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TITLE:

An In Vitro Model for Studying Tau Aggregation Using Lentiviral-Mediated Transduction of Human Neurons

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KEYWORDS:

tau, neuron, neural stem cells, Alzheimer's disease, frontotemporal dementia, neurodegeneration, lentivirus, tauopathy

SUMMARY:

This protocol details a procedure in which human neuronal cultures are transduced with lentiviral constructs coding for mutant human tau. Transduced cultures display tau aggregates and associated pathologies.

ABSTRACT:

Aberrant aggregation of the protein tau is pathogenically involved in a number of neurodegenerative diseases, including Alzheimer's disease (AD). Although mouse models of tauopathy have provided a valuable resource for investigating the neurotoxic mechanisms of aggregated tau, it is becoming increasingly apparent that, due to interspecies differences in neurophysiology, the mouse brain is unsuitable for modeling the human condition. Advances in cell culture methods have made human neuronal cultures accessible for experimental use in vitro and have aided in the development of neurotherapeutics. However, despite the adaptation of human neuronal cell cultures, in vitro models of human tauopathy are not yet widely available. This protocol describes a cellular model of tau aggregation in which human neurons are transduced with lentiviral-derived vectors that code for pathogenically mutated tau fused to a yellow fluorescent protein (YFP) reporter. Transduced cultures produce tau aggregates that stain positively for thioflavin and display markers of neurotoxicity, such as decreased axonal length and increased lysosomal volume. This procedure may be a useful and cost-effective model for studying human tauopathies.

INTRODUCTION:

Pathological aggregation of the microtubule-associated protein tau is a defining feature of many

neurodegenerative diseases, including AD, frontotemporal dementia (FTD), Pick's disease, and progressive supranuclear palsy (PSP)[1]. In a nondiseased state, tau binds to and stabilizes microtubule filaments in neuronal axons[2]. However, disease-associated hyperphosphorylation of tau promotes tau aggregation, dissociation from microtubules, and neuronal toxicity[3]. The toxic effects of aggregated tau may involve aberrant activation of cholinergic[4] and glutamatergic receptors[5] resulting in the dysregulation of intracellular calcium and, eventually, cell death. In animal models, the reduction of brain tau improves pathology in AD mice[6] and in mouse models of repetitive mild traumatic brain injury[7].

Mounting evidence demonstrates that the structure and binding affinity of mouse-derived tau are distinct from human-derived tau and that mouse tau is unsuitable for modeling human tauopathies[8]. However, human cell tauopathy models are not widely commercially available. The overall goal of this work is to describe an in vitro model of tau aggregation in which human neurons are transduced with lentiviral-derived vectors containing mutant human tau constructs[9]. Tau aggregate causing lentiviral constructs encodes for the tau repeat domain harboring P301L and V337M mutations fused to a YFP reporter (Tau-RDLM-YFP) while control constructs code for the wild-type (Wt) tau repeat domain fused to a YFP reporter (Tau-Wt-YFP). Neuronal cultures transduced using this method express approximately nine times more tau than nontransduced cultures. Although the amount of tau expression overexpressed is roughly equal between Tau-RDLM-YFP- and Tau-Wt-YFP-transduced cells, only neurons transduced with Tau-RDLM-YFP display aggregates. Cultures transduced with Tau-RDLM-YFP stain positively for thioflavin and display reductions in axonal length and synaptic density. Therefore, this cellular model may be a useful tool for studying tau aggregation in vitro.

PROTOCOL:

1. Preparation of media and reagents

1.1. Thaw the basement membrane matrix coating for culture plates at 4 °C (do not allow the basement membrane matrix to warm up or it will solidify). Make 1 mL aliquots and store them at -20 °C or -70 °C.

1.2. Reconstitute basic fibroblast growth factor (bFGF) in sterile phosphate-buffered saline (PBS) at 10 µg/mL and make 10 µL aliquots. Store them at 4 °C.

