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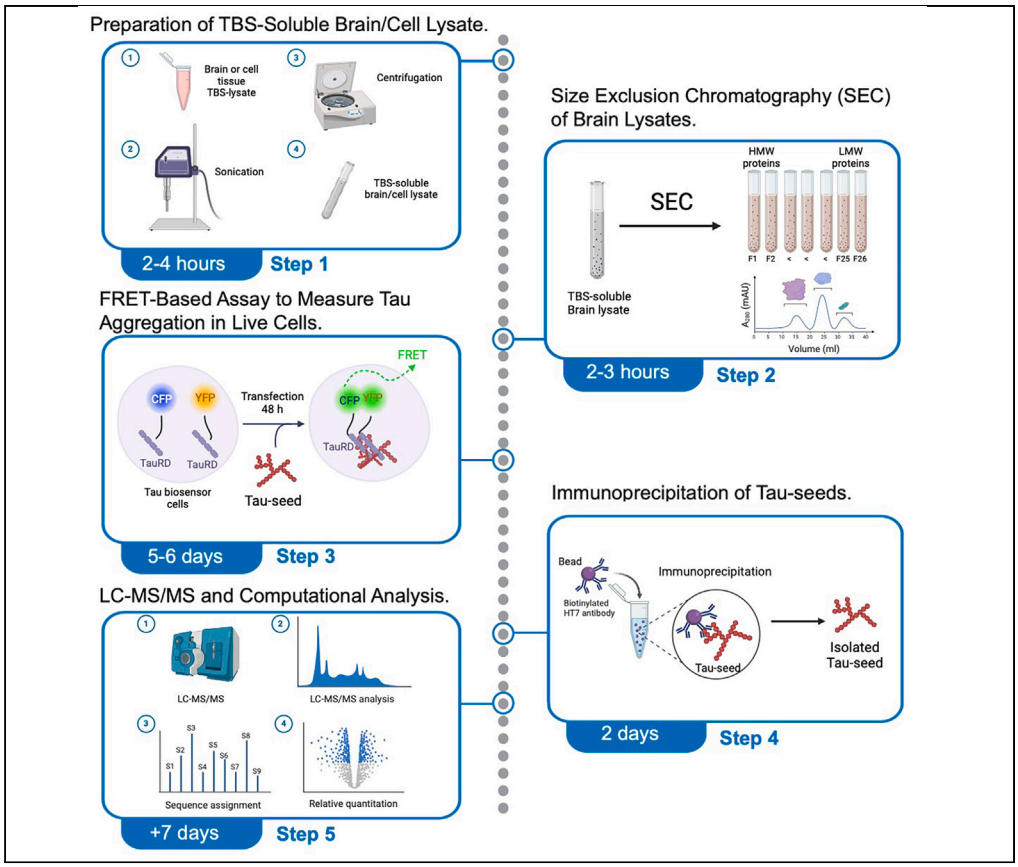
Protocol for the isolation and proteomic analysis of pathological tau-seeds



The aggregation and spreading of "tau-seeds" are key for the development and progression of tauopathies, including Alzheimer's disease. Here we describe the steps to isolate and analyze biochemically active tau-seeds from human, mouse, and cell origin. We detail the procedure to isolate soluble tau-seeds by size exclusion chromatography and seeding assay. The isolated tau-seed can be further analyzed to determine the interactome by mass spectrometry. This workflow identifies protein-protein interactors of tau-seeds, providing a useful tool for finding new therapeutic targets.

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

Pablo Martinez, Henika Patel, Yanwen You, Emma H. Doud, Amber L. Mosley, Cristian A. Lasagna-Reeves

clasagna@iu.edu

Highlights

Steps for Tau-seed isolation (from human and mouse brains, cell lines, etc.)

Protocol includes size exclusion chromatography, tau-IP, and a seeding assay

Isolated Tau-seed can be analyzed by mass spectrometry to identify interactors

New therapeutic targets could be found by interactors associated with tau-seeds

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Protocol for the isolation and proteomic analysis of pathological tau-seeds

Pablo Martinez,[1,2,5] Henika Patel,[1,2] Yanwen You,[1,2] Emma H. Doud,[3] Amber L. Mosley,[3,4] and Cristian A. Lasagna-Reeves[1,2,4,6,*]

[1]Stark Neurosciences Research Institute, Indiana University School of Medicine, Indianapolis, IN, USA
[2]Department of Anatomy, Cell Biology & Physiology, Indiana University School of Medicine, Indianapolis, IN, USA
[3]Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN, USA
[4]Center for Computational Biology and Bioinformatics, Indiana University School of Medicine, Indianapolis, IN, USA
[5]Technical contact
[6]Lead contact
*Correspondence: clasagna@iu.edu
https://doi.org/10.1016/j.xpro.2024.103185

SUMMARY

The aggregation and spreading of "tau-seeds" are key for the development and progression of tauopathies, including Alzheimer's disease. Here we describe the steps to isolate and analyze biochemically active tau-seeds from human, mouse, and cell origin. We detail the procedure to isolate soluble tau-seeds by size exclusion chromatography and seeding assay. The isolated tau-seed can be further analyzed to determine the interactome by mass spectrometry. This workflow identifies protein-protein interactors of tau-seeds, providing a useful tool for finding new therapeutic targets.

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

BEFORE YOU BEGIN

Tau-seeds, a key agent responsible for tau aggregation in several neurodegenerative diseases, can be isolated from various sources. This protocol details the isolation and characterization of tau-seeds with high seeding activity, a crucial factor in disease progression.[2,3] Furthermore, it is vital to choose the correct source of tau for your specific study. Tau-seeds can be isolated from many sources such as different mouse and cell tau models, human brain tissue from different tauopathies, etc. Alternative models can also be useful for proper tau-seed isolation. This includes, for instance, *Drosophila melanogaster* and *C. elegans* tau models. Analysis of the isolated tau-seed can be compared to monomeric tau isolated from the very same brain, providing an important advantage when analyzing different tau species from the same individual or case. Using this method, we previously identified the presynaptic protein Bassoon as a key tau-seed interactor that stabilizes and helps tau-seed to propagate throughout the brain.[1]

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Biotin mIgG1 (2 μg per 100 ng of human tau)	BioLegend	400104
Biotin HT7 (2 μg per 100 ng of human tau)	Invitrogen	MN1000B

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Protease phosphatase inhibitor	Thermo Scientific	A32959
cOmplete, mini, EDTA-free protease inhibitor cocktail	Sigma	11836170001
Sodium chloride	Acros Organics	327300025
1 M Tris pH 8.0	Invitrogen	AM9856
Lipofectamine 2000	Thermo Scientific	11668019
Streptavidin magnetic beads	Invitrogen	88817
IP elution buffer	Pierce	1858606
Urea	Sigma-Aldrich	U1250
Trifluoroacetic acid (TFA)	Sigma-Aldrich	302031
2-chloroacetamide (CAA)	Sigma-Aldrich	C0267
TMTpro labels	Thermo Scientific	A52045
Triethylammonium bicarbonate (TEAB), 1 M	Thermo Scientific	90114
50% hydroxylamine	Thermo Scientific	90115
Acetonitrile (ACN)	Fisher Scientific	A955
Formic acid (FA)	Sigma-Aldrich	695076
0.1% formic acid in water	Fisher Scientific	LC452
0.1% formic acid, 80% acetonitrile	Fisher Scientific	LS122
Tris(2-carboxyethyl)phosphine (TCEP)	Thermo Scientific	20490
Ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate (CAS 6381-92-6)	Fisher Scientific	BP120-1
Penicillin-streptomycin (10,000 U/mL)	Gibco	15140122
20% SDS	Invitrogen	AM9820
Critical commercial assays		
Pierce detergent compatible Bradford assay kit	Thermo Scientific	1863028
Experimental models: Cell lines		
Tau RD P301S FRET biosensor	ATCC	CRL-3275
HEK 293T	ATCC	CRL-3216
Tau RD P301S CFP	UT Southwestern/Marc Diamond	N/A
Tau RD P301S YFP	UT Southwestern/Marc Diamond	N/A
Software and algorithms		
FlowJo v10	BD Biosciences	RRID:SCR_008520
Proteome Discoverer 2.5	Thermo Scientific	https://www.thermofisher.com/
Other		
TBS	Corning	46-012-CM
PBS	Corning	46-013-CM
1 mL Hamilton gas tight syringe	Hamilton	81365
500 µL Hamilton gas tight syringe	Hamilton	81265
Eppendorf 5424 R refrigerated centrifuge	Eppendorf	Eppendorf 5404000014 RRID:SCR_019852
ÄKTA pure 25 L	GE/Cytiva	GE/Cytiva 29018224 RRID:SCR_023461
Fraction collector F9-C	GE/Cytiva	29027743
Superose 6 increase 10/300 GL	GE/Cytiva	29091596
Amicon 2 mL 3 K ultrafiltration filter	Millipore Sigma	UFC200324
2 mL sample loop	Idex	9055-028
1.1 mL 8-tube strips in rack	USA Scientific	1212-8410
BD LSRFortessa X-20	BD Biosciences	N/A
BD HTS	BD Biosciences	N/A
Falcon 100 mm TC-treated cell culture dish	Corning	353003
Nunc Edge 96-well, Nunclon Delta-treated, flat-bottom microplate	Thermo Scientific	167425
Conical 50 mL tubes	Corning	CLS430290
V-bottom plate	Greiner Bio-One	651101

