Video Article

In Vitro Aggregation Assays Using Hyperphosphorylated Tau Protein

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Abstract

Alzheimer's disease is one of a large group of neurodegenerative disorders known as tauopathies that are manifested by the neuronal deposits of hyperphosphorylated tau protein in the form of neurofibrillary tangles (NFTs). The density of NFT correlates well with cognitive impairment and other neurodegenerative symptoms, thus prompting the endeavor of developing tau aggregation-based therapeutics. Thus far, however, tau aggregation assays use recombinant or synthetic tau that is devoid of the pathology-related phosphorylation marks. Here we describe two assays using recombinant, hyperphosphorylated tau as the subject. These assays can be scaled up for high-throughput screens for compounds that can modulate the kinetics or stability of hyperphosphorylated tau aggregates. Novel therapeutics for Alzheimer's disease and other tauopathies can potentially be discovered using hyperphosphorylated tau isoforms.

Video Link

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Introduction

Alzheimer's disease (AD) is one of a large collection of neurodegenerative disorders known as tauopathies. The quintessential pathology underlying tauopathy is the neurofibrillary tangles, NFTs, in neurons, astrocytes and microglia [1-4]. The NFT density correlates with cognitive impairment [3,5] and neuron loss [6]. NFT contains primarily hyperphosphorylated tau protein (referred to as "p-tau" henceforth) that forms straight or paired helical filaments (PHF) [7,8]. Tau is a microtubule associated protein thought to facilitate axonal transport that is essential for neuronal signaling and trafficking [9,10]. Each tau molecule contains 2 to 3 phosphates in normal brain, but the phosphoryl content increases by several folds in tauopathy patients [11]. Multiple kinases are likely to contribute to tau hyperphosphorylation including GSK3β (glycogen synthase kinase 3β) and CDK5 (cyclin-dependent kinase 5) [12,13], but the direct trigger for the pathological phosphorylation remains elusive [14]. Abnormal phosphorylation in or near the microtubule-binding motifs dissociates tau from the microtubule [15], and causes tau mis-localization to the somatodendritic compartment, where p-tau oligomerizes into straight or paired helical filaments that can eventually polymerize into NFT inclusions. The close tie between tau hyperphosphorylation, NFT formation, and neurodegeneration led to a prevalent hypothesis that p-tau tangles elicit apoptotic and other cytotoxic responses, and thus is the underlying cause for tauopathy neurodegeneration [16,17]. Drug screens and early clinical tests based on this premise have been launched [18]. However, this hypothesis faces challenges [19,20]. For example, SantaCruz *et al.* showed that cognitive functions of transgenic mice can be improved by suppressing the expression of a mutant human tau, even though NFTs continued to form from existing tau molecules [21]. In a *Drosophila* model, NFT was shown to sequester the toxic cytosolic tau to protect the underlying neuron cells [22,23]. Clearly, the pathogenesis role of NFT, if any, will greatly impact the direction of tauopathy therapeutics development.

In high concentrations, recombinant or normal brain tau protein spontaneously but slowly polymerizes into a PHF-like structure *in vitro*, as indicated by the binding of several β-sheet preferred fluorescent dyes, electron microscopy, and light scattering spectroscopy [24-27]. Adding heparin or arachidonic acid, an abundant fatty acid in human brain, drastically accelerates PHF formation in tau isoform- and inducer concentration-dependent manners [28-32]. Intriguingly, hyperphosphorylated tau purified from AD brains or prepared by exhaustive *in vitro* phosphorylation reactions aggregates faster and more efficiently [26,33-35]. These results are in excellent agreement with the pathological roles of p-tau. An *in vitro* system based on the aggregation of p-tau may thus serve as a powerful tool for AD drug screening.