1.3. To a new, unopened, 500 mL bottle of DMEM/F12 with glutamine, add B27 (10 mL), N2 (5 mL), and penicillin-streptomycin (5 mL). Place 50 mL of this neural stem cell (NSC) media in a conical tube and add 10 µL of 10 µg/µL (2 µg/mL final) bFGF. Store the NSC (+)bFGF media at 4 °C.

1.4. To make (-)bFGF media, use the same recipe as described in step 1.3 but do not add bFGF. This media will be used to differentiate NSCs to neurons and to maintain neuronal cultures after differentiation.

2. Lentiviral constructs

NOTE: Before beginning work with lentiviral constructs, ensure that the lab has been approved to use biosafety level-2 (BSL-2) agents. Furthermore, BSL-2 culture hoods, personal protective equipment (PPE), and disposal methods must be used when working with lentiviral vectors.

2.1. Obtain tau constructs packaged into lentivirus from a preferred source (see Sanders et al.[10] for construct information).

3. Culturing human neural stem cells

NOTE: NSCs are typically seeded at 100,000–150,000 cells/cm^2 and most commercially available NSCs are sold as 1 x 10^6 cells/vial. This protocol has been optimized for 10 cm cell culture dishes (although other sizes of dishes may be used); therefore, if commercially available NSCs are being used, the NSCs may need to be expanded by first being cultured in six-well dishes in order to result in enough cells to seed 10 cm dishes. This protocol can alternatively be adapted for a variety of cell culture dish sizes (but it does not contain instructions for passaging NSCs as these protocols are available elsewhere[11,12]).

3.1. For the preparation of cell culture plates, remove one aliquot of frozen basement membrane matrix coating for cell culture plates and allow it to thaw at 4 °C (aliquots can be placed at 4 °C overnight the day before the cells are to be seeded). Add 385 µL of basement membrane matrix coating to 5 mL of DMEM/F12 media + penicillin-streptomycin.

NOTE: 5 mL of DMEM/F12 media + penicillin-streptomycin is enough to coat a single 10 cm dish (1 mL of this basement membrane matrix coating solution is sufficient to coat one well of a six-well dish). Alternatively, if the cells are to be fixed and immunostained, or stained for thioflavin, culture cells on glass coverslips in 24-well culture dishes.

3.1.1. Keep the media and the matrix coating cold before and while adding them to culture dishes. Add the basement membrane matrix coating to culture dishes and place the dishes in an incubator (at 37 °C) for 1 h. For optimal coating, do not incubate the basement membrane matrix for more than or less than 1 h. After 1 h, aspirate the basement membrane matrix coating from the cell cultures dishes. Make sure this coincides with the NSCs being ready to be plated.

NOTE: If cells are not being thawed from frozen stocks, skip step 3.2 and continue with step 3.3.

3.2. If cells are being thawed from frozen NSC stocks, take a vial of frozen cells and warm it in a water bath heated to 37 °C by moving the vial back and forth in the water. Once thawed, spray the vial with 70% ethanol and place it into a cell culture hood. Transfer the cells to ~10 mL of DMEM/F12 + penicillin-streptomycin media and centrifuge the tube at room temperature at 1,000 x *g* for 5 min. Aspirate the media and resuspend the cells in NSC media.

3.3. Dilute the cells in NSC media to obtain the appropriate seeding density for the cell culture vessel that is being used.

NOTE: For example, if NSCs are being plated onto a six-well plate—one well on a six-well culture dish has a surface area of 9 cm^2—900,000 to 1,350,000 cells are required to seed. So, one vial containing 1,000,000 cells can be resuspended in 2 mL of media and added to a single well of a six-well dish.

3.4. Add enough cells suspended in NSC media to seed the basement membrane matrix-coated culture dishes (100,000 cells/cm^2) and change the media every other day (if using a frozen stock, change the media the day after plating and every other day thereafter). Grow the cells until they are 75%–80% confluent.

