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Flow Cytometry Analysis and Quantitative Characterization of Tau in Synaptosomes from Alzheimer's Disease Brains

Karen Hoppens Gylys and **Tina Bilousova**

Abstract

Synaptosomes, resealed nerve terminals that form when tissue is homogenized in isotonic medium, are a model system that has been a key source of knowledge about neurotransmission. Synaptosomes contain mitochondria, cytoskeletal proteins, and release neurotransmitters; many have postsynaptic elements. Cryopreservation at the time of autopsy makes it possible to prepare synaptosomes from human samples. Flow cytometry is a powerful analytic technique that precisely measures fluorescence on a cell-by-cell basis, and also indicates particle size and complexity with a routine parameter that measures light scattering. We describe here a procedure for flow cytometry analysis of tau in synaptosomes, a procedure that enables (1) "purification" of synaptosomes from the P-2 fraction (crude synaptosomes) by gating on particle size, and (2) quantitative measure of tau immunofluorescence in individual terminals. Application of flow cytometry to study of synaptosomes has yielded important information, not possible with routine biochemistry, about synaptic pathology in Alzheimer's disease.

Keywords

Flow cytometry; HT7 antibody; Tau fragment; FACs; Cytoskeleton; Neurodegeneration

1 Introduction

The synapse is the functional unit of the brain, and synapse loss is thought to be an early correlate of cognitive deficits in Alzheimer's disease (AD) [1]. However, synaptic boutons are poorly visualized at the level of light microscopy, and immunodetection of proteins of interest by EM are primarily qualitative and face technical issues that include epitope masking and fixation artifacts. Therefore many researchers have used the biochemical synaptosome preparation to study synaptic proteins and function. Synaptosomes are resealed nerve terminals formed when fresh brain tissue is gently homogenized in isotonic sucrose, forming spherical particles that contain mitochondria and the organelles for neurotransmitter release (Fig. 1a). Synaptosomes are primarily presynaptic with some adherent postsynaptic structures, and the preparation has been used extensively in pharmacology since the 1960s to study in vitro receptor binding and neurotransmitter release [2]. Classical protocols for purification include Ficoll discontinuous gradient purification of the initial crude synaptosomal pellet, also called the P2. Even with additional density gradient purification, synaptosome preparations are impure and contain free vesicles, mitochondria, myelin, and

membrane fragments; quantified by flow cytometry, purified synaptosome fractions contain ~83 % synaptosomes labeled by a voltage-sensitive dye [3]. Gradient purification requires extra time and reduces viability, which has led to the use of the P2 fraction for study of functional processes not affected by the usual contaminants [2].

Flow cytometry, also referred to as fluorescence-activated cell sorting (FACS), is a widely used analytic instrument that provides rapid and simultaneous analysis of multiple characteristics of single cells, including cell size, complexity, and the fluorescence emitted by a wide range of dyes and fluorophore-conjugated antibodies. Flow cytometry offers the additional advantage of quantifying two parameters for fluorescence; both the positive fraction (% positive) and the brightness of fluorescence (relative fluorescence, RFU) are precisely measured for each particle. Several thousand cells/particles can be analyzed per second by suspension in a stream of sheath fluid that passes through multiple detectors [4]. Since synaptosomes are analogous to cells, flow cytometry analysis of synaptosomes represents a powerful approach to the study of synapses, and the earliest studies used Percoll gradient-purified synaptosomes [3, 5, 6]. However, reasoning that analysis of the P2 offered significant advantages in terms of faster preparation time and higher tissue yield, our lab developed procedures for flow cytometry analysis of P2 fraction beginning in 2000 [7]. Importantly, we have been able to demonstrate by a variety of methods that analysis of particles based on size and including only particles between 0.5 and 1.5 μm allows focus on a population of synaptosomes that is ~95 % pure [8–10], superior to the ~80 % purity obtained with gradient purification [3, 9].