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Tris-HCl, 1 M pH 8.5	Thermo Scientific	S61038AP
Tween 20	Fisher Scientific	BP337-500
Rotator	ELMI	RM-2L
Mini centrifuge	Benchmark Scientific	C1012
Magnetic rack	Invitrogen	12321D
Trypsin-EDTA 0.05%	Gibco	25300-054
Opti-MEM	Gibco	31985070
FBS	Fisher Scientific	10-437-028
DMEM culture media	Invitrogen	11995-065
Dounce homogenizer	Thomas Scientific	885300-0007
Sonicator	Qsonica	Q900
Trypsin-LysC	Promega	V5072
Pierce C18 spin columns	Thermo Scientific	89870
Optima water	Fisher Scientific	W6500
ThermoMixer or similar heat/shaking block	Eppendorf	EP5382000023
EASY-nLC 1200 (or other nano-LC)	Thermo Fisher Scientific	LC140
Aurora Ultimate, 25 cm column	IonOpticks	AUR3-25075C18
Column oven	Sonation Lab solutions	PRSO-V2
Nanospray Flex source	Thermo Fisher Scientific	ES1072
Orbitrap Eclipse Tribrid	Thermo Fisher Scientific	FSN04-10001
FAIMS Pro (optional)	Thermo Fisher Scientific	FMS02-10001
Vortex-Genie 2	Scientific Industries	SI-0236

MATERIALS AND EQUIPMENT

SEC buffer

Reagent	Final concentration	Amount
5 M NaCl	50 mM NaCl	5 mL
1 M Tris pH 8.0	50 mM Tris	25 mL
Ultra-pure water	N/A	add until total 500 mL
Total	N/A	500 mL

Note: Prepare fresh buffer for each use. Filter the buffer at 0.22 μm.

FACS buffer

Reagent	Final concentration	Amount
FBS-heat inactivated	2%	2 mL
EDTA disodium salt dihydrate	5 mM	186 mg
PBS	1X	add until total 100 mL
Total	N/A	100 mL

Note: Filter the buffer at 0.22 μm. Store at 4 C or lower temperature.

DMEM-based cell culture media

Reagent	Final concentration	Amount
DMEM	1X	220 mL
Penicillin/Streptavidin	1%	2.5 mL
GlutaMax	1%	2.5 mL
FBS-heat inactivated	10%	25 mL
Total	N/A	250 mL

8 M Urea		
Reagent	Final concentration	Amount
Urea	8 M	4.8 g
100 mM Tris-HCl, pH 8.5	N/A	up to 10 mL
Total	N/A	10 mL
Note: Store at 25°C for up to 3 months.		

TCEP 1 M Solution		
Reagent	Final concentration	Amount
tris(2-carboxyethyl)phosphine (TCEP)	1 M	2 g
Ultra-pure water	N/A	6.9 mL
Total	N/A	6.9 mL
Note: aliquot in 1 mL and 100 µL aliquots and stored at −80 for up to 2 years. It can also be purchased as a solution.		

TCEP 100 mM Solution		
Reagent	Final concentration	Amount
tris(2-carboxyethyl)phosphine (TCEP), 1 M	100 mM	100 µL
Ultra-pure water	N/A	900 µL
Total	N/A	1 mL
Note: Aliquot and store at −20 for up to 2 months. Do not freeze/thaw aliquots.		

2-chloroacetamide (CAA)		
Reagent	Final concentration	Amount
2-chloroacetamide (CAA)	500 mM	48.3 mg
Ultra-pure water	N/A	1 mL
Total	N/A	1 mL
Note: Aliquot and store at −20 for up to 2 months. Do not freeze/thaw.		

Trypsin-LysC stock solutions		
Reagent	Final concentration	Amount
Promega Trypsin/LysC	0.1 µg/µL	100 µg
Resuspension Buffer (provided with trypsin)	1 mL	1 mL
Total	N/A	1 mL
Note: Aliquot and store at −80 for up to 1 year. Do not freeze/thaw.		

TMTpro		
Reagent	Final concentration	Amount
TMTpro 5 mg reagent (Thermo Fisher)	0.5 mg dry	5 mg
Acetonitrile	N/A	250 µL
Total	N/A	250 µL
Note: Purchase and utilize the correct TMT reagent for the experimental design. For 5 mg purchased aliquots, resuspend and divide into 10 tubes, immediately speed vac. It can be stored dry at −80 for up to 1 year. Do not freeze/thaw. Keep dry.		

TEAB		
Reagent	Final concentration	Amount
TEAB 1 M	100 mM	1 mL
Ultra-pure water	N/A	9 mL
Total	N/A	10 mL
Note: Store at 25°C for up to 1 year.		

Hydroylamine, 5%		
Reagent	Final concentration	Amount
Hydroxylamine 50%	5%	50 μL
Ultra-pure water	N/A	450 μL
Total	N/A	500 μL

Note: Store at 25°C for up to 1 day.

STEP-BY-STEP METHOD DETAILS

Preparation of TBS-Soluble brain lysate

Timing: 2–4 h

This section contains the steps to prepare detergent-free TBS-based lysate from human and mouse brain tissue, and cell lines. This protocol is also adaptable to any other tau model.

1. Sample preparation.
   a. If using fresh tissue, dissect the brain region of interest on an ice-cold surface.
   b. If using frozen tissue, allow it to thaw on ice until just pliable.
   c. Weigh the tissue and record the weight.

   Note: If using cell cultures, check confluency before proceeding to homogenization.

2. Homogenization.
   a. Transfer the tissue to a pre-chilled Dounce homogenizer on ice.

      Note: other types of homogenizations can be used (e.g., bead beater).

   b. Add 10 volumes of ice-cold TBS per milligram of tissue (e.g., 1 mL TBS for 100 mg of tissue).

      Note: If using cell cultures, use 15 μL of TBS per 1 cm^2 of surface area of the plate (e.g., for a 12-well plate (3.5 cm^2 of area per well), use ~50 μL of TBS). Volume per well can be adjustable depending on the required protein concentration for a particular assay.

   c. Add protease/phosphatase inhibitor cocktail according to the manufacturer's instructions.
   d. Homogenize the tissue using 10–15 strokes with a loose-fitting pestle.
   e. For further disruption, sonicate the lysate on ice using a sonication bath for 60 s with 50% power, 5 s of pulses, and 3 s of resting periods to prevent overheating.

      Note: For cell cultures, set sonication bath with 30% power, 5 s of pulses, and 3 s of resting periods.

3. Transfer the homogenate to a centrifuge tube and centrifuge at 20,000 g for 15 min at 4°C.
4. Carefully collect the supernatant (TBS-soluble fraction) into a new tube. Keep the pellet if interested in further analysis.
5. Use a Bradford or bicinchoninic acid (BCA) assay to determine the protein concentration of the lysate.
6. Aliquot the lysate and store it at −80°C for long-term storage. Avoid repeated freeze-thaw cycles.

   Note: Maintain a cold environment throughout the procedure to minimize protein degradation.

   Note: Adjust the homogenization and centrifugation parameters depending on the tissue type and your specific needs.

Note: Optimize the sonication settings to achieve sufficient disruption without damaging the proteins.

Size exclusion chromatography (SEC) of brain lysates

Timing: 2–3 h

This section contains steps to separate brain lysate by protein size.

7. Sample preparation.

Note: This step can be performed in parallel with the next step, system preparation.

a. Decide the amount of total protein to separate by fractionation. Typical loading amounts of the Superose 6 Increase 10/300 GL column are.
   i. Mouse brain lysate: 5 mg total protein.
   ii. Human brain lysate: 1 mg total protein.

⚠ CRITICAL: It's often desirable to maximize the amount of purified material to increase the sensitivity of downstream analyses and the ease of performing them. However, loading too much protein into the column may cause issues for the separation, such as band-broadening, aggregation/precipitation, tailing, etc. It is therefore recommended to stay below ~5 mg loading for a 10 mm diameter column unless there is a specific problem to be addressed. If output material is insufficient at 5 mg loading, multiple separations of 5 mg can be performed then combined downstream, or use a larger diameter column. It is up to the experimenter to choose a good balance between sample availability, required output material, and other technical constraints, as the loading amount affects all subsequent analyses.

b. Centrifuge the brain lysate at 10,000 g for 10 min at 4°C. This removes large particles that can clog the column.
c. Calculate the volume of the sample to be loaded onto the column, base on protein concentration.