NOTE: The cells will likely reach 75%–80% confluence shortly after plating, but this may take longer if frozen stocks are used.

3.5. Once the NSCs are 75%–80% confluent, begin neuronal differentiation by removing the NSC (+)bFGF media and replacing it with NSC (-)bFGF media. Culture the cells in this media (replacing the media every other day) for at least 4 weeks.

NOTE: The cells will continue to divide for a few days following the withdrawal of bFGF; therefore, cells may reach 90-100% confluence. After culturing for 4 weeks, the cells will have achieved a neuronal fate and will be ready for the treatment of lentivirus.

4 Transduction and maintenance of neuronal cultures

4.1. To transduce neurons with lentivirus, use a titer count of 3.4 x 10^5 transducible units/cell (a 100% confluent 10 cm dish will contain ~10 million neurons). Dilute the transducible units to the necessary concentration in cell culture media and add them to the cells (use the normal volume of media for the cell culture dish). Two days after adding the lentivirus, wash the cells 1x with fresh (-)bFGF media (without lentivirus) and continue culturing in (-)bFGF media as usual.

4.2. For the post-lentiviral treatment, feed the transduced neurons by changing the cell culture media ([-]bFGF media) every other day. Maintain the cells for ~8 weeks after transduction. Routinely visualize the cells under a light microscope to ensure viability.

NOTE: Sparseness or dendritic breakages are signs that the cells are no longer viable.

5. Imaging of cells

5.1. Live-cell imaging

5.1.1. After transduction (4 days after the addition of lentivirus), observe YFP signals under a fluorescent microscope capable of live-cell imaging. Take the cell culture dishes out of the

incubator and make sure the culture wells remain sterile by keeping the lid on top of the culture dishes. Visualize the cells using a 10x objective with an excitation wavelength of ~514 nm and an emission filter of ~527 nm.

NOTE: Aggregates are typically visible in transduced cell cultures 6–8 days after the application of lentivirus.

5.2 Fixed-cell staining (β-tubulin III labeling and thioflavin staining)

5.2.1. To fix the cells in paraformaldehyde (PFA), remove the culture media from transduced neurons grown on glass coverslips. Wash the cells 1x with PBS, remove the wash, and add 300–500 µL (if using 24-well plates) of 4% PFA (diluted in PBS) to the coverslips for 20 min at room temperature. Remove the PFA and wash the coverslips 2x with PBS.

5.2.2. Prepare a blocking solution consisting of PBS, 3% bovine serum albumin (BSA), and 0.3% Triton X-100. Add 300–500 µL of the solution to the wells and incubate the coverslips for 2 h at 4 °C. After 2 h, dilute primary antibodies (at an antibody concentration of 1:500) in blocking solution and add 300–500 µL of primary antibody solution to each well. Place the plate on a rocking or rotating platform (at low speed) overnight at 4 °C.

5.2.3. The next day, remove the primary antibody solution and wash the coverslips 3x for 5 min each with PBS. Dilute secondary antibodies in a blocking buffer solution (at a concentration of 1:1,000) and add secondary antibody solution to each well. Select the appropriate secondary antibody by using a fluorescent tag that does not overlap with the YFP signal (CY-3, for example). Incubate the cells at room temperature for 2 h on a rocking or rotating platform. After 2 h, remove the secondary antibody solution and wash the coverslips 3x for 10 min each with PBS.

5.2.4. Wash the coverslips in double-deionized water (DDW) for 10 min. Remove the DDW and add 300 µL/well of 0.015% thioflavin-S diluted in 50% ethanol for 10 min. Remove the thioflavin solution and wash the coverslips 2x for 4 min each with 50% ethanol, followed by one 4 min wash with 30% ethanol and two washes for 5 min each with 30% ethanol. Wash the coverslips 1x with DDW prior to mounting.

NOTE: The thioflavin staining described in step 5.2.4 is optional.

