An update to: Tracking TAU in neurons: How to grow, fix and stain primary neurons for the investigation of TAU in all developmental stages

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Method Article

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Abstract

Primary murine neurons are a well-established tool for investigating TAU in the context of neuronal development and neurodegeneration. However, culturing primary neurons is usually time-consuming and requires multiple feeding steps, media exchanges, proprietary media supplements, and/or preparation of complex media. Here we describe i) a relatively cheap and easy cell culture procedure for the cultivation of forebrain neurons from embryonic mice (E13.5) based on a commercially available neuronal supplement (NS21), ii) a protocol for the cultivation of hippocampal and cortical neurons from postnatal (P0-P3) animals, as well as iii) basic fixation and immunofluorescence techniques for the staining of neuronal markers and endogenous TAU. We demonstrate a staining technique, which minimizes antibody consumption and allows for fast and convenient processing of samples for immunofluorescence microscopy of endogenous TAU in primary neurons. We also provide a protocol that enables cryopreservation of fixed cells for years without measurable loss of TAU signal. In sum, we provide reliable protocols enabling microscopy-based studies of TAU in primary murine neurons.

Introduction

The microtubule-associated protein TAU is sorted into the axon of mature neurons (Binder et al., 1985). In the central nervous system (CNS), four different isoforms are expressed in rodents and six in humans, respectively. The relative amounts of these isoforms are developmentally regulated, change with neuronal maturation, and are differentially localized within neurons (Bachmann et al., 2021; Bullmann et al., 2009; Goedert et al., 1989; Zempel et al., 2017). TAU is phosphorylated at different sites, which depends on the developmental stage, pathological processes, and compartmentalization (for review, see Zempel & Mandelkow, 2014). In addition, spine and dendrite maturation is also highly dependent on TAU and differs for the TAU isoforms (Zempel et al., 2017). Thus, studying TAU in a neuronal and cellular context is crucial. Primary murine forebrain neurons have proved to be an essential tool for studying TAU during development and modeling pathological processes (Bachmann et al., 2021; Bell et al., 2021; Iwata et al., 2019; Li et al., 2011; Schützmann et al., 2021; Tjiang et al., 2022; Zempel et al., 2013). However, culturing primary murine neurons according to standard protocols is time-consuming and – especially when investigating mature, synaptically active, and well-polarized neurons is necessary – requires multiple feeding steps with expensive media supplements of proprietary formulations.

Here we describe two protocols developed for convenient and reproducible long-term culturing of primary murine neurons from E13.5 mice and P0-P3 animals (for other procedures and rat cultures from E16.5 mice or E18 rats: see Zempel et al., 2017):

1) A protocol for forebrain cultures isolated from E13.5 mice that requires only two steps (plating and one feeding step) and is based on the neuronal supplement NS21 (Chen et al., 2008), which detailed formulation is known and commercially available but can also be manufactured in any standard cell biology laboratory. Our NS21-based culturing method with the modification outlined below has proven to be cost-efficient and reproducible. In addition, it generates cultures that achieve sufficient maturity to

investigate TAU distribution, isoform expression, and posttranslational modification in the context of neuronal development and degeneration.

2) A protocol for cultivating neonatal hippocampal and cortical neurons, isolated from mice at postnatal day (P) 0-3 that supports neuronal viability without the need for glial feeder layers and is suitable for monitoring synaptogenesis and dendritic spine analysis dependent on TAU.

Of note, both culturing methods allow for seeding densities that are amenable to microscopy analysis of endogenous TAU or suitable for live-cell imaging techniques. In addition, we describe a standard fixation and staining procedure suitable for investigating endogenous TAU and its phosphorylation status in primary neurons. This procedure uses low antibody amounts and allows fast and convenient processing (i.e., blocking, staining, washing) of samples for immunofluorescence microscopy.

Reagents

1. Cultivation of forebrain neurons from embryonic mice (E13.5)

1.1. Materials and animals

1.1.1. Animals

Note: Animal experiments require authorization by the responsible supervising authority. All our animal experiments agree with the local regulations, but special permissions may be needed depending on the state or country. Our experiments were approved by the Animal Welfare Officers of the University of Cologne, the veterinary inspection office of Cologne, and the local government (Landesamt für Natur, Umwelt und Verbraucherschutz/LANUV, North Rhine-Westphalia, Recklinghausen, Germany).

The number of embryos can vary depending on the strain of mice used: For example, FVB/N mice give more offspring compared to C57BL/6J .

