGlut1 and PSD95.
    c. Optimize and apply the same threshold for 'quality' and 'mean puncta intensity' across all images/sections/genotypes analyzed.

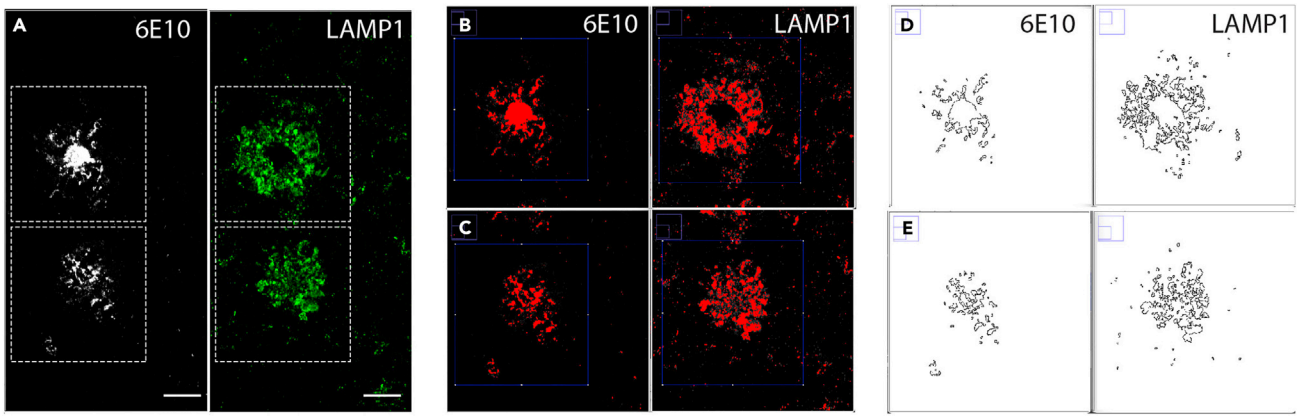

Figure 5. Assessment of LAMP1 immunofluorescence as a proxy for measurement of dystrophic neurites in APP/PS1 cortex

(A) Representative images of the halo of LAMP1$^+$ dystrophic membranes (green, right panel) that surround 6E10$^+$ plaques in 12 mo *APP/PS1* cortex. (B–E) Individual plaques are quantified for the ratio of LAMP1$^+$ area to 6E10$^+$ plaque area and categorized into 'dense-core' (B and D, referring to the upper box in A) and 'diffuse' plaques (C and E, referring to lower box in A). Representative intensity thresholding (B and C) and quantification (D and E) for area above threshold (red) on 6E10 and LAMP1 channels.

Scale bar, 20 μm.

d. Transform 'PSD95' spots into a distance vector using the 'distance transform' function followed by an in-built MATLAB-based calculation of distance between vGlut1 spots and PSD95 spots.

Note: The selected puncta of both channels are considered colocalized if the distance between vGlut1 and PSD95 centroids is $\leq$ 0.7 μm.

3. Number of colocalized puncta from each image is averaged for one section or for one animal and compared between experimental groups that contain 3 animals. To gain a good representation of synaptic abundance in CA1 and to mitigate high biological variability issues, at least 3 images per section and $\geq$ 3–5 sections per animal should be analyzed.

Note: Biological and replicate variability does exist and can affect the interpretation of the study. Normalizing group mean to littermate control samples, normalizing synaptic density to another brain region on the same section, or doing paired analysis may attenuate some issues with high variability in experiments and technical replicates.

Quantification of ThioS dense-core plaque burden using Fiji (ImageJ)

4. Set measurement for 'area' only; set scale to the correct pixel to distance (use 1 pixel = pixel size × magnification of objective).
   a. For example, 10× images using the Olympus slidescanner VS-120 with a pixel size of 6.5 μm, so 1 pixel = 0.65 μm for the output image.
5. Open a series of cortex and hippocampus tilescan of ThioS (FITC channel) in Fiji/ImageJ software. Convert output from Olympus slidescanner VS-120 into 16-bit TIFF images.
6. Select 'Image', and adjust 'Brightness and contrast' (B&C) to 0–5000 for brightness on the set settings (or until signal to noise ratio on the ThioS channel is reasonable pronounced without over exposure).
7. Check the option to 'propagate to all open images' of the current B&C setting.

Note: This may take a while depending on sample size.

8. Then convert each image from 16 bit to 8 bit for further analyze.

9. For every image, set threshold for these 8-bit images to 50–255 or immediately before the start of the signal peak.
10. Draw cortex area using the 'freehand' selection tool on one image.