5.2.5. To mount the coverslips to glass slides, place a single drop of mounting media on the "+" side of a glass slide. Remove all liquid from the well containing the glass coverslip and, using fine forceps, carefully remove the glass coverslip, keeping track of which side has the cultured cells.

5.2.6. Gently press the edge of the coverslip against a Kimwipe to remove any excess moisture. Still gripping the coverslip with fine forceps, touch the edge (with the cell side facing toward the drop of mounting media) of the coverslip to the mounting media, and then, gently place the coverslip on the mounting media (placing the cultured cells into the mounting media and

sandwiching them between the coverslip and the glass slide). Allow the mounting media to harden for 30 min prior to imaging. The slides can be stored at -20 °C for future use.

5.2.7. Image the cells using a fluorescent microscope and the appropriate excitation/emission spectra (YFP = ~514/527 nm, CY-3 = ~555/568 nm, and thioflavin S = ~390/426 nm).

6. Optional methods

6.1. Every 2 days, collect conditioned media from the cultures and store it at -20 °C for future analyses of tau species released by cultures.

REPRESENTATIVE RESULTS:

Tau-RDLM-YFP-transduced neurons were fluorescently tagged with YFP, and RDLM-transduced cultures displayed aggregates after transduction. These inclusions stained positive for thioflavin (**Figure 1**). As **Figure 1** demonstrates, this protocol produces neuronal cultures that display thioflavin-positive tau aggregates. For initial experiments, it is recommended that neuronal differentiation is confirmed by immunolabeling the neuron-specific marker β-tubulin III in cultures. Importantly, fluorescently tagged secondary antibodies should have an excitation/emission spectrum that does not overlap with that of YPF (Cy3, for example). Although yellow fluorescent tau aggregates will be visible in the absence of staining, thioflavin should also be used for imaging in order to confirm that the fluorescent signal is aggregated tau and not cellular debris. Additionally, the example in **Figure 1** does not include DAPI staining to label the cell nuclei as the excitation/emission of DAPI overlaps with that of thioflavin-S; alternative nuclei stains should be used with thioflavin-S if desired.

FIGURE AND LEGEND:

Figure 1: Thioflavin staining of transduced cultures. Neurons transduced with tau-RDLM-YFP lentivirus were fixed and immunolabeled with the neuron-specific marker β-tubulin III (red). Additionally, tau aggregates (YFP signal in green) were stained for thioflavin (blue). The scale bars are 25 µm.

DISCUSSION:

This protocol describes the generation of an in vitro model of human tauopathy that exhibits silver-stain-positive aggregates and thioflavin-positive neurofibrillary tangles (NFTs). Moreover, transduced cells display tau-induced pathologies such as morphological defects, reduced synaptogenesis, and an increased lysosomal volume. The main advantage of this protocol is that it provides an accessible and cost-effective model of neuronal tauopathy, which can be used for drug screening studies, as well as for the analysis of tau toxicity. This model fills a material need in neurodegenerative research as human tauopathy cell lines are not yet widely available and the use of transgenic tau from mouse-derived neurons requires animal breeding and is limited due to differences in neuronal characteristics between species.

In addition to those listed above, there are a number of critical steps for the success of this

procedure. First, ensure that the correct viral titer is used because if the viral titer is too low, cultures will not form aggregates. Second, make sure that NSC cultures are properly maintained and do not exceed ~80% confluence. Overgrowth of NSCs will cause premature differentiation. Last, wait a full 4 weeks for neuronal cultures to differentiate after withdrawing the bFGF media from the NSCs. This duration is necessary to ensure maturation of the neurons.