Note: If the volume is > 1000 μL, the sample *must* be concentrated before loading. If the volume is > 500 μL, it is *recommended* to concentrate the sample before loading. If necessary, concentrate the required volume of lysate to 250–500 μL using an ultrafiltration device with molecular weight cut off of 3 kDa (Amicon Ultra, Sigma UFC200324). The precise final volume is not important as the whole volume will be injected, but overconcentration may induce precipitation/aggregation; so try to keep the final concentration below ~10 mg/mL.

8. System preparation.
   a. This method consumes ~72 mL of SEC buffer. Check there is a sufficient volume of buffers in the bottle. Freshly prepared SEC buffer is preferred.
   b. Install a 2 mL sample loop on the chromatography system. Loop volume must be at least 2x the injected sample volume.
   c. Equilibrate the column (GE Superose 6 Increase 10/300 GL, GE/Cytiva 29091596) with 1.5–2 column volumes (36–48 mL) of SEC buffer or more until a stable baseline is achieved.
   d. Prepare the fraction collection plate.
      i. Arrange 1 mL 8-tube strips in a 96-well rack.
      ii. Label each individual collection tube in case they get separated.

iii. Prepare 50X protease inhibitor by dissolving two tablets of protease inhibitor (cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail, Sigma 11836170001) in 400 μL SEC buffer. The solution may require briefly warming to 37°C and/or vortexing for complete dissolution.
iv. Add 20 μL 50X protease inhibitor to each tube, such that when 1 mL fractions are collected, the final concentration will be 1X.

e. On the chromatography system, create a method that elutes the injected sample with 1.5 column volumes (CV) (36 mL) of SEC buffer at 0.3 mL/min flow rate, collecting 1 mL fractions. Nothing should elute in the first 6 fractions (0–6 mL), which can be omitted.

9. Inject the prepared sample and collect fractions.

⚠ CRITICAL: Be careful not to inject ANY air into the system. It may be easier to prepare an excess sample for injection and leave the remainder in the syringe after injection. If there is a small amount of air in the syringe, tilt the syringe slightly upwards during injection so bubbles rise to the plunger, away from the needle.

Note: HMW tau-seed is expected to elute in the void volume of the column. The void volume of Superose 6 Increase 10/300 GL is ~8 mL. Void volume of the column can be determined via injection of blue dextran.

10. Aliquot 200 μL of each fraction into 8-strip PCR tubes, if desired. Store fractions at −80°C.
11. Wash the column by injecting 2 mL of 1% SDS, then elute with water or SEC buffer.

FRET-based assay to measure tau aggregation in biosensor cells

Timing: 5–6 days

This section contains the steps to analyze the tau seeding activity of SEC fractions of brain lysates. This identifies the fractions containing tau-seed from which we will immunoprecipitate tau interactors. This protocol can accommodate any other seed-competent starting material such as cell lysates, mouse[4] and human brain tissue homogenates,[5] etc.

Culturing of biosensor cells

Timing: 1–2 days

Growing biosensor cells required for testing material for the tau-seeding activity.

12. Remove the cells from liquid nitrogen and thaw the cell stock in a 37°C-heat bath.
13. Pipette the contents into a conical tube with 10 mL warm DMEM.

Note: Always warm the DMEM at 37°C before adding to cells.

14. Centrifuge at 500 g for 5 min and aspirate the supernatant using a glass pipette.
15. Resuspend the cell pellet in 10 mL DMEM and transfer it to the 100 mm cell culture plate using a pipette controller.
16. Incubate at 37°C with 5% CO2.

Note: Passage cells every 2–3 days, to keep confluence at or below 70%–80%. Doubling time is ~24 h under these conditions. Use these steps to passage cells if required

17. Aspirate the media from the cell plate using a glass pipette.
18. Add 500 μL trypsin to the plate (drop-by-drop evenly around the plate).
19. Swirl the plate and tap gently to dislodge the cells from the plate.

20. Add about 2 mL of warm DMEM media to the trypsin in the plate to inactivate the trypsin.
21. Using a 1 mL pipette, dislodge all the cells and transfer them to a 50 mL conical tube tube.
22. Centrifuge at 500 g for 5 min and aspirate the supernatant using a glass pipette.
23. Resuspend the cell pellet in 20 mL DMEM and divide it into two to add 10 mL per 100 mm cell culture plate using a pipette controller.

Day 1: Cell plating

Timing: 30 min

Plate biosensor cells into a 96-well plate to perform the seeding assay.

24. Aspirate media from the 100 mm dish and wash cells with PBS.
25. Trypsinize using ~1 mL of trypsin-EDTA 0.05% (Gibco 25300-054) and incubate for 1–2 min.
26. Quench trypsin with ~2 mL cell culture media.
27. Transfer the cells to a conical tube and centrifuge at 500 g for 5 min.
28. Aspirate the media keeping the cell pellet, and resuspend cells in a 1–2 mL cell culture medium. Break up cell clumps.
29. Mix 10 μL of cell suspension with 10 μL Trypan Blue and determine the cell concentration using a cell counter.
30. Dilute cell suspension to a concentration of 269,000 cells/mL (35,000 cells per 130 μL) in culture media.
31. Transfer 130 μL cell suspension to each well in the 96-well cell culture plate (Thermo 167425).
32. Incubate at 37°C with 5% CO2 for 1 day.

Note: The seeding quantification is sensitive to cell density. To minimize inter-assay variation, ensure an accurate cell count by averaging multiple measurements and resuspending cells frequently while dispensing into wells.

Day 2: Transfection

Timing: 1 h

Transfect tau-seeds into biosensor cells to induce aggregation.

Note: Consider using at least two technical replicates due to assay variability. Prepare a small excess of volume at each step to accommodate pipetting.

33. Determine the number of samples and replicates, and add the following controls.
    a. Biosensor cells only (no treatment).
    b. Mock transfection (add transfection reagent without sample).
    c. Assay negative control (e.g., WT or KO Tau samples).
    d. Assay positive control (e.g., 9–11-month-old PS19 brain lysate).
34. For each well, prepare 10 μL of transfection reagent by diluting 1.25 μL of Lipofectamine 2000 in 8.75 μL Opti-MEM and incubate 5 min at 25°C.
35. Add 10 μL of transfection reagent to 10 μL of each fraction and incubate for 20 min at 25°C.
36. Add 20 μL transfection complex to each well containing plated cells.
37. Incubate for 2 days at 37°C with 5% CO2.

Day 4: Harvest cells

Timing: 1 h

Collect and prepare cells for analysis by flow cytometry

38. Prepare compensation control cells.
    a. Trypsinize and collect cells from HEK 293T, Tau RD P301S CFP, Tau RD P301S YFP, and Tau RD P301S FRET Biosensor cell lines.
    b. Pellet with a centrifuge at 500 g and resuspend in flow cytometry buffer.
39. Harvest transfected biosensor cells.
    a. Gently aspirate media (150 µL) without detaching cells using a multichannel pipette.
    c. Add 50 µL trypsin-EDTA 0.05% and incubate for 5 min at 25°C.
    d. Quench trypsin with 100 µL culture media.
    e. Transfer cells to a 96-well V-bottom plate (Greiner Bio-One 651101) using a multichannel pipette.

    Note: If some cells are still attached to the plate, rapidly eject media onto the surface with the multichannel to detach them. Try to collect most of the cells.

    Pause point: If cells cannot be analyzed on the same day, they should be fixed with 100 µL 2% PFA for 10 min at this stage. Cells can then be stored for at least 24 h. Fixed compensation cells can be stored for at least 1 month.

    f. Pellet the cells by centrifugation at 500 g for 5 min.
    g. Discard the supernatant and add 25 µL flow cytometry buffer. The cell pellet does not need to be resuspended; the pellet will be resuspended by BD HTS before injection.
40. Add compensation control cells harvested in step 38 to an empty well in the plate, so that they can be run together with the samples.
41. Store cells covered at 4°C in the dark until ready for run.

Flow cytometry

Timing: 1 h per 96 wells + 30 min first time setup

Analyze biosensor cells using flow cytometry.

Note: Use a flow cytometer with the following filter lines or similar.

Flow cytometer channels

Channel name	Excitation	Mirror	Filter	Measured signal
FITC	488	505 LP	530/30 BP	YFP
BV421	405		450/50 BP	CFP
BV510	405	505 LP	525/50 BP	FRET

Note: Refer to manufacturer instructions for details of instrument operation, startup, shut-down, and cleaning.