Note: Avoid folded areas or minor regions with noisy background.

11. Measure and record drawn area.
12. Select 'Analyze particles' with a parameter of '20-infinity (unit is in μm^2)'.
    a. Save analysis results for each image before closing window.
13. Loop above steps 10–12 for all open images for cortical plaque burden analysis, then proceed on another brain region (i.e., hippocampus) or next series of samples.
14. Summarize plaque number and abundance by binning on size and map a distribution of area of 'ThioS+ plaques' (particles) once all data are collected.

Quantification of the area of LAMP1-marked neuronal dystrophy using Fiji (ImageJ)

15. Randomly choose 3 representative fields of view for a trial analysis (212 μm × 212 μm × 15 μm z-stacks at 1024 × 1024 pixel resolution) containing around equal number of 6E10-positive plaques spanning from at least 3 sections per animal in the prefrontal cortex using the 6E10 channel.
16. Use FIJI (imageJ) to analyze the maximum intensity projection of these confocal images.
17. For quantification of dystrophic neurite area using LAMP1 or RTN3, set a fixed fluorescent threshold for either LAMP1 or RTN3 channel whose area is considered as LAMP1 or RTN3-marked neuritic dystrophy per plaque basis.
18. For each plaque, calculate and sum 6E10 immuno-positive area using the FIJI 'set threshold' and 'analyze particle' tool (Figures 5B–5E). Then analyze the dystrophy impact as a ratio of plaque burden by calculating the ratio of summed area of both channels (LAMP1+ or RTN3+ area divided by the respective 6E10+ plaque burden) on per plaque basis.
19. For a binary quantitative categorization of plaque species for LAMP1-marked neuronal dystrophy (examples shown in Figure 5), dense-core plaques are defined as plaques that contain a single or solid (usually bright) 6E10-positive core area $\geq$ 100 μm^2, and diffuse plaques are defined as the complement of these dense-core plaques that are characterized as those devoid of compact 6E10-positive area $\geq$ 100 μm^2.

LIMITATIONS

This protocol outlines streamlined procedures from tissue preparation to assessment of soluble Aβ and plaque composition and related pathological changes such as neuronal dystrophy in brains of amyloidogenic mouse models of AD. As such, it does not focus on post-mortem human brain samples, nor cover analysis of other hallmarks of AD, such as pathological Tau and neuroinflammation, although this versatile pipeline can be easily converted to assess such changes. For the immunofluorescence and imaging sections, the protocol is fine-tuned to detect changes mostly after, not before, deposition of amyloid plaque deposition using thinly sectioned brain sections.

TROUBLESHOOTING

Problem 1

Common cryosection issues, related to steps 10–14.

Potential solution

- Sections curl on the edges and/or fall off the section: this is often due to residual sucrose in the sulci and fissures of the brain. Be sure to pat dry carefully with a kimwipe tissue before coating the brain hemisphere with mounting media.

- Brittle sections (horizontal cracks): block or cutting temperature may be too cold. Blocks in TFM or OCT taken out of −80°C as well as the cryosection knife/blade will need to be equilibrated inside the cryostat to the correct chamber temperature before proceeding on sectioning.
- Wrinkly and smooshed sections: internal chamber temperature may be too warm. We usually process OCT blocks at −20°C and TFM blocks with fixed tissues at −25°C for ~10 μm–15 μm. Make sure the knife and chuck mounted with the tissue block reach the same chamber temperature when cutting.
- Consecutive split sections (vertically split): check if hair from the brush tip is stuck on the cryosection knife; change/clean knife/blade.
- During immunofluorescence or immunohistochemistry experiments, if some sections lift/fold up in corners on the microscopy slides, this may be due to some mounting media left underneath the section. Hydrating, unfold the folds on the sections then dehydrate again by airdrying the sections again for 2 h for the sections to all fully adhere to the slides before restarting the experiment. That is, if the experiments following involves a complete dehydration of the tissue slides (for example, by using Xylene), then having the sections adhere perfectly to the slides is crucial because unattached portion of brain section may expand/shrink unproportionally to the rest of the section throughout dehydration process. Otherwise, the minor folds can be corrected by a moistened art brush before blocking buffer incubation.

Problem 2

Issues with tissue sections, related to steps 10–14.