(Taketo et al., 1991)

1.1.2. Cell culture media and reagents

Prepare all solutions in a sterile manner and under sterile conditions. For washing use sterile DPBS (D8537, Merck) or HBSS (14175095, Thermofisher Scientific).

1. Coating solution: Prepare a stock solution of 10mg/ml Poly-D-Lysine (P7886, Merck) in ddH_2O, aliquot to 1 ml and store at -20°C. Before use, thaw on ice and dilute to 20 µg/ml using sterile DPBS.

Dilution can be stored up to 4 weeks at 4°C.

2. (10x) 0.5% Trypsin (P10-024100, PanBiotech): aliquot and store at -20°C. Dilute to 1x before usage with DPBS.

3. 10% Fetal Bovine Serum (FBS, Biochrom AG, S0615) in HBSS

4. Neuron plating media (NPM):

· Neurobasal media (21103-049, Thermofisher Scientific)

· 1% FBS

· 1x Antibiotic-Antimycotic Solution (AA, Merck, A5955)

· 1x GlutaMAX (Thermofisher Scientific 35050061)

· 2% B27 (17504044, Thermofisher Scientific) or 1x NS21 (P07-20001, PanBiotech).

Alternatively, it is possible to use DMEM but with inferior results. Store medium at 4°C for up to 1 month or aliquot and freeze for long-term storage.

5. Neuron maintenance media (NMM):

· Neurobasal medium

· 1x GlutaMAX

· 1x AA

· 1x NS21

· Optional: add 5-10% astrocyte conditioned medium (ACM) to enhance neuronal survival.

Store at 4° for up to 1 month.

6. Cytosine β-D-arabinofuranoside (AraC, Merck, C1768): Dissolve 10 mg in 1 ml ddH_2O, filter sterile, aliquot, and store at -20°C. Avoid multiple freeze-thaw cycles of AraC aliquots.

7. Multi-well plates and coverslips: We use preferentially 24- or 6-well cell culture plates (VWR) and 12- or 25-mm coverslips (VWR).

2. Cultivation of hippocampal and cortical neurons from postnatal mice (P0-P3)

2.1. Materials and animals

2.1.1. Animals

Note: Animal experiments require authorization by the responsible supervising authority. All our animal experiments are approved by the Animal Welfare Officers of the University of Cologne and RWTH Aachen University, and the local government (Landesamt für Natur, Umwelt und Verbraucherschutz/LANUV, North Rhine-Westphalia, Recklinghausen, Germany).

For optimal results, animals should not be older than 3 days postnatal (P3) because neuronal yield and viability will decrease drastically with the increasing maturity of postnatal primary neurons. Additionally, brain dissection becomes more complex for older animals, and residual meninges will impair cultures by glial/endothelial overgrowth.

2.1.2. Cell culture media

1. 1x Trypsin-EDTA: Dilute Trypsin-EDTA (0.5% Trypsin-EDTA (10x), no Phenolred, 15400054, Thermofisher Scientific) to 1x in HBSS, add 10 mM HEPES and 1x AA before usage.

2. 10% Fetal Bovine Serum (FBS, Biochrom AG, S0615) in HBSS

3. 5 mM β-Mercaptoethanol: Dilute 3.49 µl β-Mercaptoethanol in 10 ml Neurobasal, filter- sterilize.

4. DNase I (Sigma-Aldrich, 11284932001): Prepare a 4 mg/ml stock solution using ddH_2O, sterile filter, aliquot, and store at -20°C. CAVE: Do not vortex DNase solutions as this will decrease enzyme integrity/activity!

5. HBSS: Add 1% 1M HEPES buffer and 1x AA for 500 ml HBSS before usage.

6. Borate buffer: Dissolve 123.7 mg of boric acid (61,84 g/mol, #P010.1, Roth), 100.6 mg of Sodium borate (CAVE: Toxic! 201.22 g/mol, #221732, Sigma-Aldrich) in 50 ml ddH_2O (overnight end over end shaking), sterilize by filtration.

7. 1mg/ml Poly-L-Lysin (Sigma-Aldrich, P2636-100MG): Weigh approx. 2 mg of PLL in 15 ml Falcon tubes and store at -20°C until use. On the day of the dissection, take a PLL aliquot and add the respective volume of borate buffer (e.g., 2.4 mg PLL + 2.4 ml of borate buffer), dissolve for 15 minutes at 37°C in the incubator, filter sterile.