Note: First-time setup: Settings adjustment is only required for the first experiment; subsequent experiments can reuse the voltage and acquisition settings from a template. Compensation should still be done for every batch.

Note: Refer to the analysis step for gating details.

42. Adjust channel voltages (see Figure 1).

a. Collect ~10^6 Tau RD P301S FRET Biosensor cells into ~1 mL flow cytometry buffer and run using a tube sampler.
b. Adjust FSC and SSC voltages such that the cell population is visible on the FSC vs. SSC plot. If it's difficult to determine the correct population, compare with flow cytometry buffer without cells as a control, or gate from the fluorescent population in BV421 or FITC to identify the FSC/SSC population.

Optional: Set the FSC threshold to exclude small debris.

c. Create a gate for Cells, then Single Cells (See data analysis step for details of gating strategy).
d. While monitoring the population of Single Cells, adjust FITC and BV421 voltage as high as possible without saturating any events in each channel (<0.01% of the population), then decrease voltage slightly.

⚠ CRITICAL: Always keep the PMT voltage and output signal within the linear range of each fluorescence channel (FITC, BV421, BV510)

e. Use a positive control sample to adjust BV510 voltage.
f. While monitoring the population of Single Cells, adjust BV510 voltage as high as possible without saturating any events in the channel (<0.01% of the population), then decrease voltage slightly.

Note: If BV510 of the FRET-positive population is saturated during acquisition, decrease the voltage for subsequent experiments.

43. Switch instrument to HTS sampler.
44. Set HTS acquisition settings.
    a. Create Cells and Single Cells gates (refer to analysis step for gating details).

Optional: Create a FRET gate to monitor results in real-time.

b. Add sample wells and plates to acquisition settings.
c. Set the experiment to collect 10,000 events in the Single Cells gate.

Note: Increasing the number of events may help if seeding activity is low.

d. Adjust loader settings.

Loader Settings

Parameter	Value
Throughput Mode	High
Sample Flow Rate	1.0 µL/s
Sample Volume	10 µL
Mixing Volume	15 µL
Mixing Speed	150 µL/s
Number of Mixes	5 (maximum)
Wash Volume	800 µL (maximum)
Enable BLR	disable

⚠ CRITICAL: In the High Throughput mode of the BD HTS, care must be taken to not introduce bubbles or clumps into the sampler due to the low volumes involved. Improper loading can cause bubbles, failure to disperse the pellet, and/or unstable

flow, leading to loss of usable data. It is recommended to test the loader settings on a few spare wells to ensure proper operation before running the whole plate. If issues arise, adjust the Mixing Volume, Mixing Speed, and/or Number of Mixes and test on spare wells.

Note: We rely on the mixing function of the BD HTS to disperse the cell pellet before injection. If pellets are not sufficiently dispersed by the HTS, increase Mixing Volume and/or Mixing Speed. Alternatively, use a multichannel pipette to manually disperse the pellet.

Note: Note that in High Throughput mode, the BD HTS always removes 20 µL from the well, regardless of what Sample Volume is set to. Sample Volume determines how much of the removed 20 µL is collected as data, the remainder is discarded. Since 10 µL is the maximum Sample Volume in High Throughput mode, only 50% of the cells in a well can be analyzed. To maximize the number of analyzed cells while preventing bubbles during mixing and injection, ensure each well contains 25 µL of fluid. For very low seeding activity, do not use high throughput mode, since it will be more difficult to quantify rare events.

45. Run the plate(s).
46. Export data into .fcs files.

Data analysis

Timing: 1 h

Quantify the seeding activity measured by flow cytometry using FlowJo.

47. Import the .fcs files into FlowJo and optionally label each with the corresponding Sample ID.
48. Create a gating tree (see Figure 2).
    a. Gate the cell population using SSC-A vs. FSC-A and label it as 'Cells'.
    b. From the 'Cells' population, use FSC-H vs. FSC-W to exclude doublets (events with high width) and label them as 'Single Cells FSC'.
    c. From the 'Cells' population, use SSC-H vs. SSC-W to exclude doublets (events with high width) and label them as 'Single Cells SSC'.
    d. Make a boolean "AND" gate of "Single Cells FSC" and "Single Cells SSC" and label it as "Single Cells."

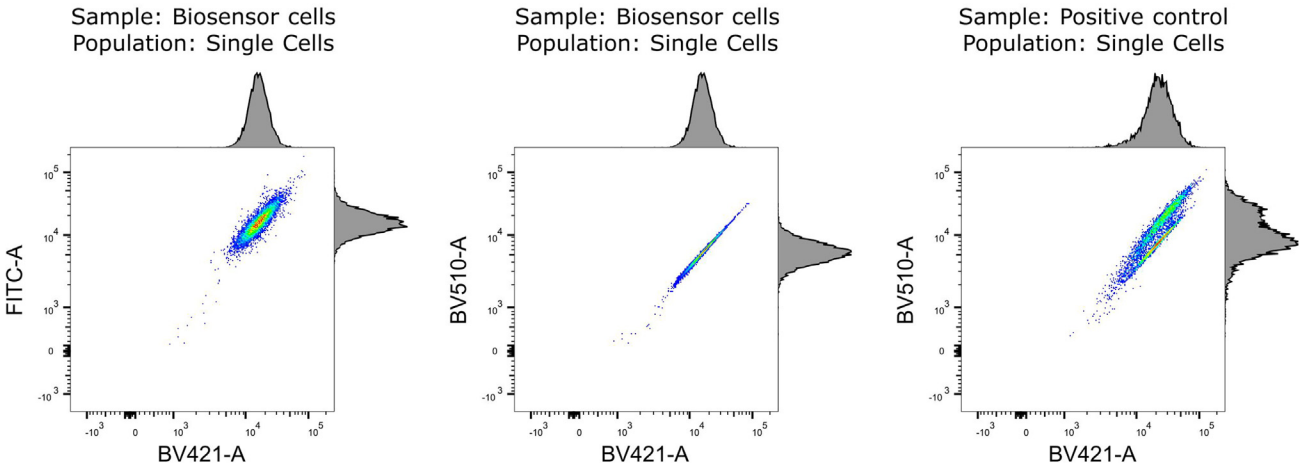

Figure 1. Voltage adjustment

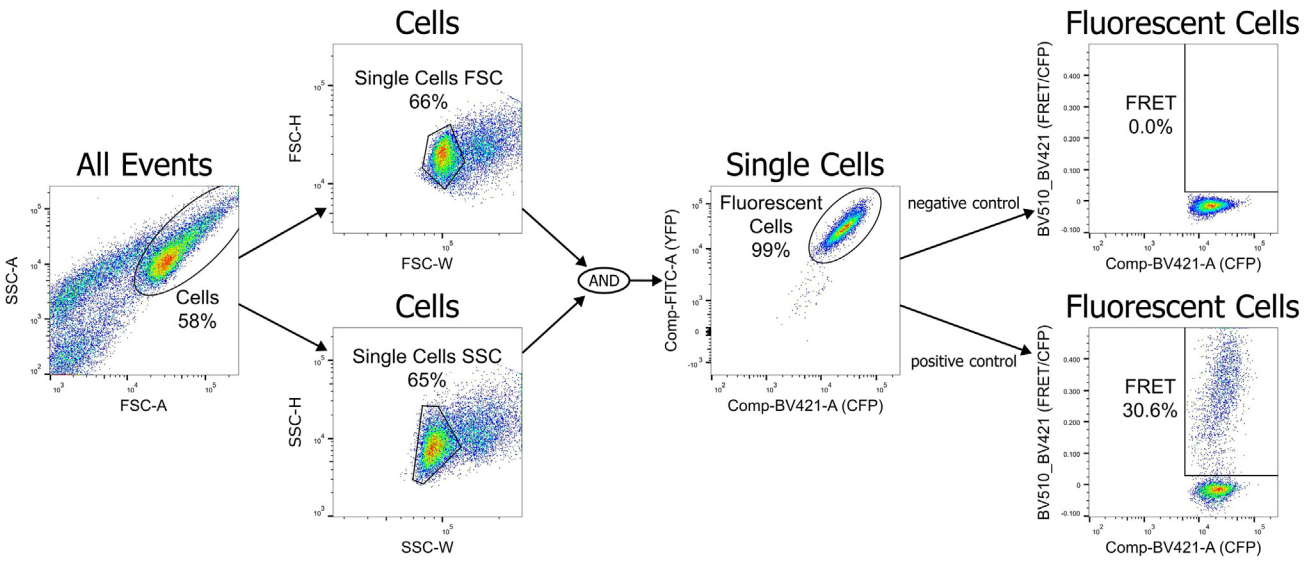

Figure 2. Gating strategy for quantification of seeding activity

9 months-old PS19 full lysate was transfected into the tau biosensor cells. After two days cells were collected for flow cytometry analysis.