Although the procedure described above is an efficient method for producing an in vitro tauopathy model, there are some limitations associated with this protocol. First, as described previously, this method produces NFTs in human induced pluripotent stem cell-derived neurons but not in rat embryonic (E18)-derived neurons. Even after using three times more virus to transduce rat neurons than was used for human neurons, rodent cell cultures remained negative for thioflavin staining, which suggests that this method cannot be adapted for rodent cultures. The differences between transduced mouse cells and human neurons may be due to the increased propensity of human tau toward aggregation[13]. The second limitation of this procedure is that although tau aggregates are formed in cultures, changes in tau phosphorylation between Tau-RDLM-transduced cells and Tau-Wt-YFP-transduced cultures were undetectable using protein PHF-1 (which detects tau phosphorylated at Ser 396/Ser 404) and CP13 antibodies (which detect tau phosphorylated at Ser 202). These findings suggest that tau aggregation in Tau-RDLM-YFP cultures due to P301L and V337M involves a mechanism that is independent of hyperphosphorylation, or the level of endogenous tau phosphorylation is under the detectable level by immunoblot. Because of the lack of differences in PHF-1 and CP13 immunoreactivity between Tau-RDLM-YFP and Tau-Wt-YFP cultures, it is unclear whether or not this model would be useful for studies analyzing the effects of tau kinase/phosphatase activity on pathology. However, both PHF-1 and CP13 antibodies recognize regions outside of the repeat domain harboring the mutations; therefore, additional antibodies raised against various tau phosphorylation sites may be useful for future studies.

In addition to cell culture assays, this model may be a valuable tool for studies beyond the cell culture paradigm. For instance, exosomes isolated from the media of transduced cells contain toxic tau species. Exosomes are small secretory vesicles released from nearly every cell type, and exosomes have been implicated in tau propagation[14]. Tau-RDLM neuronal exosomes contain human tau which is detectable by western blot[9], and these exosomes are sufficient to produce tau inclusions in the naïve mouse brain. These inclusions are immunoreactive for antibodies specific for human tau (K9JA), but it is unclear whether or not the inclusions include aggregated mouse tau. The finding described here that the procedure does not produce thioflavin-positive staining aggregates in rodent neurons suggests that the inclusions observed in the mouse brain are composed entirely of human tau, although future studies are required to confirm the composition of in vivo deposits.

Given the apparent role of exosome-derived tau in tauopathies, the model described here may be a useful resource for examining the role of the exosome in mediating tau-induced neurodegeneration. In conclusion, this procedure produces an in vitro model of human tauopathy that has significant advantages over transgenic mouse neuronal systems and can be employed for a variety of preclinical analyses.

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DISCLOSURES:

The authors have nothing to disclose.

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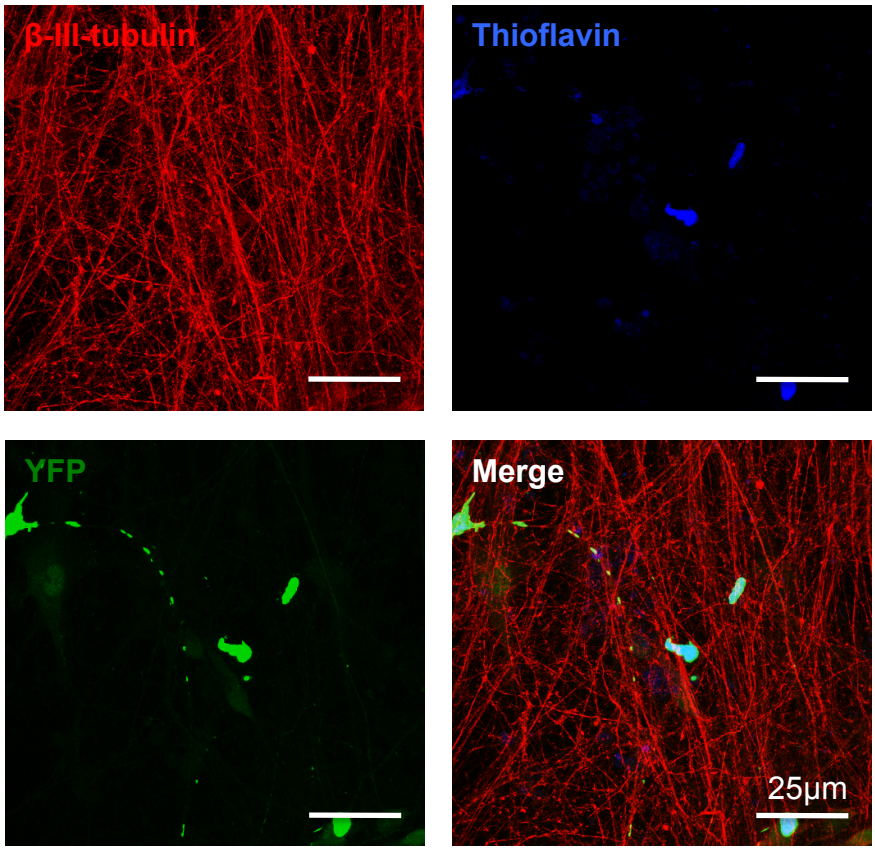
Figure 1