A number of workers have studied synaptosomes prepared from human tissue obtained during surgery or at the time of autopsy [11–13], in which case it is cryopreserved in isotonic sucrose using protocols that have been previously optimized [14]. In some circumstances, including studies of tau release, we have expanded these cryopreservation strategies to include cryopreservation of P2 pellets after isolation. The structural and functional integrity of synaptosomes prepared from cryopreserved postmortem tissue has been repeatedly demonstrated by our group and more recently by others [15]; for example, 99 % of size gated synaptosomes label with Fluo-4NW, a calcium-sensitive dye that indicates intact membranes and esterase activity [16]. We have recently shown depolarization-dependent release of tau from human AD synaptosomes [17], and a number of other authors have also measured functional endpoints using cryopreserved postmortem human synaptosomes [12, 18, 19]. The combined techniques of flow cytometry and synaptosome preparation offer quantitative answers to questions it is not possible to ask with routine biochemistry or pathology techniques, and we report here a detailed protocol for flow cytometry analysis of immunolabeled synaptosomes from human AD samples.

2 Materials

2.1 Solutions for Brain Tissue Cryopreservation and Synaptosome-Enriched (P2) Fraction Preparation

Prepare all the solutions using deionized water.

For prolonged storage all water-based stock solutions need to be sterilized by filtration through 0.45 μm filters.

1. 0.2 M ethylene diamine tetraacetic acid (EDTA) stock solution: weigh 0.744 g of EDTA disodium salt (FW 372.2), add 8 ml of water, adjust the pH to 8.0 with constant stirring (*see* Note 1); when EDTA salt is completely dissolved adjust volume to 10 ml with water. Store the solution at room temperature (stable for more than 1 year).
2. 0.2 M ethylene glycol tetraacetic acid (EGTA) stock solution: weigh 0.761 g of EGTA (FW 380.4), add 8 ml of water, adjust the pH to 8.0 with constant stirring (*see* Note 1); when EGTA is completely dissolved adjust volume to 10 ml with water. Store the solution at room temperature (stable for more than 1 year).
3. 0.1 M phenylmethylsulfonyl fluoride (PMSF) stock solution: dissolve 0.174 g of PMSF (FW 174.2) in 10 ml of 100 % ethanol (protect from light). Divide into 200 μl aliquots and store at −20 °C (stable for more than 1 year).
4. 0.1 M sodium pyrophosphate decahydrate (NaPP) stock solution: weigh 4.46 g of $Na_4P_2O_7 \cdot 10H_2O$ (FW 400.1) and dissolve in 100 ml of water. Store at 4 °C (stable for 3–4 weeks).
5. 1 M sodium fluoride (NaF) stock solution: dissolve 0.42 g of NaF (FW 41.99) in 10 ml of water. Store at 4 °C (stable for more than 1 year).
6. 1.5 mM pepstatin A stock solution: dissolve 5 mg of pepstatin A (FW 685.9) in 5 ml of 100 % Methanol. Divide into 100 μl aliquots and store at −20 °C, avoid freeze–thaw cycles (stable for more than 1 year).
7. 20 mM leupeptin stock solution: dissolve 10 mg of leupeptin (FW 475.6) in 1 ml of water. Divide into 100 μl aliquots and store them at −20 °C, avoid freeze–thaw cycles (stable for more than 1 year).
8. 1.5 mM aprotinin stock solution: dissolve 10 mg of aprotinin in 1 ml of water. Divide into 100 μl aliquots and store them at −20 °C, avoid freeze–thaw cycles (stable for more than 1 year).
9. Trypsin inhibitor (from soybean): dissolve 100 mg of trypsin inhibitor in 10 ml of water. Divide into 200 μl aliquots and store them at −20 °C, avoid freeze–thaw cycles (stable for more than 1 year).
10. 1 M Tris–HCl, pH 8.0: dissolve 12.1 g of Tris base in 90 ml of water. Adjust pH to 8.0. Bring up the volume to 100 ml with water. Divide into 1 ml aliquots and store at −20 °C (stable for more than 1 year).
11. Cryopreservation buffer (0.32 M sucrose, 2 mM EDTA, 2 mM EGTA, 0.2 mM PMSF, 1 mM NaPP, 5 mM NaF, 10 mM Tris–HCl, pH 8.0): to make 100 ml of