49. Spillover Compensation (see Figure 3).

Note: Use biexponential scaling for each parameter to visualize the distributions properly.

a. Use YFP cells to set compensation values for "% FITC in BV421" and "% FITC in BV510"

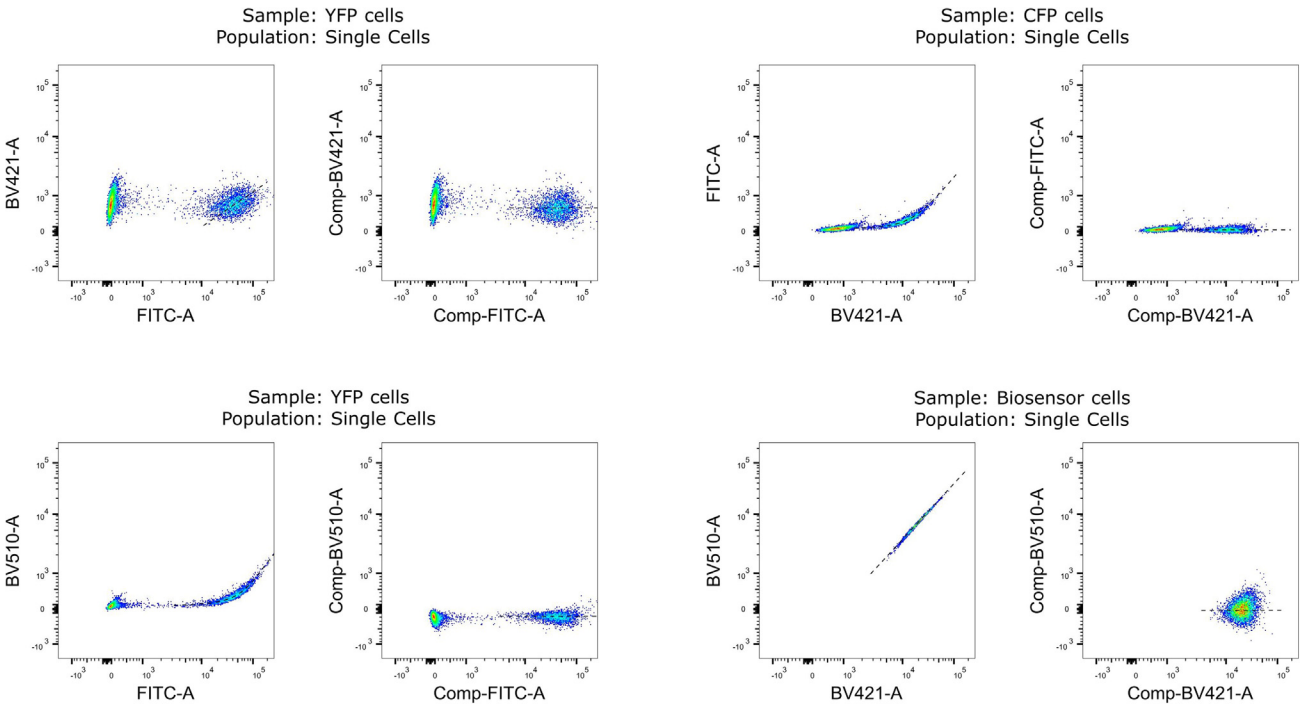

Figure 3. Spillover compensation

Example of event distribution before and after compensation. CFP, YFP, and biosensor FRET cell lines were used for spillover compensation. Parameters without a "Comp" prefix represent uncompensated parameters.

i. Observing the Single Cells population on a plot of Comp-BV421-A vs. Comp-FITC-A, adjust "% FITC in BV421" until the FITC+ population does not correlate (no co-variance between Comp-BV421-A and Comp-FITC-A), i.e., the population should appear as a horizontal ellipse.
ii. Observing the Single Cells population on a plot of Comp-BV510-A vs. Comp-FITC-A, adjust "% FITC in BV510" until the FITC+ population has no correlation (no co-variance between Comp-BV510-A and Comp-FITC-A).

b. Use CFP cells to set compensation value for "% BV421 in FITC."
i. Observing the Single Cells population on a plot of Comp-FITC-A vs. Comp-BV421-A, adjust "% BV421 in FITC" until the BV421+ population has no correlation (no co-variance between Comp-FITC-A and Comp-BV421-A).

c. Use biosensor cells treated with mock transfection to set compensation value for "% BV421 in BV510"
i. Observing the Single Cells population on a plot of Comp-BV510-A vs. Comp-BV421-A, adjust "% BV421 in BV510" until the BV421+ population has no correlation (no co-variance between Comp-BV510-A and Comp-BV421-A).

Note: Depending on the voltage settings, the required value for % BV421 in BV510 may seem very high. This is correct and not an error. This is due to the high spectral overlap between CFP and YFP emissions in the BV510 channel. For correct MFI computation, the FRET-negative population must be close to 0 on Comp-BV510-A, even if the value for "% BV421 in BV510" seems excessively high. If MFI is not required, then this compensation parameter is optional.

d. Check compensation settings again, since later settings may slightly affect previous compensation settings. Adjust as necessary until all criteria are simultaneously satisfied.
e. Do not attempt to set compensation values for "% BV510 in FITC" or "% BV510 in BV421."

50. Quantification (see Figure 2).
a. From the Single Cells population on a plot of Comp-FITC-A vs. Comp-BV421-A, create a gate around the main population, excluding low fluorescence debris, and label it as "Fluorescent Cells".
b. Create a derived parameter with the formula Comp-BV510-A/Comp-BV421-A and name it "BV510_BV421".
c. From the "Fluorescent Cells" population, plot BV510_BV421 vs. Comp-BV421-A and draw a rectangular gate around the FRET-positive population, and label it as "FRET". Use the positive control to locate the population and the negative control to exclude the negative population from bleeding into the gate.
51. Export a data table with the following columns.
a. Keyword: PLATE NAME.
b. Keyword: WELL ID.
c. Keyword: Sample ID.
d. Statistic: Frequency of Parent; Population: FRET, labeled as "% FRET".
e. Statistic: Median; Population: FRET; Parameter: Comp-BV510-A, labeled as "MFI".
f. Formula: % FRET * MFI, labeled as "Integrated FRET".
52. Use "Integrated FRET" as the measure of seeding activity. Select the fraction(s) with the highest seeding activity for immunoprecipitation.

Enrichment of tau-seeds by immunoprecipitation

Timing: 2 days

This section covers the steps to enrich biochemically active tau-seed and its interactors by immunoprecipitating human tau from SEC fractions with seeding activity.

MS/MS data will be used quantitatively. Perform subsequent sample preparation steps with accuracy and consistency in mind.

53. Sample Preparation.
    a. Thaw SEC fractions samples on ice. Aliquot a small portion of each sample as "IP input" for further analysis.
    b. Divide each sample into two 1.5 mL microcentrifuge tubes labeled "Isotype control" and "IP".
54. Antibody incubation.
    a. Add 2 μg of either biotinylated HT7 or mIgG1 antibody for every 100 ng of total Tau protein (measured by hTau ELISA) to each sample tube.
    b. Adjust the final volume to a minimum of 350 μL with TBS buffer.
    c. Wrap the cap with Parafilm and incubate on a rotator at 4°C end-to-end for 18 h.
55. Bead preparation.
    a. Vortex the Streptavidin magnetic beads bottle vigorously for 1 min.
    b. Transfer the required amount of bead suspension (20 μL, corresponding to 200 μg) to a separate 1.5 mL tube for each sample.
    c. Add TBST buffer to reach a final volume of 1 mL. Vortex briefly and spin down in a mini centrifuge.
    d. Place the tube on a magnetic rack for 1 min and discard the supernatant without disturbing the beads.
    e. Repeat this washing step twice with 1 mL TBST each time.
    f. After the final washing step, resuspend the beads in TBST to the original volume used in step 54b.
56. Immunoprecipitation.
    a. Vortex the beads, and add 20 μL of the bead suspension every 2 μg of HT7 antibody to each sample tube from step 54 (containing the overnight incubated fractions and antibodies).
    b. Incubate on a rotator at 4°C for 4 h.
57. Washing.
    a. Briefly spin down the tubes to collect the solution at the bottom.
    b. Place the tubes on the magnetic rack for 1 min and carefully take the supernatant ("IP flow-through").
    c. Add 1 mL of TBST, gently mix the beads by pipetting, and spin down briefly.
    d. Repeat steps b and c two more times with TBST.
    e. Perform one additional wash with TBS (without Tween-20).
58. Downstream analysis.
    a. Resuspend beads in TBS and transfer them to new 1.5 mL tubes. Finish the washing step. This step helps avoid protein contamination from the plastic surface.
    b. Once finished the last wash with TBS, leave beads in the tubes (beads only, there's no buffer in the tube), and wrap the cap with parafilm, beads can be stored at −80°C and submitted for MS. Or beads can be resuspended in 1x Western Blot sample loading buffer, then heated at 95°C for 5 min for follow up analysis.
59. Optional: Elution of tau-seeds.
    a. Resuspend the beads in 50 μL of 0.1 M glycine (pH 2.8) and incubate at 25°C for 10 min. Flick the tubes every 1–2 min to keep the beads resuspended.
    b. Spin down the tubes briefly and place them on a magnetic rack for 1 min.
    c. Transfer the supernatant (containing the eluted tau-seeds) to a new tube.
    d. Neutralize the eluate by adding 5 μL of 1 M Tris-HCl (pH 8.0) to adjust the pH to 7.0–7.5.
    e. The eluate and beads can be used for further assays like AFM.