Given the close association between tau aggregation and the progressive neurodegeneration of AD, as well as the recent failure in drug development targeting the Aβ plaque, another key histological marker of AD [36-38], the interest in discovering drugs that control tau aggregation is rising. Indeed, several groups have already begun drug screens at different throughput, using *in vitro* tau aggregation reactions as the primary assay. A number of chemicals were found to exhibit inhibitory or reversal activities on tau aggregation *in vitro* [39-42]. However, all current tau aggregation regulator screens use unmodified tau that misses the key pathological mark of phosphorylation, raising a concern for the specificity and efficacy of using these compounds in AD treatment.

One of the major hurdles of developing aggregation assays for biochemical characterization and AD drug screening is the production of sufficient quantities of the pathophysiologically relevant hyperphosphorylated tau protein. Using the Zippers-Assisted Catalysis system in which the 1N4R

isoform of tau and the GSK-3β kinase are co-expressed in *E. coli* as leucine zipper fusion proteins, we have overcome this challenge (Sui *et al.*, submitted; see **Figure 1** for the final products of tau and p-tau; also see [43] for preliminary mass spectrometry characterization of p-tau). From a panel of nine antibodies specific for different phosphorylation sites of tau, positive signals were seen in eight positions (data not shown). Below, we describe protocols and instrumentations that can differentiate the aggregation kinetic differences between unmodified tau and p-tau species. These assays were modified from published protocols that measured the increase of fluorescence of thioflavin T (ThT) or thioflavin S (ThS) upon amyloid (tau aggregates) binding [26]. In the first "terminal", no-dye approach, aggregation reactions are assembled and incubated in the absence of the amyloid dye. At different time points, an aliquot of each reaction is removed and mixed with equal volume of the ThT-containing buffer to stop aggregation and allow ThT to bind tau aggregates. Fluorescence is measured by an IAP FluoroMax-2 fluorometer. In the second "with-dye" continual monitoring assay, ThT or ThS is included in the aggregation reactions. Fluorescence can be measured continuously throughout the entire experiment manually or using a multi-plate reader. In addition, we describe an assay that uses a near-physiological concentration of tau and p-tau for aggregation in the continual measurement mode. The effect of phosphorylation remains readily detectable. Below, we will describe step-by-step operation procedures, and show representative results of these assays. Discussion of some of the pros and cons of each approach, as well as potential drug screening applications will follow.

At a high concentration, tau aggregates into amyloid-like structures spontaneously. However, in the laboratory, tau fibrillization is typically accelerated by such inducers as heparin (average molecular weight, 6,000 g/mol) and arachidonic acid. Examples shown herein include 30 μM heparin. The formation of tau amyloid aggregates is monitored by the fluorescence resulting from amyloid binding by thioflavin T (ThT) or thioflavin S (ThS). Upon binding to tau aggregates, ThT exhibits a red shift in fluorescence (excitation: 450 nm; peak emission: 485 nm). ThS, on the other hand, has weak emission at 510 nm (excitation at 450 nm) before amyloid binding, but this fluorescence increases significantly in the presence of an amyloid protein such as the aggregated tau [44]. Both dyes work well in detecting tau and p-tau aggregation. Because of the strong and relatively broad emission peak of ThT (see **Figure 2**), there is only 30% reduction in the fluorescence unit at 510 nm. For convenience, we use the same combination of excitation/emission wavelengths (*i.e.*, 450 nm/510 nm) to monitor tau aggregation when using either dye.

Tau aggregation can be done in the presence or absence of the dye, depending on the purpose of the assay and the availability of tau protein. Both modes of reactions are shown below. In addition, we demonstrate the operation of two different instruments – a single-sample fluorometer (ISA-SPEX FluoroMax-2) and a multi-plate reader (SpectraMax M2). Readers should be able to adapt these protocols to suit their specific needs and instrument availability.