[1]EDTA and EGTA compounds, especially in the free acid form, are almost insoluble in water. Place magnet stirring bar to the EDTA (EGTA) water mixture and place the beaker on the magnet stirrer hot plate. Stir constantly, carefully titrate with sodium hydroxide (10 N NaOH) and continuously measure pH. Stop when pH reaches 8.0 and continue stirring until EDTA/EGTA is fully dissolved in water.

cryopreservation buffer, weigh 11 g sucrose and dilute it in 90 ml of deionized water (*see* Note 2), add 100 µl of 0.2 M EDTA, 100 µl of 0.2 M EGTA, 200 µl of 100 mM PMSF, 1 ml of 100 mM NaPP, 500 µl of 1 M NaF, and 1 ml of 1 M Tris–HCl, pH 8.0 stock solution. Adjust volume to 100 ml with water; keep the solution on ice until use.

12. P2 buffer (0.32 M sucrose, 1.5 µM pepstatin A, 0.75 µM aprotinin, 8 µM leupeptin, 1 µM trypsin inhibitor, 2 mM EDTA, 2 mM EGTA, 0.2 mM PMSF, 1 mM NaPP, 5 mM NaF, 10 mM Tris–HCl, pH 8.0): to make 100 ml of P2 buffer, weigh 11 g sucrose and dilute it in 90 ml of deionized water, add protease and phosphatase inhibitors using stock solutions—100 µl of 1.5 mM pepstatin A, 50 µl of 1.5 mM aprotinin, 40 µl of 20 mM leupeptin, 200 µl of 0.5 mM trypsin inhibitor, 100 µl of 0.2 M EDTA, 100 µl of 0.2 M EGTA, 200 µl of 100 mM PMSF, 1 ml of 100 mM NaPP, 500 µl of 1 M NaF, and 1 ml of 1 M Tris–HCl, pH 8.0.

2.2 Solutions and Reagents for P2 Immunostaining and Flow Cytometry

1. Phosphate buffered saline (PBS): 0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride, pH 7.4 (*see* Note 3).
2. 4 % paraformaldehyde (PFA) stock solution: weigh 2 g of paraformaldehyde, move it into 50 ml centrifuge tube, add PBS to reach 50 ml total volume, close and seal the tube with Parafilm and incubate overnight into water bath (55 °C). Next day let the solution to cool down to room temperature, recheck pH and adjust it to 7.4 if required. Filter the solution through 0.45 µm filter and store at 4 °C (stable for 6 months).
3. Fixation buffer (0.25 % PFA in PBS, pH 7.4): add 625 µl of PFA stock solution to 9 ml 375 µl of PBS, vortex. Prepare fixation buffer fresh right before fixation step.
4. 10 % Tween 20 stock solution: add 1 ml or 100 % Tween 20 (*see* Note 4) to 9 ml of water, invert tube several times to mix, store at room temperature, protect from light (the solution is stable for a month).
5. Permeabilization buffer (0.2 % Tween 20 in PBS): add 1 ml of 10 % Tween 20 stock solution to 49 ml of PBS. Invert tube or slightly vortex to mix.
6. Immunostaining/Blocking Buffer (2 % fetal bovine serum (FBS) in PBS, pH 7.4): add 40 µl of FBS to 960 µl of PBS, pH 7.4. Invert tube or slightly vortex to mix.

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[2]Sucrose solution without protease/phosphates inhibitors can be made in advance and either kept on ice or frozen at −20 °C for prolonged storage. Immediate cryopreservation of human brain autopsy samples is very important in order to minimize tissue degradation and obtain high quality synaptosomal fraction, thus all the possible advance preparation should be made to be able to start cryopreservation procedure as soon as the autopsy samples arrive to the laboratory.

[3]To standardize PBS composition we routinely use commercially available PBS tablets.

[4]Tween 20 (100 %) is highly viscous liquid, in order to accurately measure 1 ml of the reagent cut the very tip portion of pipette tip (around 3–5 mm), fill the pipette slowly, after release tween 20 to water, wash pipette tip a couple of times by pipetting up and down to make sure that a whole 1 ml of tween 20 is added to the solution.