Note: Poly-D-Lysine coating used for embryonic neuron culture (see **2.1.2**) should work too for postnatal primary cultures (not tested).

8. Neuron plating medium (NPM):

· MEM (31095029, Thermofisher Scientific)

· 10% FBS

· 0.6% Glucose

· 1x AA

Store medium at 4°C for up to 1 month or aliquot and freeze for long-term storage.

9. Neuron maintenance medium (NMM):

· Neurobasal-A medium (10888022, Thermofisher Scientific)

· 1x GlutaMAX (35050061, Thermofisher Scientific)

· 1x B27 (17504044, Thermofisher Scientific) or 1x NS21 (P07-20001, PanBiotech)

· 0.2% Horse serum

· 5 µM β-Mercaptoethanol

· 1x AA

· Add 5-10% conditioned medium from primary rat or mouse neuronal cultures (prepared from embryonic tissues) to enhance neuronal survival. We use primary rat neuron conditioned medium (conditioned for 10-20 days) and have not tested other conditioned media, but know from similar experiments that primary murine neuron or astrocyte cultures should work as well.

Store at 4° for up to 1 month.

Important note: Neurobasal-A is optimized for postnatal/adult primary neuron culture and different from Neurobasal (used for embryonic neuron cultures, see **2.1.2.**)!

10. 1mM Cytosine β-D-arabinofuranoside (AraC), stock solution (C6645, Sigma-Aldrich): Dissolve in ddH_2O, sterilize by filtration, store aliquots at -20°C. Supplement culture medium at day 2 post-seeding with 2.5 µM AraC (1:400) to inhibit glial overgrowth.

11. Multi-well plates and coverslips: We use preferentially 24- or 12-well cell culture plates (VWR) and 12 or 18 mm coverslips (VWR).

12. Paraffin wax (327204, Sigma-Aldrich, melting point 50°C-60°C).

3. Fixation and staining of primary neurons for endogenous TAU trafficking

3.1. Materials

3.1.1. Fixation solution

1. 4% (w/v) sucrose: dissolve 4 g of Sucrose (S0389, Merck) in 100 ml DPBS, store at room temperature

2. Fixation solution: Dilute 37% formaldehyde (FA) in 4% Sucrose. Standard fixation uses 3.7% FA, to minimize cell shrinkage during fixation, 7.4% FA can be directly added to the cells (1:1 mix of FA and cell culture medium).

3. Storing solution for fixed cells: Store coverslips in multi-well plates filled with 60% glycerol diluted in DPBS at -20°C.

3.1.2. Blocking and staining solutions

1. Blocking and permeabilizing solution (B+P): Dilute 5% Bovine Serum Albumin (BSA, A7906, Merck) and 0.5% Triton-X100 (X100, Merck) in DPBS.

2. Washing solution: All washing steps are carried out with DPBS if not stated otherwise.

3. Mounting medium: Aqua Polymount (08381-120, Polysciences)

4. Antibodies: Dilute antibodies directly in DPBS. Use the following dilutions for primary antibodies:

· Rabbit anti-total TAU (K9JA, Dako): 1:1000

· Rabbit anti-pTAU (Epitope present in axon; PHF1, generously provided by P. Davies, AEC, NY): 1:200

· Mouse anti-pTAU (Epitope present in dendrites; 12E8, Prothena Bioscience): 1:1000

· Secondary fluorescent antibodies from Thermofisher Scientific (Alexa Fluor Dyes): 1:2000

5. Phalloidin: Dilute Phalloidin (e.g., AlexaFluor 647 Phalloidin, A122287, Thermofisher Scientific) according to the manufacturer's guide. Directly add it to the secondary antibody mixture for f-actin

counterstaining (dilute 1:40).

6. NucBlue (R37605, Thermofisher Scientific): use according to the manufacturer's protocol (2 drops per ml medium; incubation for 20 minutes).

Optional: add NucBlue directly to the B+P solution.

Equipment

Procedure

1. Cultivation of forebrain neurons from embryonic mice (E13.5)

1.1.1. Coating of cell culture plates for neurons

1. Transfer one coverslip into each well of a 24- or 6- well culture plate.

2. Add 500- 1000 µl of coating solution (20 µg/ml Poly-D-Lysine in DPBS) into each well.

3. Incubate plate at 37°C for at least 2 hours (we recommend overnight incubation), e.g., in a humidified cell culture incubator.