Name of Material/ Equipment

Description

10 cm culture dishes

15 ml tubes

16% paraformaldehyde

24 well culture plates

50ml tubes

70% ethanol in spray bottle

B27 supplement

Basement membrane matrix (Matrigel)

Basic FGF

Bovine serum albumin

Cell culture incubator

Centrifuge

DMEM-F12 culture media with glutamine

Ethanol (50% concentration or higher)

Flourescently labeled secondary antibodies

Fluorescent microscope

Glass coverslips

Glass slides

Human neural stem cells

Lentiviral vectors

Mounting media

N2 supplement

Penicillin-Streptomycin

Phosphate buffered saline

Primary antibodies

Rocking or rotating platform

Sterile cell culture hood

Thioflavin S

Triton X-100

Water bath

Company	**Catalog Number**
company	*catalogue number*
Thermofisher	12556002
Biopioneer	CNT-15
Thermofisher	50-980-487
Thermofisher	930186
Biopioneer	CNT-50
Various sources	NA
Thermofisher	17504044
Corning	356231
Biopioneer	HRP-0011
Sigma	A7906
Various sources	NA
Various sources	NA
Thermofisher	10565042
Various sources	NA
Various Sources, experiment dependent	NA
Various sources	NA
Thermofisher	1254581
Thermofisher	12-550-15
Various sources	NA
Various sources	custom order
Thermofisher	P36934
Thermofisher	17502048
Thermofisher	15140122
Thermofisher	14190250
Various Sources, experiment dependent	NA
Various sources	NA
Various sources	NA
Sigma	T1892-25G
Thermofisher	BP151-100
Various sources	NA

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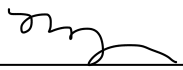
Signature:	
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Full journal titles for each reference have been added.

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There are 8 key words/phrases in the manuscript

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The manuscript has been revised so that there is no more than 1 note per step.

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Step one has been deleted and the Table of Materials revised.

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The bottle is 500 mL. This information has been added to the manuscript.

8. 3.1: Please write this step in the imperative tense.

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15. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc.

We have added discussion regarding the representative results to the manuscript.

“As figure 1 demonstrates, this protocol produces neuronal cultures that display thioflavin positive tau aggregates. For initial experiments, it is recommended that neuronal differentiation be confirmed by immunolabeling the neuron specific marker beta tubulin III in cultures. Importantly, fluorescently tagged secondary antibodies should have an excitation/emission spectra that does not overlap with that of YPF (Cy3 for example). Although yellow fluorescent tau aggregates will be visible in the absence of staining, thioflavin should also be used for imaging in order to confirm that the fluorescent signal is aggregated tau and not cellular debris. Additionally, the example in figure 1 does not include DAPI staining to label cell nuclei as the excitation/emission DAPI overlaps with that of Thioflavin S, alternative nuclei stains should be used with Thioflavin S if desired.”