7. Washing buffer (0.1 % tween 20 in PBS): add 500 μl of 10 % Tween 20 stock solution to 49 ml of PBS. Invert tube or slightly vortex to mix.
8. Tau/ptau-specific antibodies and isotype control antibodies.
9. Presynaptic marker-specific antibody (for example, SNAP25, or Synaptophysin, or VGluT1-specific antibody).
10. Isotype control antibodies.
11. Zenon kits (*see* Note 5). Particular Zenon kits need to be selected based on the primary antibody host, isotype and suitable fluorophore type.
12. Polystyrene beads with 0.75, 1.5 and 4.5 μm in diameter.

3 Methods

All the procedures were carried out on ice unless specified. Unfixed human tissue is considered biohazardous material; always follow corresponding biosafety protocols to protect yourself and environment (*see* Note 6).

3.1 Human Brain Tissue Cryopreservation

1. Make cryopreservation buffer and keep it chilled on ice (*see* Subheading 2.1, **step 11** and Note 2).
2. Weight fresh brain tissue (*see* Note 7) and calculate the volume of cryopreservation buffer based on 1:10 mass to volume ratio (10 ml of cryopreservation buffer for 1 g of fresh tissue).
3. Place brain tissue into a petri dish and add 2 ml of ice cold cryopreservation buffer. Mince the tissue into 1–3 mm fragments using a prechilled scalpel.
4. Transfer the minced tissue and buffer into a 15 ml or 50 ml caped centrifuge tube and add the rest of the cryopreservation buffer with inhibitors.
5. Placed the tube into Styrofoam boxes with insulating material for slow freezing to −80 °C (*see* Note 8).

[5]The ways to attach fluorescent tag to antibodies of interest is not limited to Zenon labeling technology; classical covalent primary antibody–fluorophore conjugates, secondary antibody conjugates etc. can be used. In our hands, Zenon kits perform extremely well: the labeling procedure is very fast (15 min), minimum amount of antibody is required for labeling reaction (starting from 1 μg), and Zenon reagents are isotype-specific which allows an additional flexibility for multicolor experiments. A limitation of Zenon kits, which needs to be taken into account, is that Fab fragment/antibody interactions are not as strong as covalent bonds, therefore flow cytometry needs to be performed fairly soon after the immunostaining step.

[6]Unfixed human tissue is considered hazardous material because it can potentially contain some infectious pathogens. Thus, always use personal protective equipment and work within a biosafety cabinet; it is especially important for the homogenization step when risk of aerosol formation is high. Aerosols can also be released during centrifugation steps, always use sealed centrifuge cups/rotors to protect yourself and environment.

[7]Keeping good notes about tissue weight is important, because based on this information you will estimate amount of buffer for homogenization of tissue during P2 fraction preparation later on. P2 preparation may be performed in months, sometimes even years after original cryopreservation of brain tissue.

[8]We use paper towels as insulation material, wrapping each tube into multiple layers of paper towels before placed in a Styrofoam box.

3.2 Synaptosomal (P2)-Fraction Preparation and Cryopreservation

1. Remove a tube contained cryopreserved brain tissue from −80 °C freezer and place in warm (37 °C) water bath for 1–2 min to defrost perimeter.
2. Mix with 5 volumes of ice cold P2-buffer and shake vigorously until tissue is free.
3. Centrifuge the mix for 2 min at 1000 × *g*, 4 °C to pellet minced brain tissue.
4. Mix the pellet with 10 volumes of P2-buffer, based on the weight of fresh tissue before cryopreservation (1:10 mass to volume ratio; *see* Subheading 3.1, **step 2** and Note 7).
5. Homogenize tissue using a Teflon/glass homogenizer (clearance 0.1–0.15 mm) by eight gentle up and down strokes at 800 rpm. Save 200–500 μl of homogenate for P2-quality assay (*see* Note 9).
6. Centrifuge the rest of homogenate for 10 min at 1000 × *g*, 4 °C to isolate cell debris and nuclei (P1-fraction).
7. Collect supernatant (S1), place it into the clean pre-weighed centrifuge tube and centrifuge for 20 min at 10,000 × *g*, 4 °C to obtain crude synaptosomal P2 pellet (Fig. 1b).
8. Carefully discard supernatant (S2) and weight P2 pellet (*see* Note 10).
9. Resuspend P2 pellet in 10 (1:10 mass to volume ratio) volumes of P2-buffer.
10. Divide resuspended P2 fraction into 500 μl aliquots.
11. Placed the tube into Styrofoam boxes with insulating material for slow freezing to −80 °C (*see* Note 11).