Note: Be aware of not letting the beads dry throughout the IP process.

Note: The "IP input," "flow through," "eluted sample," and beads can be used for various downstream assays like Western blot.

Note: Samples can be split at this point if analyses other than mass spectrometry are performed.

Liquid chromatography with tandem mass spectrometry (LC-MS/MS)

This protocol uncovers tau interactors by identifying proteins enriched by immunoprecipitation using LC-MS/MS.

Note: MANY different LC-MS/MS setups will work for this experiment. The key is to have appropriate mass spectrometry resolution for the isobaric channels. A time of flight or lower-resolution instrument may allow for up to 9 different isobaric tags (i.e. up to 9 samples multiplexed), while a high-resolution instrument allows for the full set of 18 labels.

Protein digestion, peptide cleanup, and labeling with tandem mass tag

Timing: 2–3 days, stopping points noted throughout

The following sections describe typical digestion, peptide cleanup, and labeling with isobaric tags for mass spectrometry multiplexing of samples.[6] The current limitations of the multiplexing strategy are that only up to 18 samples can be combined at a time. Immunoprecipitated proteins are digested into peptides which can be multiplexed (bar-coded) and analyzed via liquid chromatography-mass spectrometry.

60. Denature proteins and reduce and alkylate cysteines.
    a. Cover the beads with 30 μL of 8 M Urea, 100 mM Tris pH 8.5.

⚠ CRITICAL: Do not overheat (>35°C) proteins and beads in the presence of urea as this can carbamylate amino acid side chains and peptide N-termini.

    b. Add 1.5 μL of 100 mM TCEP solution (5 mM final concentration) and incubate with shaking at 32°C for 15 min.
    c. Add 0.6 μL of 500 mM CAA solution (10 mM final concentration), vortex briefly, and spin down briefly. Incubate at 25°C, protected from light for 30 min.

Note: Alternative reduction and alkylation reagents (such as DTT or iodoacetamide) can be utilized, but the fixed modifications used for data analysis should be consistent with this step.

61. Perform 2-step LysC-trypsin digest.
    a. Add 28 μL 50 mM Tris, pH 8.5 to dilute samples to 4 M Urea and add 4 μL of 0.1 μg/ μL LysC/trypsin stock solution.
    b. Incubate with 600 RPM shaking at 35°C for 2 h.
    c. Add 60 μL 50 mM Tris, pH 8.5 to dilute samples to 2 M Urea.
    d. Incubate 600 RPM at 35°C for 12–16 h.
    e. Add 6 μL of 10% trifluoroacetic acid to acidify and quench digestion, vortex, and spin briefly.

Note: This 2-step digestion protocol is used with the Promega mass spectrometry grade LysC-Trypsin (V5072). Other sources of LysC and trypsin can also be used, but proteases should be consistent within and across experiments as variation in digestion will create variation in the peptides identified. If immunoprecipitation is being done with <1 mg of starting protein, less protease can be used (0.1–0.2 μg).

62. Cleanup peptides using reverse phase spin columns.
    a. Place the spin column in a 2 mL tube. Prepare spin columns by adding 200 μL of acetonitrile, 0.1% FA, and spinning at 1500 g for 1 min. Repeat. Discard flowthrough.

b. Equilibrate spin columns by adding 200 μL of 0.5% TFA in water, spinning at 1500 g for 1 min. Repeat. Discard flowthrough.
c. Load the acidified digest to the top of the resin. Centrifuge at 1500 g for 1 min.
d. Re-apply the flowthrough to the top of the resin. Centrifuge at 1500 g for 1 min. Save this as a 'flowthrough' to troubleshoot any issues with the protocol.
e. Move the spin column to a fresh 2 mL tube and apply 200 μL of 0.5% TFA in water. Centrifuge at 1500 g for 1 min. Repeat. Save this as 'wash.'
f. Move the spin column to a Lo bind Eppendorf tube. Apply 20 μL of 70% acetonitrile, 0.1% FA. Centrifuge at 1500 g for 1 min. Repeat in the same tube.
g. Vacuum centrifuge to dry cleaned-up peptides. These can be stored at −80°C for up to 2 years.

63. Label with Isobaric tags.
    a. Resuspend peptides in 25 μL of 100 mM TEAB by vortexing at 25°C for 5 min. Briefly centrifuge to get liquid to the bottom of the tube.
    b. Allow TMT reagents to come to 25°C.
    c. Resuspend TMT aliquots (0.2–0.5 mg) in 25 μL acetonitrile by pipetting up and down and immediately add each tag to the relevant sample. Mix peptide and TMT label and allow to react at 25°C for 1 h.

⚠ CRITICAL: TMT will hydrolyze in the presence of water and so should be used immediately after resuspension. Larger quantities of TMT labels can be purchased, resuspended in acetonitrile, aliquoted into 0.2 or 0.5 mg aliquots, and immediately dried. These can be stored at −80°C for up to one year in our hands. Older reagents may be partially hydrolyzed and result in less efficient labeling of peptides.

Optional: Check TMT labeling efficiency by adding 0.5 μL–15 μL 0.1% FA and injecting on trap column Ultimate 3000/QE Plus.

Note: Any instrument setup can be used for this. TMT or TMTpro should be searched for as a variable modification on lysines and peptide N-termini to confirm > 90% of peptides are labeled.

64. Quench TMT labeling by the addition of 3 μL of 5% hydroxylamine (final hydroxylamine concentration of 0.3%) and incubate at 25°C for 15 min.

Carefully consider labeling and mixing strategies. TMTpro reagents allow for multiplexing up to 18 samples at a time. In the case of immunoprecipitation, it may be useful to mix in multiple ways to compare across different genotypes or against different controls. If possible, run the corresponding IgG and HT7 samples in the same injection for optimal identification and quantification, for downstream analyses.

Nano-liquid chromatography-mass spectrometry

Timing: 3–24 h

This section describes the use of an orbitrap tribrid instrument for MS2-based quantification of the TMTpro-labeled immunoprecipitated proteins.

A portion of the mixed and cleaned-up TMT-labeled peptides are separated by reversed-phase nano chromatography and then analyzed by high-resolution mass spectrometry. This allows for the identification of peptides as well as the quantification of those peptides from each sample.

65. Resuspend mixed dried peptides in Buffer A (25–100 μL) depending on LC-MS being used.

66. LC-MS/MS methods. LC Buffer A = 0.1% FA in water; Buffer B = 0.1% FA, 80% Acetonitrile. Sample Loading will be 2n+2 μL where n = the volume being injected.

Time	Flow (nL/min)	%B
0	400	8
160	400	35
170	400	95
172	400	95
180	400	5

Mass spectrometer Global settings: LC, expected peak width 30 s, advanced peak determination on, default charge state 2, Easy-IC on, static spray voltage, 1800 V, ion transfer tube 350°C, FAIMS mode standard resolution, static.

FAIMSpro settings: 3 × 1.5 s cycles of −45 CV, −55 CV, −65 CV. Each LC-MS experiment with identical MS and MS2 settings.

Note: if not using FAIMSpro then use a 4 sec cycle time with the MS/MS parameters below.

MS1 settings	
Orbitrap resolution	120000
Scan Range (m/z)	400–1600
RF lens (%)	30
AGC target	standard
Maximum inject time	50 ms
Data type	Profile
Polarity	positive

Filters of Monoisotopic peak determination: peptide, intensity threshold of 2.5e4, charge states 2–6, dynamic exclusion: use common settings, exclude after 1 time for 60 s with mass tolerance of 5 ppm low and high, exclude isotopes.

MS2 settings	
Isolation mode	Quadrupole
Isolation width (m/z)	0.7
Activation type (fixed)	HCD
HCD collision energy	34
Orbitrap resolution	50000
AGC target	Custom, 200%
Maximum inject time	dynamic
Scan range	Define first mass
First mass (m/z)	100

TMT will utilize slightly different settings than TMTpro. Double-check which reagents you are using and modify your MS/MS method accordingly.