4. Wash twice thoroughly with DPBS (see **Note 1**).

5. Store plates in the final washing solution for use on the same day.

6. For long-term storage (up to 3 weeks): let plates dry completely at room temperature, store at 4°C.

1.1.2. Dissection of embryonic forebrain

Before starting: Sterilize preparation tools (one large and one small scissors, one large and two small forceps) using ethanol. It is essential to work as fast and sterile as possible, using pre-cooled HBSS + 1x AA for dissection. In addition, it is crucial to work on ice during the whole dissection procedure.

1. Euthanize a pregnant mouse at day 13.5 of gestation, dissect mouse embryos and keep heads on ice.

2. Remove scalp of dissected heads.

3. Extract the entire brain carefully and remove meninges with forceps (thoroughly!); it is crucial to remove the meninges completely to avoid endothelial cell growth.

4. If necessary: Remove olfactory bulbs.

5. Gently divide the brainstem and cortical hemispheres.

6. If necessary: Remove remaining meninges patches.

7. Transfer cortical hemispheres of each animal in different reaction tubes (filled with HBSS) and store them on ice. Note: Cortices from different animals can be pooled to increase yield.

8. Optional: Use other parts of the brain, e.g., brain stem, to culture glial cells.

1.1.3. Cell isolation and plating

Transfer dissected brains to a sterile clean bench and proceed with cell isolation.

1. Carefully remove supernatant and wash twice using pre-warmed HBSS.

2. Add 1x Trypsin and incubate for 7 minutes at 37°C, swirl 2-3 times during incubation.

3. Inactivate Trypsin with 10% FBS in HBSS by doubling the volume.

4. Discard the supernatant and dilute cells with HBSS.

5. Gently but thoroughly triturate cells using a 1 ml pipette.

6. Count cells using Trypan Blue (1450022, BioRad).

7. Dilute cells to desired density using pre-warmed (37°C) NPM (see **Notes 2 and 3**):

a. For a 24-Well plate: seed 200.000 cells/ well in 500 µl.

b. For a 6-Well plate: seed 800.000 cells/ well in 1 ml.

8. Transfer plate immediately into a humidified cell culture incubator, at 37°C and 5% CO_2.

1.1.4. Neuronal maintenance

1. Grow mouse neurons for 4 days after plating in a humidified cell culture incubator at 37°C and 5% CO_2

2. Freshly prepare NMM with 1 µg/ml AraC (see **Note 4**).

3. Double the volume of the cell culture medium by adding NMM including AraC (see also **Note 5**). Cells can now be grown for at least 4 weeks, depending on the desired analysis (see **Note 6**).

2. Cultivation of hippocampal and cortical neurons from postnatal mice (P0-P3)

2.1.1. Coating of coverslips for neurons

1. Prepare coverslips for top-down culture: Heat paraffin in a blue-cap glass bottle (VWR, e.g., 215-1514) in a water bath at 70-80°C (CAVE: do not microwave or cook, Paraffin wax is ignitive! Only use a water bath! Loosen blue cap!). Meanwhile, put sterile coverslips side-to-side into a petri dish and add 3 drops of liquid and sterile Paraffin warmed to ~70°C onto each coverslip with a sterile glass Pasteur pipette in a tripod-shape. Let dry under the hood until the drops get white and solid for at least 10 minutes. Store coverslips sealed with parafilm at room temperature.

2. Put five 12 mm coverslips in each well of a 30 mm petri dish.

3. Add 90 µl for 12 mm (200 µl for 18 mm coverslips) of coating solution (1 mg/ml Poly-L-Lysine in borate buffer) to each coverslip.

4. Incubate coverslips at 37°C for at least 2-3 hours (best is overnight), e.g., in a humidified cell culture incubator.

5. Wash twice with sterile ddH_2O, once with 1x HBSS (without phenol red!; see **Note 1**).

6. Dry coverslips for 5 minutes under a sterile hood.

7. Add 4 ml plating medium per petri dish containing the coated coverslips and store them in the incubator at 37°C until needed.

8. For long-term storage (up to 3 weeks): let coverslips dry completely at room temperature under the sterile hood, wrap in aluminum foil, and store at 4°C.

2.1.2. Dissection of hippocampi/ cortices

Before starting: Sterilize preparation tools (scissors (big and small), one big and two small forceps) using ethanol. It is essential to work as fast and sterile as possible, using pre-cooled HBSS + 1x AA for dissection. All dissection steps should be performed on ice. We regularly use the pooled hippocampi of at

least three animals per genotype, which yields (in the case of three animals each) sufficient material for ten 12 mm or three 18 mm coverslips.