3.3 Immunostaining of P2 Fraction

1. Remove a P2 fraction aliquot from −80 °C and thaw immediately in warm (37 °C) water bath for 1–2 min.
2. Transfer P2 fraction into a tube containing equivalent volume of ice-cold PBS and gently mix by pipetting up and down several times, avoid using vortex before fixation step. 10 cm
3. Centrifuge for 4 min at 4000 × *g* (4 °C) to pellet P2 fraction.
4. Decant the supernatant and resuspend the pellet in fixation solution, incubate for 1 h at 4 °C.

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[9] In addition to synaptosomal P2 fraction, we routinely save small portions of all other fractions (homogenate, P1, S1, and S2) to be able to monitor quality of P2 fraction by comparison of synaptic marker levels between those fraction by Western blotting. Moreover, the P1 fraction, with cell nucleus elements, can be used for genotyping.

[10] Transferring of the P2 pellet to a new tube or a weigh boat to determine weight can lead to loss of material. To avoid this, we use set of centrifuge tubes with known mass and weigh P2 without transferring to another container.

[11] The described double-cryopreservation protocol is not only adequate for maintenance of synaptosomal structures but also their functional integrity for functional assays, for example depolarization-induced tau release [17]. Based on our unpublished data, functional integrity of synaptosomes highly depends on postmortem interval (time between patient death and cryopreservation of brain tissue) but not so much on second cryopreservation of P2 fraction.

5. Centrifuge the sample for 4 min at 4000 × *g* (4 °C) to pellet P2 fraction.
6. Decant supernatant.
7. Resuspend fixed P2 fraction in 500 μl permeabilization buffer (*see* Note 12).
8. Place the tube into the water bath (37 °C) and incubate for 15 min, invert the tube every 5 min.
9. Add 500 μl of PBS and centrifuge for 4 min at 4000 × *g* (4 °C) to pellet P2-fraction. Decant supernatant.

   Steps 10–12 *Subheading 3.3* (*Antibody conjugation using Zenon kit*) *should be performed in parallel with* steps 7 *and* 8 *Subheading 3.3.*
10. Mix 18 μl of Immunostaining/Blocking Buffer with 1 μg of specific antibodies or isotype control antibodies for each immunostaining reaction (*see* Note 13).
11. Add 4.2 μl of corresponded Zenon kit Solution A (Fab fragments with conjugated Alexa dye) to the antibodies and incubate for 5 min at room temperature, protected from light.
12. Add 4.2 μl of Zenon kit Solution B (Stop solution) to the antibody/solution A mixture and incubate for additional 5 min at room temperature, protect from light.
13. Place 5 μl of P2 fraction (from **step 9**, Subheading 3.3) to a new tube
14. Add 25 μl of corresponded Zenon kit conjugated antibodies (from **step 12**, Subheading 3.3) or 25 μl of Immunostaining/Blocking Buffer (blank) to the tube, mix well and incubate for 30 min at room temperature, protect from light (*see* Note 14).
15. Add 1 ml of washing buffer to each reaction, mix well and centrifuge for 4 min at 4000 × *g* (4 °C).
16. Repeat the washing **step 2** times, protect from light.
17. Resuspend immunostained P2-fraction in washing buffer and transfer to FACS tubes, protected from light.

3.4 Flow Cytometry (FC) Analysis

Periodical checking and monitoring of cytometer performance (*laser alignment*, *laser time delay etc.*) *is beyond the scope of this chapter*.

[12]If P2 immunostaining and flow cytometry can't be performed on the same day, the fixed P2 fraction can be resuspended in 250 μl of PBS and kept at 4 °C overnight. Next day add 250 μl of 0.4 % Tween 20 in PBS to achieve the correct concentration of Tween 20 for permeabilization. Continue to follow the protocol from Subheading 3.3, **step 8**.

[13]Usually more than one P2 sample needs to be labeled with antibodies in a single experiment. In this case the Antibody/Zenon kit conjugation reaction can be scaled up based on how many individual staining reactions will be performed, and then the Antibody/Zenon kit conjugation mixture is divided between multiple immunostaining reactions (25 μl per one reaction).