Protocol

1. Preparation of Reagents

1. Prepare aggregation buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA). Store at RT, stable for months. Supplement 1 mM dithiothreitol (DTT) before use.
   NOTE: a HEPES-based buffer (10 mM HEPES, pH 7.5, 0.1 mM EDTA, 5 mM DTT) also produces similar results in tau aggregation.
2. Prepare thioflavin T or thioflavin S stock solution (3 mM, dissolved in aggregation buffer), and filter by 0.22 μm sterile filter unit. Store at –20 °C in a tube covered by aluminum foil, stable for months.
3. Prepare heparin stock solution (300 μM, dissolved in aggregation buffer). Store at –20 °C, stable for months.
4. Prepare dithiothreitol (DTT) stock (1 M, dissolved in water). Aliquot into 1.5 ml tubes. Store at –20 °C. Before aggregation assays, thaw the 1 M solution at RT. From this 1,000x stock, prepare an aliquot of 100 mM working stock with deionized water. Leave on ice until ready.
5. Remove tau from –80 °C freezer. Thaw on ice. Adjust tau to predetermined concentration with the aggregation buffer. Spin in a microcentrifuge at 20,800 x g for 10 min at 4 °C to remove pre-formed large aggregates. This pre-spinning step increases consistency in the subsequent fluorescence measurement of each batch of the protein prep. Transfer the supernatant to another tube; leave on ice until ready to assemble the aggregation reaction.

2. No-dye, Terminal Assay

NOTE: The aggregation reaction of this assay is done in the absence of the fluorescent dye. After mixing all components, the reaction is allowed to proceed to pre-determined time points. Aliquots are then taken out of the aggregation reaction and mixed with ThT or ThS for amyloid binding before fluorescence reading. The initial volume of the aggregation reaction depends on the number of time points needed. This approach may require a large amount of tau protein, but is fast, straightforward, and can be done in a fluorometer or a multi-well plate reader (see Discussion). Below is the step-by-step operation, using the ISA SPEX FluoroMax-2 compact spectrofluorometer for fluorescence quantification.

1. Set up the aggregation mixture in 1.5 ml Eppendorf tubes as in **Table 1**. Each column represents the ingredients needed for a 100 μl reaction, which is enough for one time-point measurement. Adjust the amount for the entire aggregation mix based on the time points needed for the particular experiment. Add additional 10% of each component to give room for pipetting error. The typical reaction containing heparin shown here can be replaced by arachidonic acid or aggregation buffer. Add DTT to 1 mM to the reaction mixture. If the entire reaction lasts more than one day, supplement fresh DTT everyday (1 mM) to ensure a reducing environment.
2. Invert the tube a few times to mix. Place each reaction in a 37 °C incubator or water bath. Agitation is not needed for tau aggregation.
3. Before measuring the fluorescence, turn on the spectrofluorometer (lamp first, then computer).
   NOTE: The xenon arc lamp that can be used right away. However, for best results allow the machine to warm up for about 10 min before reading fluorescence.
4. Start the software on the computer.
   1. Choose *Real Time Display* mode in *Instrument Control Center*, set excitation wavelength to 450 nm (slit to 2 nm) and emission wavelength to 510 nm (slit to 5 nm). Close *Real Time Display* mode window to return to the *Instrument Control Center*.
   2. Choose *Constant Wavelength Analysis*, press Add>> key in the upper frame to add wavelengths sets. Set acquisition parameters of *Standard Error* to 1 and *Maximum Trials* to 3, then click *Add*. Click *Go!* to open the *Data Display* window.

3. In the *Data Display* window, click *Start Acq* to open the *New Sample dialog box*. Choose “unknown” for *sample type*.

5. To every 100 µl aggregation mixture, add 98 µl aggregation buffer and 2 µl 3 mM thioflavin T. Pipet several times to mix.
6. Transfer the entire mixture to a cuvette (FCA3, exterior dimension, w x l x h = 12.5 mm x 12.5 mm x 45 mm). Place the cuvette in the sample holder in the sample-compartment and close the lid. Click *Run* to collect the fluorescence data. Record the data.
7. Remove the cuvette and decant the solution. Rinse the cuvette by distilled water 3 times. Dry by blowing air in and outside the cuvette.