1. Euthanize the pub by decapitation using a sharp scissor.

2. Place head in a dish filled with HBSS +1x AA kept on ice.

3. Remove scalp of dissected heads.

4. Carefully remove the skull using fine forceps.

5. Extract the entire brain carefully.

6. *Optional:* To reduce the risk of contamination, transfer brains to a fresh dish filled with HBSS+1x AA.

7. Gently divide the brainstem and the cortical hemispheres.

8. Gently remove the midbrain and thalamic tissue from each hemisphere.

9. Carefully remove meninges (thoroughly!); it is crucial to remove the meninges completely to avoid the growth of glial/endothelial cells.

10. Dissect the hippocampus or cortex and collect in a 15 ml falcon tube (filled with 5 ml HBSS+1xAA, on ice). Hippocampi/ cortices can be pooled or processed individually.

2.1.3. Cell isolation and plating

Transfer dissected hippocampi/ cortices to a sterile clean bench and proceed with cell isolation.

1. Carefully remove supernatant and wash 3 times using 5 ml of HBSS.

2. Add 5 ml of Trypsin solution (maximum of 3 half-cortices or 6 hippocampi) and incubate for 25 minutes at 37°C, swirling several times during incubation.

3. Discard the supernatant and wash tissue three times with 5 ml of HBSS.

4. Add 2 ml NPM and 2 µl DNase I (improves dissociation by reducing shear forces).

5. Gently but thoroughly triturate cells using three fire-polished glass pipettes of decreasing diameter (ranging from original size (but polished) to reduced size that allows still smoothly aspirating fluid, but avoiding pressure differences due to high resistance of too small polished pipettes).

6. Count cells using Trypan Blue (1450022, BioRad).

7. Dilute cells to desired density using pre-warmed (37°C) NPM (see **Notes 2 and 3**) and plate at the desired density directly onto the coverslips in Petri dishes:

Generally, distribute hippocampal neurons from 3 animals onto ten 12 mm coverslips or three 18 mm coverslips, respectively. Cortical cultures are more complex/sensitive to plating density, so the optimal cell density is between 30.000 to 60.000 cells/12 mm coverslip.

8. Transfer neurons immediately into a humidified cell culture incubator, at 37°C and 5% CO_2. Let cells attach for 45 - 60 minutes.

9. Afterwards, flip 12 mm coverslips (neurons facing down) and transfer to a 24-well plate filled with NMM. For 18 mm coverslips use 12-well-plates filled with 1 ml of NMM.

2.1.4. Neuronal maintenance

1. Grow mouse neurons for 2-3 days after plating in a humidified cell culture incubator at 37°C and 5% CO_2.

2. Freshly prepare NMM with 2.5 µM AraC (see **Note 4**).

3. Double the volume of the cell culture medium by adding pre-warmed NMM including AraC (see also **Note 5**). Cells can now be grown for up to 1 month, depending on the desired analysis (see **Note 6**).

3. Fixation and staining of primary neurons for endogenous TAU trafficking

3.1.1. Fixation and staining of neurons

At the desired stage of TAU development, neurons can then be treated (see **Note 7**) and fixed for immunofluorescence analysis.

1. Remove cell culture medium completely and immediately add 3.7% fixation solution (see **Note 8**).

Alternatively: directly add 7.4% FA onto the cells (with medium still in the wells, doubling the total amount of liquid, thus resulting in a final concentration of 3.7% FA).

2. Incubate for 30 minutes at room temperature (see **Note 9**).

3. Wash cells once with DPBS.

4. **Pause step:** It is possible to directly store the cells for up to 2 years after fixation using 60% glycerol diluted in DPBS, store plates at -20°C. Let cells adjust to room temperature before continuing with the staining procedure.

5. Wash coverslips two times with DPBS.

6. Add B+P solution for 5- 10 minutes at room temperature.

Optional: add 1 drop of NucBlue to the B+P solution incubate for 10 minutes at room temperature. Omit NucBlue in the last washing step of step 14.

7. Wash twice with DPBS.

8. Prepare a parafilm-coated lid and add a drop of antibody solution (30 µl for 12 mm coverslip and 80-100 µl for 25 mm coverslip) for each coverslip to be stained.

9. Transfer coverslip with cells facing down to the parafilm-coated plate and place the well-plate lid in a moist chamber (containing wet towels) for incubation.