[14]In case of dual labeling experiments, the corresponding Zenon/Antibody conjugates can be added simultaneously (25 μl of each per reaction), but all volumes of blanks, isotype controls, and single labeled samples then need to be adjust to 50 μl.

1. Run size calibration standards, we use polystyrene beads (0.75, 1.5, and 4.5 μm) and adjust the forward scatter (FSC) and the side scatter (SSC) detectors to place the populations of interest (synaptosome sizes are between 0.75 and 1.5 μm) on scale, adjust FSC threshold to exclude noise and apply FSC-based size gate (Fig. 2a).
2. Run controls: unstained samples (blanks), negative (isotype) controls, and positive controls (samples labeled with antibodies against presynaptic markers; we routinely use SNAP25, synaptophysin or VGluT1 antibodies) and samples of interest to confirm and/or optimize the settings by adjusting voltage on corresponding detectors (Fig. 2b, c and Note 15).
3. Adjust fluorescent compensation by running single and multi-labeled samples, if multiple color data will be collected from each sample.
4. Collect from 5000 to 10,000 events within the size gate from each experimental sample.
5. Analyze the data (*see* Fig. 2d, e for an example).

References

1. Gouras GK, Tampellini D, Takahashi RH, Capetillo-Zarate E. Intraneuronal beta-amyloid accumulation and synapse pathology in Alzheimer's disease. Acta Neuropathol. 2010; 119:523–541. DOI: 10.1007/s00401-010-0679-9 [PubMed: 20354705]
2. Dunkley PR, Jarvie PE, Robinson PJ. A rapid Percoll gradient procedure for preparation of synaptosomes. Nat Protoc. 2008; 3:1718–1728. DOI: 10.1038/nprot.2008.171 [PubMed: 18927557]
3. Wolf ME, Kapatos G. Flow cytometric analysis of rat striatal nerve terminals. J Neurosci. 1989; 9:94–105. [PubMed: 2563283]
4. Woo J, Baumann A, Arguello V. Recent advancements of flow cytometry: new applications in hematology and oncology. Expert Rev Mol Diagn. 2014; 14:67–81. DOI: 10.1586/14737159.2014.862153 [PubMed: 24308362]
5. Wolf ME, Kapatos G. Flow cytometric analysis and isolation of permeabilized dopamine nerve terminals from rat striatum. J Neurosci. 1989; 9:106–114. [PubMed: 2563275]
6. Wolf ME, Kapatos G. Stimulation of D2 dopamine receptors decreases intracellular calcium levels in rat anterior pituitary cells but not striatal synaptosomes: a flow cytometric study using indo-1. Synapse. 1989; 4:353–370. DOI: 10.1002/syn.890040411 [PubMed: 2481345]
7. Gylys KH, Fein JA, Cole GM. Quantitative characterization of crude synaptosomal fraction (P-2) components by flow cytometry. J Neurosci Res. 2000; 61:186–192. [PubMed: 10878591]
8. Fein JA, Sokolow S, Miller CA, Vinters HV, Yang F, Cole GM, Gylys KH. Co-localization of amyloid beta and tau pathology in Alzheimer's disease synaptosomes. Am J Pathol. 2008; 172:1683–1692. DOI: 10.2353/ajpath.2008.070829 [PubMed: 18467692]
9. Gylys KH, Fein JA, Yang F, Cole GM. Enrichment of presynaptic and postsynaptic markers by size-based gating analysis of synaptosome preparations from rat and human cortex. Cytometry A. 2004; 60:90–96. DOI: 10.1002/cyto.a.20031 [PubMed: 15229861]
10. Gylys KH, Fein JA, Yang F, Wiley DJ, Miller CA, Cole GM. Synaptic changes in Alzheimer's disease: increased amyloid-beta and gliosis in surviving terminals is accompanied by decreased PSD-95 fluorescence. Am J Pathol. 2004; 165:1809–1817. [PubMed: 15509549]

[15]If synaptosomal preparations are of a good quality, and immunostaining (Subheading 3.3) and FC adjustments (Subheading 3.4, **steps 1** and **2**) are performed correctly, more than 90 % of total event number within the size gate should be positive for the presynaptic markers, and less than 5 % should show nonspecific binding to isotype control antibodies.