3. With-dye, Continual Mode Assay on a SpectraMax M2 Plate Reader

NOTE: This assay differs from the previous one in that the fluorescent dye ThT or ThS is included in the aggregation reaction. This allows continuous measurement of the same set of reactions. Because of the repetitive use of the reaction, this method is better done with an automatic multi-well plate reader (as shown below the operation of SpectraMax M2). A regular fluorometer also works but the frequency of measurement of fast aggregation reactions is somewhat limited, due to the manual nature of the operation.

1. Set up the aggregation mix in a 96-well plate (96-well black solid plate, well-volume 360 µl, flat bottom) as in **Table 2**. Each column represents the ingredients needed for a 200 µl reaction, which is enough for one time-point measurement. Mix well by pipetting several times. Supplement fresh 1mM DTT every day throughout the course of experiments.
2. Incubate the 96-well plate at 37 °C.
3. At each time point before fluorescence measurement, turn on the multi-mode microplate reader and the computer. Allow sufficient time for the machine to stabilize, about 10 min.
4. Start the software on the computer. Set temperature to 37 °C and choose Fluorescence Intensity (FI-Top Read) mode, set excitation wavelength at 450 nm and emission wavelength at 510 nm.
5. Insert the 96-well plate into the drawer and press the READ key to start measurement.
6. After reading, remove the plate and return it back to the 37 °C incubator. Copy the data and paste into an Excel spreadsheet for data analysis and plotting.

4. With-dye, Continual Mode Assay on an Compact Spectrofluorometer

1. Set up the aggregation mixture in 1.5 ml Eppendorf tubes as in **Table 3**. Each column represents the ingredients needed for a 200 µl reaction, which is enough for one time-point measurement.
2. Invert the tube a few times to mix.
3. Turn on the spectrofluorometer and set the software as in steps 2.3 and 2.4.
4. Transfer the entire mixture to a cuvette. Place the cuvette in the sample holder in the sample-compartment and close the lid. Click *Run* to collect the fluorescence data. Record the data.
5. Continue reading at appropriate intervals by clicking *Run* and recording the data. If the aggregation is to be monitored at a high frequency (*e.g.*, every 30 or 60 sec), leave the reaction in the cuvette and in the machine until either the measurement is finished, or when there is sufficient time to swap reactions or cuvettes.
6. Remove the cuvette and decant the solution. Rinse the cuvette by distilled water 3 times. Dry by blowing air in and outside the cuvette.

Representative Results

Using recombinant tau and p-tau (**Figure 1**), we established two different protocols to compare the kinetics of aggregation of tau and p-tau, taking advantage of the strong fluorescence emission of ThT and ThS upon binding to amyloidal protein aggregates, including tau and p-tau (**Figure 2**). With or without the fluorescent dye in the aggregation reaction, we observed consistent enhancement of tau aggregation by hyperphosphorylation (**Figures 3–5**). This stimulation is independent of heparin (data not shown). In a typical reaction, tau and p-tau oligomerize at fast rates within the first 30 min before slowing down significantly (**Figures 3 and 5**), with p-tau exhibiting higher fluorescence units throughout the course of the experiment. Including ThT in the aggregation reactions causes significant retardation in the rate of aggregation (**Figure 4**). Both isoforms approached plateau 160 hr after the reactions had started. ThS, on the other hand, does not cause appreciable slowdown of aggregation (**Figure 5**).

