Labeling Protein Aggregates

Reference

1. Afreen, S., & Ferreira, A. (2022). The formation of small aggregates contributes to the neurotoxic effects of tau$_{45\text{-}230}$. *Neurochemistry international*, *152*, 105252. https://doi.org/10.1016/j.neuint.2021.105252

Kit

1. Molecular Probes Protein Labeling Kit, Cat# MP30006

Solutions

1. Arachidonic acid solution: 4 mM Arachidonic Acid prepared in ice cold 200 proof ethanol
2. Aggregation buffer: 10 mM HEPES, 100 mM NaCl, 5 mM DTT
3. Fixative: 4% Paraformaldehyde prepared in PBS w/ 4% Sucrose, pH 7.4 w/ NaOH
4. Blocking Buffer: 10% Bovine Serum Albumin prepared in PBS

Abbreviations

1. AA: Arachidonic Acid
2. NaCl: Sodium Chloride
3. DTT: Dithiothreitol
4. EtOH: Ethanol
5. DI water: Deionized water
6. RT: Room Temperature
7. PBS: Phosphate Buffered Saline
8. PFA: Paraformaldehyde
9. BSA: Bovine Serum Albumin

Protocol

1. Make FRESH arachidonic acid solution (Cat# 90010.1 from Cayman Chemical – stored at -20°C once opened).
    a. Wash AA designated pipet with 10 rinses of ice cold 200 proof ethanol.
    b. Put 811 µL of ethanol in autoclaved Eppendorf on ice.
    c. Add 10 µL arachidonic acid (stored at -20°C) to ethanol.
    d. Final concentration = 4 mM.
    e. Rinse off pipet with 10 more rinses of ethanol.
2. Combine aggregation buffer with tau protein to make 500 µL:
    a. HEPES pH 7.4 to final concentration of 10 mM (20 µL 250 mM stock).
    b. NaCl to final concentration of 100 mM (20 µL 2.5 M NaCl).
    c. DTT (from 4°C) to final concentration of 5 mM (10 µL of 250 mM stock DTT).
    d. Tau to final concentration of 4 µm.
    e. dH_2O up to 500 µL.

3. Tilt to mix, do not pipet.
4. Add protein mixture to wells.
    a. Add 239 µL of each protein to 2 wells (one will be used as unaggregated control and the other will be aggregated).
    b. Then add 9.38 µL of either AA or EtOH (EtOH is added to the unaggregated wells) to each well to induce.
5. Incubate for 24 hours (may vary) at RT.
6. Airfuge aggregates at 100,000 x g for 15 minutes.
7. Mark expected location of pellet on tubes and carefully remove/discard supernatants.
8. Resuspend pellet in 20 µl of aggregation buffer over ice.
9. Begin Molecular Probes MP30006 protein labeling protocol.
    a. Prepare 1M Sodium Bicarbonate (Component B).
        i. Add 1 ml of DI water, pipet to dissolve.
        ii. Store in -20°C after use.
    b. Transfer resuspended pellet containing aggregated proteins into reaction tube (Component C) (~20 µl).
    c. Add 1/10 volume of 1M Sodium Bicarbonate, pipet to mix (~2 µl).
    d. Dissolve AlexaFluor 488 dye (Component A) in 10 µl of DI water giving a concentration of [11.3 nmol/µl].
        i. Dye cannot be reused once dissolved in water.
    e. Add 1 µl of reactive dye to resuspended protein aggregates in reaction tube (Component C), gently tap to mix thoroughly.
    f. Incubate at RT for 1 hour.
    g. Label the spin filter tubes accordingly.
    h. Gently shake gel resin.
    i. Add 800 µl of resin to upper chamber of spin filter.
    j. Centrifuge the spin filter at 16,000 x g for 15 seconds (includes spin up time).
    k. Decant buffer in collection tube.
    l. Gently add protein-labeled solution onto center of resin bed surface.
    m. Centrifuge 16,000 x g for 1 minute.
    n. Collection tube will contain the dye-labeled protein aggregates in approximately 60-100 µl of buffer.
        i. Resin will change to yellow-green color.
        ii. Collection tube liquid will be predominantly clear.
10. Use 60 mm dish previously plated with cells, aspirate media and replenish with 1 ml fresh media.
11. Add the labeled protein aggregates to dish and gently swirl to mix.
12. Incubate at 37°C overnight.
13. Next day, rinse coverslips once in PBS and fix cells for 15 min in 4% PFA (4% sucrose) at RT.
14. Remove PFA and rinse 3 x with PBS. These can be stored at 4°C in PBS.

15. Day 1 - In a 30- or 60-mm dish, permeabilize the cells with 0.3% Triton X-100 prepared in PBS for 4 min at RT.

16. Rinse 3 x 5 min with PBS.
    a. Skip steps 1 and 2 if using antibodies with extracellular epitopes to visualize protein membrane. Just rinse once in PBS.
17. With the coverslips placed separately on wax sheet, cells-up, block non-specific binding with 100 µL of 10% BSA (previously prepared in PBS) per coverslip for 1 h at RT. Cover the wax sheet to minimize evaporation.
18. Prepare the primary antibody solution (100 µL per coverslip) at the correct concentration in 1% BSA (dilute the 10% BSA with PBS). NOTE: If using more than one primary antibody, make sure they were prepared from different species.
19. Remove the coverslips from the blocking solution and place on a wax sheet in a humid container (can be made out of a Petri dish with wet filter paper under the wax sheet).
20. Cover each coverslip with 100 µL primary antibody solution.
21. Store overnight at 4°C.
22. Day 2 - Rinse 3 x 5 min with PBS.
23. Prepare secondary antibody solution (100 µL per coverslip) at the appropriate concentration in 1% BSA (in PBS). Always make 50 µL more than you need per secondary antibody (or avidin) and spin in benchtop centrifuge for 5 min and pipet from the top.
    a. NOTE: If using more than one secondary, make sure they're from different species and can be seen under different filters… ex: fluorescein vs rhodamine.
24. Place coverslips cells-up in a humid box as described above.
25. Cover each coverslip with 100 µL secondary antibody solution.
26. Incubate at 37°C for 1 h.
    b. If secondary antibody is not biotinylated, proceed to step 28.
27. If secondary is biotinylated, rinse after incubation 3 x 5 min in PBS then add the avidin for 1 h at 37°C.
28. Rinse 2 x 5 min with PBS.
29. Carefully remove paraplast dots with forceps and rinse 1 x 5 min with PBS.
30. Mount coverslips on clean labeled microscope slides using ~5-10 µL slow-fade mounting media.
31. Allow coverslips to dry in the dark overnight at RT.
32. Next day, rinse coverslips with MilliQ water to dissolve dried salts.
33. Store the coverslips at 4°C.