11. Battista N, Bari M, Finazzi-Agrò A, Maccarrone M. Anandamide uptake by synaptosomes from human, mouse and rat brain: inhibition by glutamine and glutamate. Lipids Health Dis. 2002; 1:1. [PubMed: 12617751]
12. Kanekiyo T, Cirrito JR, Liu C-C, Shinohara M, Li J, Schuler DR, Shinohara M, Holtzman DM, Bu G. Neuronal clearance of amyloid-β by endocytic receptor LRP1. J Neurosci. 2013; 33:19276–19283. DOI: 10.1523/JNEUROSCI.3487-13.2013 [PubMed: 24305823]
13. Rausch JL, Johnson ME, Li J, Hutcheson J, Carr BM, Corley KM, Gowans AB, Smith J. Serotonin transport kinetics correlated between human platelets and brain synaptosomes. Psychopharmacology (Berl). 2005; 180:391–398. DOI: 10.1007/s00213-005-2178-6 [PubMed: 15726335]
14. Dodd PR, Hardy JA, Baig EB, Kidd AM, Bird ED, Watson WE, Johnston GA. Optimization of freezing, storage, and thawing conditions for the preparation of metabolically active synaptosomes from frozen rat and human brain. Neurochem Pathol. 1986; 4:177–198. [PubMed: 3561893]
15. Jones PB, Adams KW, Rozkalne A, Spires-Jones TL, Hshieh TT, Hashimoto T, von Armin CAF, Mielke M, Bacskai BJ, Hyman BT. Apolipoprotein E: isoform specific differences in tertiary structure and interaction with amyloid-β in human Alzheimer brain. PLoS One. 2011; 6:e14586.doi: 10.1371/journal.pone.0014586 [PubMed: 21297948]
16. Sokolow S, Luu SH, Headley AJ, Hanson AY, Kim T, Miller CA, Vinters HV, Gylys KH. High levels of synaptosomal Na(+)-Ca(2+) exchangers (NCX1, NCX2, NCX3) co-localized with amyloid-beta in human cerebral cortex affected by Alzheimer's disease. Cell Calcium. 2011; 49:208–216. DOI: 10.1016/j.ceca.2010.12.008 [PubMed: 21382638]
17. Sokolow S, Henkins KM, Bilousova T, Gonzalez B, Vinters HV, Miller CA, Cornwell L, Poon WW, Gylys KH. Pre-synaptic C-terminal truncated tau is released from cortical synapses in Alzheimer's disease. J Neurochem. 2015; 133:368–379. DOI: 10.1111/jnc.12991 [PubMed: 25393609]
18. Begley JG, Butterfield DA, Keller JN, Koppal T, Drake J, Mattson MP. Cryopreservation of rat cortical synaptosomes and analysis of glucose and glutamate transporter activities, and mitochondrial function. Brain Res Brain Res Protoc. 1998; 3:76–82. [PubMed: 9767122]
19. Pankiewicz JE, Guridi M, Kim J, Asuni AA, Sanchez S, Sullivan PM, Holtzman DM, Sadowski MJ. Blocking the apoE/Aβ interaction ameliorates Aβ-related pathology in APOE ε2 and ε4 targeted replacement Alzheimer model mice. Acta Neuropathol Commun. 2014; 2:75.doi: 10.1186/s40478-014-0075-0 [PubMed: 24972680]

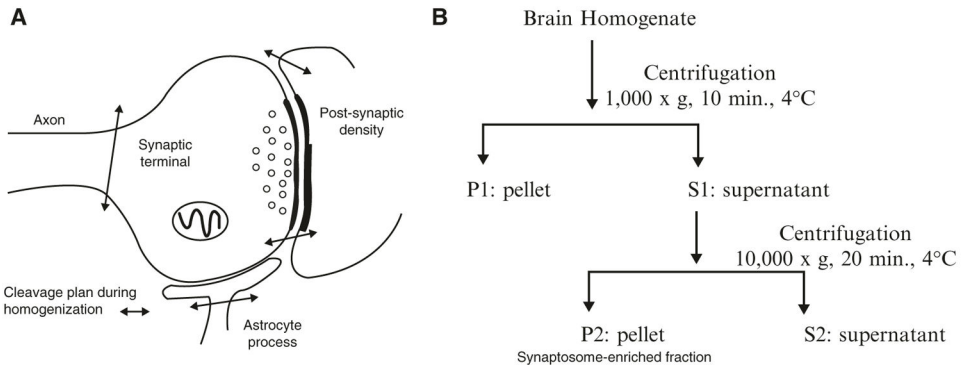

Fig. 1.