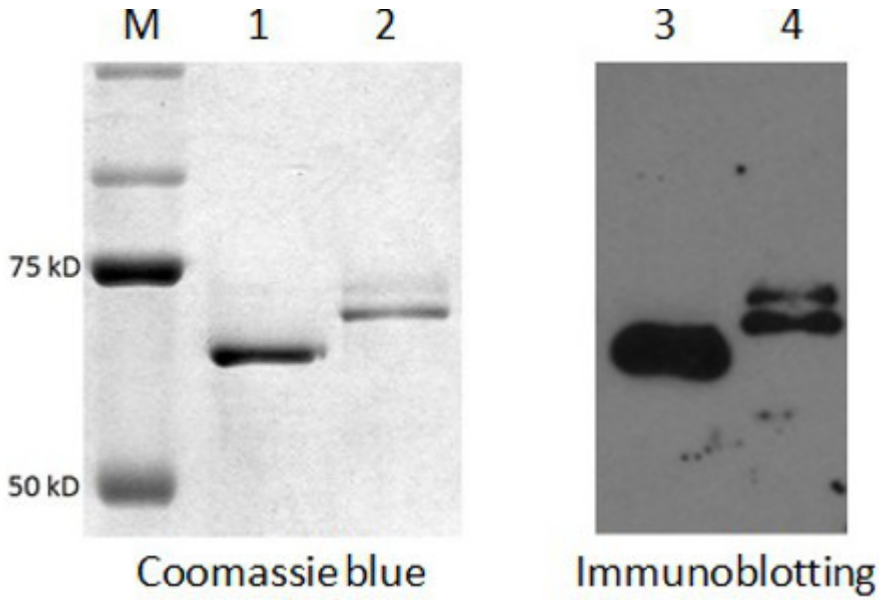

Figure 1. Purified tau and hyperphosphorylated tau (p-tau) used in this study. Samples were resolved by a 10% SDS-PAGE gel, and stained by Coomassie blue R250 (left) or probed by an anti-tau monoclonal antibody (right panel). Lane M, molecular weight marker; lanes 1 and 3, unphosphorylated tau; lane2 and 4, hyperphosphorylated tau.