Potential solution

- Unnatural 'holes' in sections when looked through a light microscope may suggest ice crystals damage in the tissue block. This may be caused by insufficient cryoprotection or frequent freeze and thaw of the blocks. Use 30% sucrose in PBS for CNS tissues and do not proceed onto the freezing process until the brains fully sink to the bottom of the 30% sucrose solution. Store tissue blocks in −80°C freezers for long term storage. Ice crystal damage can also arise from a slow freezing process, which can be improved by using thinner tissue freezing molds and using isopentane and crushed dry ice for better temperature conductance.
- Uneven dark/light blotches on the sections: this can be due to insufficient or over perfusion/fixation or penetration of cryoprotectant. Always use freshly made 4% PFA as old PFA stored at 4°C can polymerize and lower its cross-linking efficiency. For complete cryoprotection, wait at least for 24 h after switching to 30% sucrose post-fixation or until the brain hemispheres sink to the bottom of the vial.

Problem 3

Low signal during immunofluorescence, related to steps 15–34.

Potential solution

Signal/noise ratio needs to be boosted.

- Issues with low signal – Add a step of citrate antigen retrieval (see optional step under step 17) or formic acid retrieval, which can unmask previously unavailable epitopes; Increase primary antibody concentration or longer time of incubation.

  Alternatives: For thicker tissue sections, enhance permeablization (triton X-100 content) or adopt a free-floating immunofluorescence protocol to enhance antibody penetration.

- Make sure antibody solution fully covers the tissue sections for the entire duration of incubation; spin down antibodies in a table centrifuge for at least 30 s at maximum speed prior to preparing for antibody buffers to avoid precipitates in antibodies obstructing accurate labeling of antigen.

- Whenever possible with a new antibody trial, perform an antibody serial dilution assay to find the optimal antibody dilution and include non-primary (secondary antibody only) controls and knockout controls if available.

Problem 4

High-background and non-specific staining during immunofluorescence, related to steps 15–34.

Potential solution

Together with problem 3, changing the following variables should optimize signal/noise ratio during imaging:

- Issues with high noise or background – enhance the strength of blocking (for example, using 10% serum compared to 5%); make sure blocking buffer uses the same host species of the secondary antibodies (i.e., if the secondary antibody is made in Donkey, then during initial blocking step use normal donkey serum; switch to use F'ab fragmented or cross-absorbed secondary antibodies to minimize non-specific or off-target labeling from secondary antibodies; Add 30 s-1 min of TrueBlack treatment before secondary antibody staining (see optional step under step 27) to reduce non-specific tissue background and autofluorescence in aged mouse CNS.
- Whenever possible, include a no-primary secondary-only control to discern specific immunoreactivity signals from noise from non-specific secondary antibody binding.

Problem 5

Inconsistency in ELISA readouts, related to steps 35–54.

Potential solution

The same transgenic mouse brain sample diluted by different dilution factors should result in similar estimation of the concentration of the undiluted sample after interpolation of each individual diluted sample readout, but this may not always be the case. There are several potential leads for troubleshooting:

- Roof/floor effects: Ideally the diluted sample readouts should occupy the most fitted portion of the standard curve (usually in the middle portion of the standard curve between standards 2 and 5 if followed by a standard 8-standards series).
- If samples fall outside of the standard range or too close to one end, try increase/decrease the standards or the dilution factor cascades for the standard curve to well cover the spread of the sample readouts. Choose the most fitting curve and recognize the asymptotic region of the curve to be regions of inaccuracy where roof/floor effects may take place in sample optical density readouts.
- A matrix effect from adult brain tissue lysate could have played a role in inaccurate interpolation. In general, the more diluted the brain lysates are, the less likely the matrix effect plays a role in the accuracy of the soluble Aβ readouts so long as the is still within the readable range on the standard curve and not touching on the floor effect. Typically, a dilution factor of 1:10–1:20 works the best in our hands for aged AD transgenic mouse cortical tissue after ultracentrifugation preparation. Higher dilution may lower the accuracy of soluble Aβ readouts. Alternatively, a "spike in" assay can be introduced to assess the degree of matrix effect play a role in the readouts of soluble Aβ and a recovery rate of 80%–120% of the expected value should be expected.
- Remember to include all essential controls (i.e., samples from wild type littermate brains, sample buffer only control, etc.) and technical replicates in each assay.

Alternatives: Meso Scale Discovery (MSD) platform immunoassays for Aβ 1-x, Aβx-40, and Aβx-42 can be used for the best detection sensitivity and selectivity of Aβ species in tissue homogenates.[14,19]

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Youtong Huang, youtong.huang@childrens.harvard.edu

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article includes all datasets generated or analyzed during this study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101891.

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AUTHOR CONTRIBUTIONS

Y.H. and G.L. wrote and edited this manuscript; G.L. supervised the study and provided funding (1RF1AG060748 from the NIH).

DECLARATION OF INTERESTS

The authors declare no competing interests.

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