Synaptosome preparation. (**a**) Homogenization of fresh brain tissue in isotonic sucrose solution induces formation of synaptosomes—avulsion and resealing of synaptic terminals, which may contain some postsynaptic elements and sometimes astrocyte end-feet. (**b**) Schematic illustration of synaptosome-enriched (P2) fraction preparation from brain homogenate

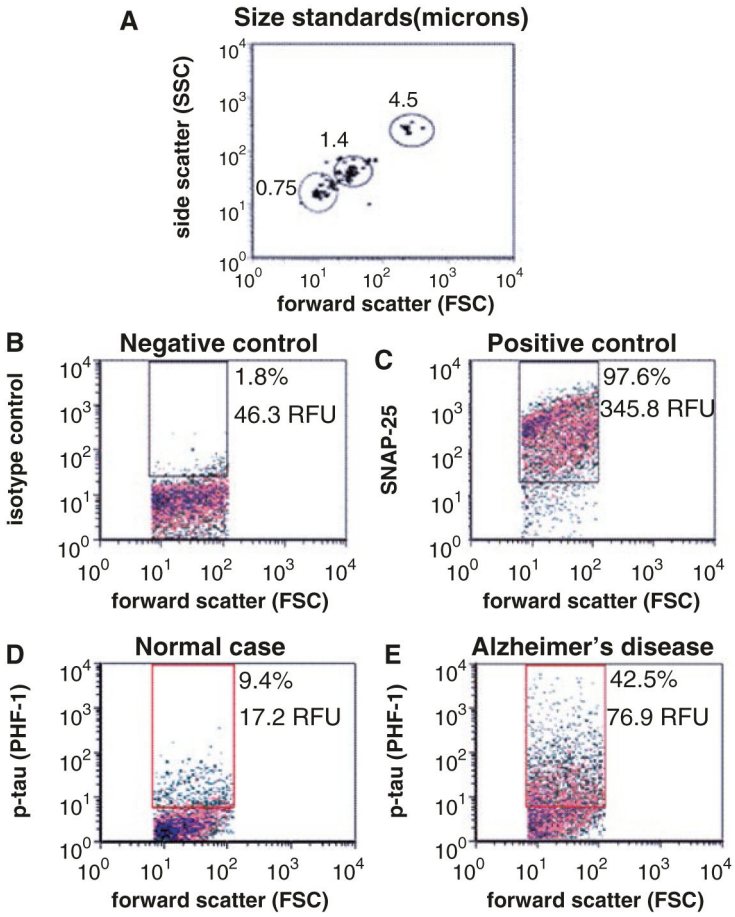

Fig. 2.

Flow cytometry analysis of synaptosomal preparations. (**a**) An example of size standard acquisition for FSC-based size gate establishment. (**b**) Background labeling in presence of isotype control antibodies (*negative control*): only 1.8 % from the total 10,000 events collected within applied size gates show positive signal with average intensity 46.3 relative fluorescence units (RFU). (**c**) Presynaptic marker (SNAP25)-positive events within the size gate (*positive control*): 97.6 % positive events with average intensity 345.8 RFU. (**d**) An example of p-tau (Ser369/Ser404 phosphorylation-dependent epitopes accessed by PHF-1 antibody) signal in a synaptosomal fraction from normal human brain cortex: 9.4 % of p-tau positive events with average intensity 17.2 RFU. (**e**) An example of p-tau (PHF-1) signal in a synaptosomal preparation from brain of a subject with a late stage of Alzheimer's disease: 42.5 % of p-tau positive events with average intensity 76.9 RFU