Data analysis

Timing: 1–5 days

This describes the use of Proteome Discoverer 2.5 to analyze TMT-labeled immunoprecipitation samples. Some alternative software exists including freely available MSFragpipe and MaxQuant. Key settings for identification and quantification are highlighted below.

67. Transfer .raw files from mass spectrometer computer to analysis computer with Proteome Discoverer 2.5 (or 2.4 and above).[7]
68. Download an updated protein database from UniProt.org[8] (NCBI can also be considered). Search taxonomy for "*Mus musculus*" (or whichever organism is being studied), select "proteomes" > "reference proteome" and download a noncompressed .fasta file. A database containing common laboratory contaminants (including human keratin and trypsin) should also be downloaded.[9] Include only reviewed sequences. Don't include isoforms, use only canonical sequences. Tau isoforms may be included.
69. In Proteome Discoverer, load the protein database by selecting> administration>Maintain fasta files> Add and select fasta file for the project.
70. Create a quantification method based on the total number of samples utilized in the experiment. Go to 'administration' and 'maintain quantification files. Click 'add' and Select TMTPro 18plex.
71. Download TMTPro lot-specific isotopic impurities from Thermo Fisher Scientific website (or from the original packaging of the reagent).
72. Add isotopic impurity information and check 'active' for any TMT label utilized in the experiment.
73. Click ok, name, and save the quantification method.
74. Go to File, create a new project, and name your proteome discoverer project.
75. In the study definition window, add the quantification method just created.
76. In the study definition window, add categorical study factors. Factor name genotype and factors called X, Y, Z. If you are comparing samples IP-ed from the same animal or case, you can add 'biological replicate' identifiers with the case or individual ID. This will calculate nested abundance ratios (see the Proteome Discoverer User Manual for a full explanation of categorical vs. biological replicates in this analysis).
77. In the input files window, 'add files' and select your .raw file, and select the quantification method in the pull-down menu.
78. In the samples window, add the categorical factors (and/or biological if comparing samples from the same individual) to describe each TMT label. Note that for biological replicates to be used in Proteome Discoverer, each categorical factor must have matching biological identifiers (i.e., case 1 (biological replicate) fraction 7 (categorical factor) vs. case 1 (biological replicate) fraction 14 (matching categorical factor will calculate the abundance ratio of any protein in Case 1 F7/Case 1 F14 in a nested fashion prior to calculating p-value). See Proteome Discoverer User Manual.
79. Select a new analysis. In the consensus window load the common template > consensusWF>CWF_Comprehnsive_EnhancedAnnotation_Rproter_Quan. In the processing step window load the common template and use the base consensus workflow of ProcessingWF_Tribrid>PWF_Reproter_Based_Quant_SequestHT_Percolator.
80. Modify the Processing workflow as follows:
    a. In spectrum files RC node, select the protein database fasta that you imported, enzyme trypsin (full), static peptide N-terminus choose TMTpro(304.207 Da), and static modification of TMTpro on Lysine.
    b. Reporter Ions quantifier node: Peak integration 20 ppm, most confident centroid, Mass Analyzer: FTMS, MS Order MS2, Activation Type HCD.

    Note: If MS3-based TMTpro quantitation is being performed, select MS3, and use fragment mass tolerance of 0.6 Da in the Sequest HT node below.

    c. Sequest HT node: Protein Database: select organism fasta and the common contaminant database. Enzyme: Trypsin (full): Max missed cleavage sites: 3, Min Peptide Length: 6 AA, Max peptide length 144. Precursor Mass Tolerance 10 ppm, Fragment Mass tolerance 0.02 Da, Max modifications per peptide: 3, Dynamic modifications of Oxidation on methionine (+15.995) and plus phospho on serine, threonine, and tyrosine (+79.966). Dynamic protein modifications should include N-terminal acetylation, N-terminal Met-loss, and N-terminal Met-loss+Acetyl. Static modifications should be peptide N-terminus: TMTpro, carbamidomethyl on Cysteine, TMTpro on Lysine.

Note: Carbamidomethyl is the alkylation result of the use of chloroacetamide. If a different alkylation reagent is used in protein preparation that should be selected as the modification on cysteine.

d. Percolator FDR node should be utilized for FDR cutoffs.
e. If phosphorylation or other PTMs are of interest, drag and select the IMP-ptmRS node should be added following percolator.

81. Modify the consensus workflow as follows:
    a. In the Reporter Ions Quantifier Node: Reporter Ion abundance base on automatic; Apply Quant Value Corrections: True; Co-isolation Threshold: 30; Average Reporter S/N Threshold: 6. Normalization Mode set to none, Scaling Mode set to none.

Note: In some IP-MS experiments, it may be helpful to normalize the bait protein. In this case, create a fasta file with just the bait protein sequence, add as described above, and select Normalization Mode: Specific Protein. Then load that protein fasta file in the proteins for normalization window.

⚠ CRITICAL: Do not use sample protein abundances to normalize between different sample groups (HT7 and IgG); in a correctly performed IP experiment, HT7 samples should have more total protein and a different composition than IgG. This difference is critical for the identification of non-specific binding and must be preserved. Normalize using other methods such as an external spike-in, or don't normalize between groups at all. Normalization within each sample group may still make sense depending on the situation.

Note: If PTMs are being investigated, add the "Modification Sites" and "Peptide Isoform Grouper" nodes to the consensus workflow.

EXPECTED OUTCOMES

The typical protein concentration of mouse brain lysate is 3–10 mg/mL. The typical protein concentration of human brain lysate is 0.8–5 mg/mL.

The performance of this protocol will allow to isolation of HMW tau-seeds from different biological samples from tauopathy models. For SEC, it is expected to elute a fraction containing HMW-tau in the void volume peak ~8 mL. No protein should elute before ~7 mL if the column is performing properly. For the majority of tau models overexpressing human tau with pathogenic mutations, seeding activity is expected to be highest from F8 to F10 (Figure 4) and dramatically lower in monomeric tau fractions (~F17) (Figure 4).[1] However, any treatment, insult, genetic manipulation, etc. could trigger modifications in the formation of tau aggregates, leading to changes in the molecular weight. In this situation, the distribution of seeding activity can change through SEC fractions.

The next step consists of the isolation of tau material from SEC fractions (Figure 5). It is expected to observe a reduction in the seeding activity in the flow through after the isolation of tau-seed by immunoprecipitation in SEC fractions.

Analyzing protein interactors of tau is the last big step that allows us to determine proteins that interact directly and indirectly with different tau species. In this context, total protein abundance in the mass spec of HT7 purified immunoprecipitation is expected to be higher than IgG, since IgG is used as a control for nonspecific binding.

LIMITATIONS

Tau-seed is inherently prone to aggregation and a small amount of aggregation/precipitation may be unavoidable. Spin down precipitates before each analysis.

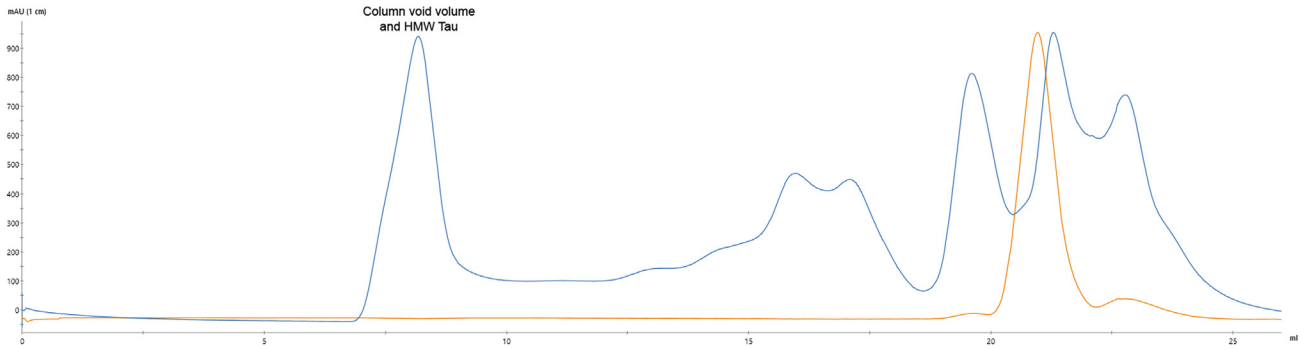

Figure 4. Chromatogram of SEC separation

TBS-soluble lysate from 9 months-old PS19 mouse was fractionated by SEC using the described methodology. The blue trace is the absorbance at 280 nm normalized to 1 cm path length. Void volume is the first peak ~8 mL, containing HMW tau. The pattern of other peaks may vary from sample to sample and lysate source. Orange is conductivity, showing the injected solvent peak around 21 mL.