10. Incubate 16 hours at 4°C.

11. Transfer coverslips back to well-plates (cells facing up) and wash 3 times with DPBS.

12. Proceed with secondary antibody staining (*optional*: include Phalloidin) as described before (see points 7. and 8.)

13. Incubate for 2 hours at 37°C in the dark in a moist chamber (see **Note 10**).

14. Transfer coverslips back to well-plates (cells facing up) and wash 3 times with DPBS. In the last washing step: add 1 drop of NucBlue to each well and incubate for 20 minutes.

15. Wash coverslip once with DPBS and twice with ddH_2O.

16. Mount coverslips on a microcopy glass slide upside down with a drop (~5 µl) of mounting medium. Take care that coverslips are mounted as plane as possible.

17. Allow mounting medium to polymerize overnight at room temperature before imaging. Microscopy slides can be stored at 4°C in the dark for at least 5 years with only a slight decrease in fluorescence intensity.

Troubleshooting

1. Coating coverslips requires at least 2-3 hours, but results are poorer (i.e., cells do not grow as well and not as long) if the coating is done for less than 12 hours. In addition, washing needs to be done with great caution, as common preparations of PDL have trace amounts of toxic substances that prevent the growth of neurons.

2. Cell culture medium must not be oxygenated. Usually, the cell culture medium contains phenol red as a pH indicator; the color must be bright red, but not purple or yellow. If the color is other than bright red, pre-incubate the medium in 5% CO_2 until the color turns bright red.

3. Cell density can be easily adjusted by changing the volume of the plating solution, but this cell/volume ratio has proven to be robust. Best conditions need to be determined for each well size and desired cell density.

4. AraC is relatively unstable; it will not resist several freeze/thaw cycles. Prepare a stock solution and make small aliquots stored at -20°C.

5. Primary neurons are vulnerable to cell culture medium replacements, especially at later stages of development. While the neurons support doubling of the culture medium after a few days of culture, more mature neurons do not support complete medium replacement already after a week. With this protocol, neurons are usually fine for up to 2 months, but if a medium replacement becomes necessary (e.g., for high-density long-term cultures), do not replace more than 20% of the maintenance medium at once.

6. Polarized distribution of TAU and differential TAU phosphorylation starts at around 3 days *in vitro* (DIV3) and is usually completed within DIV12. Mature TAU isoforms begin to appear at DIV7-14. Therefore, if studying TAU in the context of adult-like neurotransmission and age-dependent neurodegeneration is desired, cultures should be maintained for at least 14 to 21 days.

(Li et al., 2011)

7. Treatment of primary neurons with cell culture compatible substances (i.e., non-toxic substances) is not trivial; the following points must be considered: i) Primary neurons, especially after weeks of culturing, do not support medium replacement. Even fresh and completely supplemented growth medium is insufficient to sustain growth. Instead, primary neurons need to be kept in a "conditioned medium" (CM), which means that the growth medium has been conditioned by the neurons (or astrocytes) for several days. Thus, treatments of primary neurons should be conducted simply by adding substances to the CM and not by replacing the CM. To save valuable substances, it is possible to reduce the amount of CM, e.g., in a 24-well plate from 1 ml to 0.3 ml for a limited time (1-3 days is usually fine, but this depends on the age of the culture, the older, the more difficult it gets). Replacing more than 10% of CM with DPBS, adding fresh maintenance medium or other non-neuronal cell culture medium will influence TAU distribution and phosphorylation (see, e.g., . TAU distribution and phosphorylation are also temperature-sensitive (, so reasonable care must be taken to treat samples quickly and maintain them at 37°C.

Zempel et al. 2010)

Bretteville et al., 2012)

8. The standard fixation solution is 3.7% FA in DPBS. Still, slightly better results can be obtained by using a double concentrated fixation solution (i.e., 7.4% FA in DPBS) added on top of the cell culture volume to reach a final concentration of 3.7% FA. This results in slightly better fixation results, allowing for faster processing and reducing cellular stress (as media is not removed from cells, cells are not exposed to oxygen, etc.).

9. Fixation time can be shortened to 15 minutes when incubating at 37°C, achieving similar results. The fixation protocol here is optimized for the described antibodies; depending on the desired antibody staining, the fixation time and reagent may be adjusted.

10. The incubation step with the secondary antibody mixture is quite robust and can be 1 hour at 37°C, 3 hours at room temperature, or overnight at 4°C but must be kept constant for reproducible results. Coverslips have to be maintained moist for the whole staining procedure.

Time Taken

Anticipated Results

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