The purification protocol enriches tau-seeds but cannot fully separate tau-seeds from tau that do not have seeding activity. Interpret the results of the mass spec appropriately, as not all proteins detected by mass spec are necessarily part of the seed. Comparison with controls and further validation are required to verify tau-seed interactors.

The biosensor seeding assay can have significant inter-assay variability. Seeding assays performed in different batches cannot be quantitatively compared.

Pilot IP-MS samples should be run to ensure that the antibody of choice is selective and binds well enough to the target protein to be used for pull-down. In many cases, even commercially available antibodies may lack specificity or selectivity and may not bind strongly enough to be used to immunoprecipitated proteins. In this case, alternatives such as CRISPR-tagged cell lines could be utilized.

Follow-up experiments should interrogate any identified binding partners or post-translational modifications.

TROUBLESHOOTING

Problem 1

Biosensor cells exhibit cytotoxicity following transfection with tau protein lysate (related to steps 33–41).

Potential solution

It is possible to observe cell toxicity after transfecting the biosensor. This can be due to high levels of tau-seed and also other lysate proteins. Some sources of toxicity include structural components of the tau model such as chitin from Drosophila exoskeleton, larvae constituents, etc. If this is the case, reducing the amount of lysate is recommended.

Problem 2

Low seeding activity is observed in the biosensor cell line following plating (related to steps 42–46).

Potential solution

Accurately determine cell confluency before seeding. Optimize the seeding density based on the growth characteristics of the biosensor cell line and the desired assay format. Consider using a cell counter to ensure accurate cell numbers. Alternatively, use larger wells (e.g., 48 well plate) in order to collect a higher number of cells for FRET analysis.

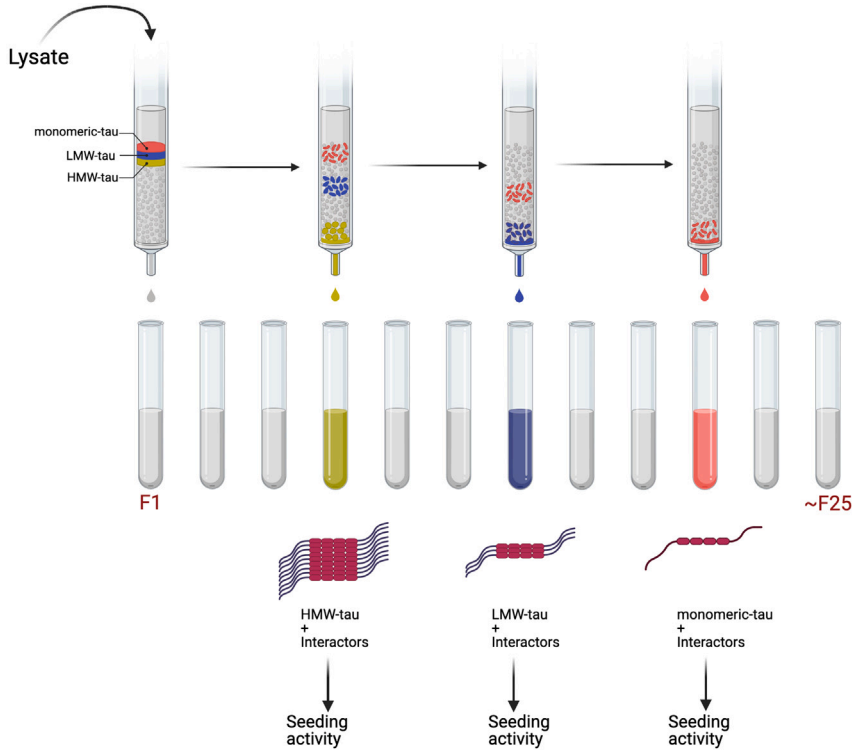

Figure 5. Schematic of size exclusion chromatography and tau species distribution

Soluble TBS-lysates are fractioned in the chromatography column. Lower fractions will contain HMW-tau complexes while higher fractions are enriched in LMW-tau protein complexes.

Problem 3

Biosensor responses are saturated, resulting in minimal or undetectable differences between samples. This can significantly limit the assay's ability to discriminate between different experimental conditions (related to steps 42–52).

Potential solution

The system may be saturated. Perform a titration experiment with varying amounts of tau lysate to establish a response curve. This will help identify the optimal transfection dose that balances sensitivity and signal saturation.

Problem 4

Low amount of immunoprecipitated tau (related to steps 53–58).

Potential solution

Check that the amount of HT7-biotin antibody is correct. Sometimes the amount of tau differs between models and human samples. Verify the amount of tau before performing IP through ELISA, for instance. Other validations include the verification of the amount of tau in the input and flow through after IP. In this case, run a western blot of both samples to check the decrease of tau levels before and after IP.

RESOURCE AVAILABILITY

Lead contact

Cristian A. Lasagna-Reeves, PhD. E-mail: clasagna@iu.edu.

Technical contact

Pablo Martinez, PhD. E-mail: martipab@iu.edu.

Materials availability

This protocol did not generate new or unique reagents.

Data and code availability

No new data or code was generated by this protocol.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.M. and C.A.L.-R.; methodology, P.M., H.P., Y.Y., and E.H.D.; investigation, P.M., H.P., Y.Y., and E.H.D.; writing – review and editing, P.M., H.P., Y.Y., E.H.D., A.L.M., and C.A.L.-R.; funding acquisition, E.H.D., A.L.M., P.M., and C.A.L.-R.; supervision P.M. and C.A.L.-R.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Martinez, P., Patel, H., You, Y., Jury, N., Perkins, A., Lee-Gosselin, A., Taylor, X., You, Y., Viana Di Prisco, G., Huang, X., et al. (2022). Bassoon contributes to tau-seed propagation and neurotoxicity. Nat. Neurosci. *25*, 1597–1607. https://doi.org/10.1038/s41593-022-01191-6.

2. Alonso, A.d.C., Li, B., Grundke-Iqbal, I., and Iqbal, K. (2008). Mechanism of tau-induced neurodegeneration in Alzheimer disease and related tauopathies. Curr. Alzheimer Res. *5*, 375–384. https://doi.org/10.2174/156720508785132307.

3. Lee, V.M., Goedert, M., and Trojanowski, J.Q. (2001). NEURODEGENERATIVE TAUOPATHIES. Neuroscience *24*, 1121–1159. https://doi.org/10.1146/annurev.neuro.24.1.1121.

4. Patel, H., Martinez, P., Perkins, A., Taylor, X., Jury, N., McKinzie, D., and Lasagna-Reeves, C.A. (2022). Pathological tau and reactive astrogliosis are associated with distinct functional deficits in a mouse model of tauopathy. Neurobiol. Aging *109*, 52–63. https://doi.org/10.1016/j.neurobiolaging.2021.09.006.

5. Jury-Garfe, N., You, Y., Martínez, P., Redding-Ochoa, J., Karahan, H., Johnson, T.S., Zhang, J., Kim, J., Troncoso, J.C., and Lasagna-Reeves, C.A. (2023). Enhanced microglial dynamics and paucity of tau seeding in the amyloid plaque microenvironment contributes to cognitive resilience in Alzheimer's disease. bioRxiv. https://doi.org/10.1101/2023.07.27.550884.

6. Li, J., Van Vranken, J.G., Pontano Vaites, L., Schweppe, D.K., Huttlin, E.L., Etienne, C., Nandhikonda, P., Viner, R., Robitaille, A.M., Thompson, A.H., et al. (2020). TMTpro reagents: a set of isobaric labeling mass tags enables simultaneous proteome-wide measurements across 16 samples. Nat. Methods *17*, 399–404. https://doi.org/10.1038/s41592-020-0781-4.

7. Orsburn, B.C. (2021). Proteome Discoverer—A Community Enhanced Data Processing Suite for Protein Informatics. Proteomes *9*, 15. https://doi.org/10.3390/proteomes9010015.

8. Bateman, A., Martin, M.J., Orchard, S., Magrane, M., Ahmad, S., Alpi, E., Bowler-Barnett, E.H., Britto, R., Bye-A-Jee, H., Cukura, A., et al. (2022). UniProt: the Universal Protein Knowledgebase in 2023. Nucleic Acids Res. *51*, D523–D531. https://doi.org/10.1093/nar/gkac1052.

9. Mellacheruvu, D., Wright, Z., Couzens, A.L., Lambert, J.-P., St-Denis, N.A., Li, T., Miteva, Y.V., Hauri, S., Sardiu, M.E., Low, T.Y., et al. (2013). The CRAPome: a contaminant repository for affinity purification–mass spectrometry data. Nat. Methods *10*, 730–736. https://doi.org/10.1038/nmeth.2557.