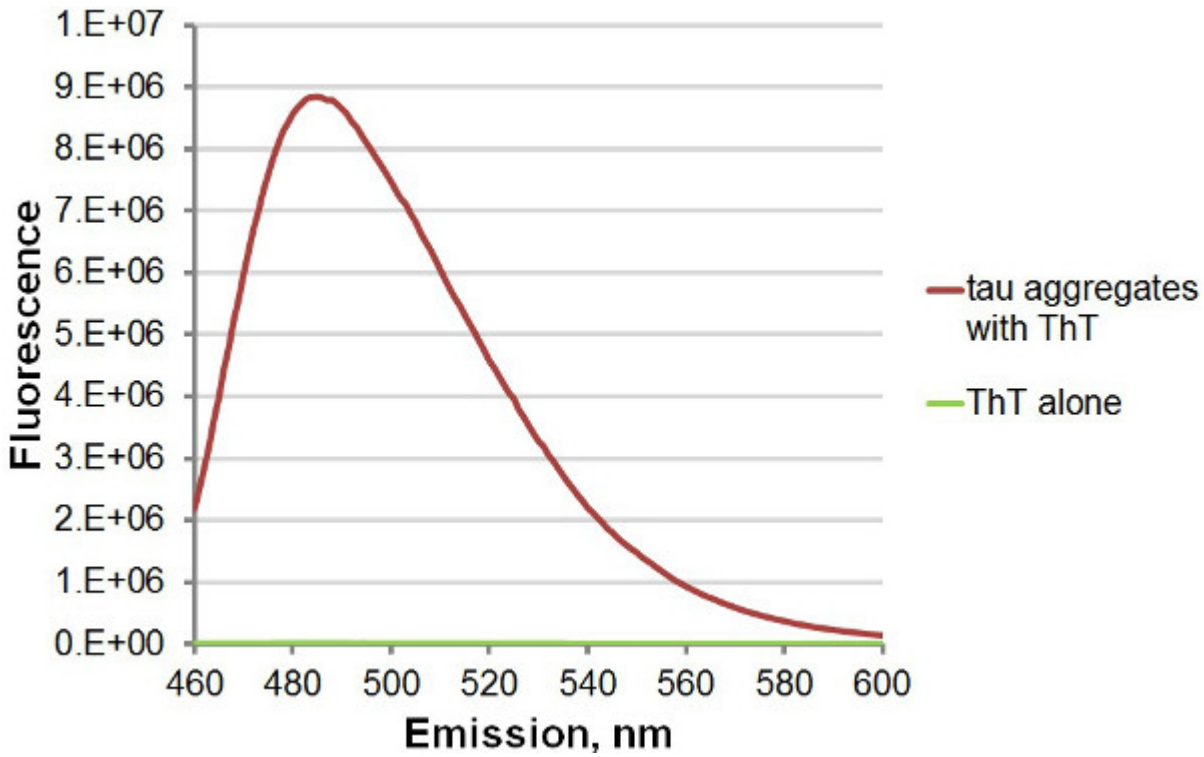

Figure 2. Emission spectra for ThT (30 µM) with or without binding tau aggregates. Emission acquisition was scanned from 460 nm to 600 nm (1 nm increment; 0.1 sec integration, 5 nm slit width), excitation at 450 nm. Tau aggregates were obtained by allowing 50 µM tau aggregation to proceed at 37 °C O/N (see **Protocol 2** for details).

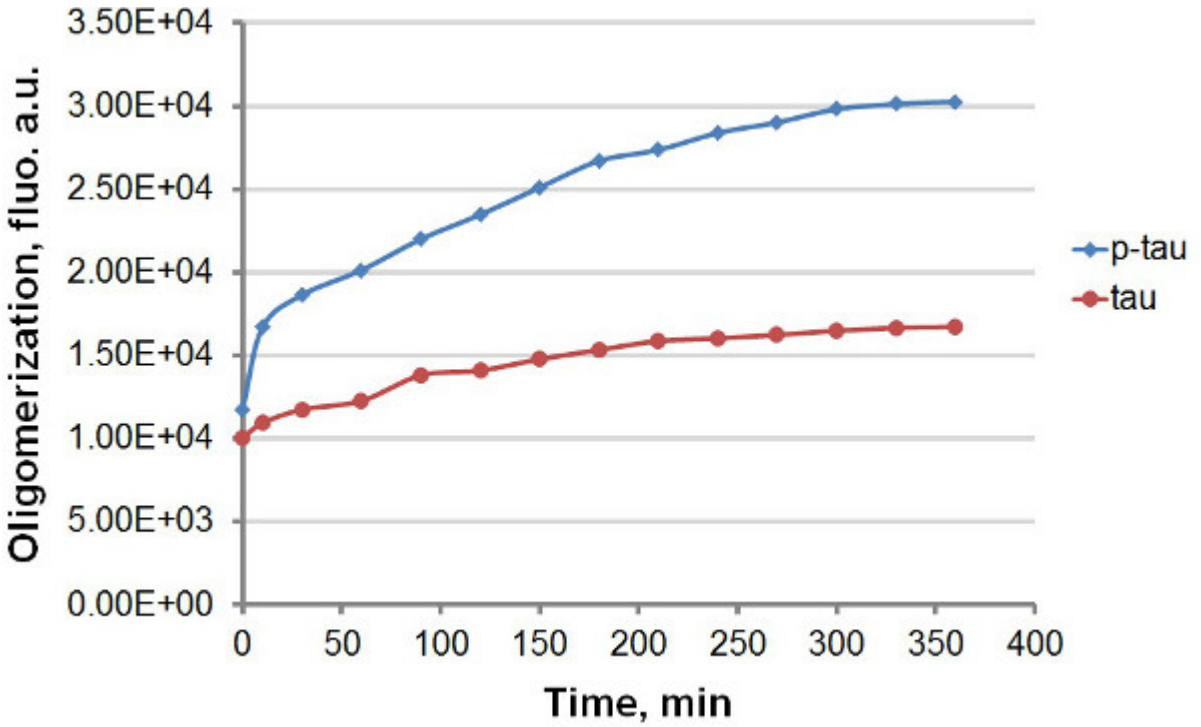

Figure 3. Aggregation curves for tau and p-tau in terminal assay. Aggregation of 50 µM tau and p-tau was completed with 30 µM heparin as the inducer. At different times after the start of the reaction, 100 µl of the reaction was removed and mixed with same volume of 60 µM ThT before fluorescence measurement. Fluorescence was measured at 450 nm excitation, 510 nm emission. “a.u.”, arbitrary units. Note that the time scale is in minutes.

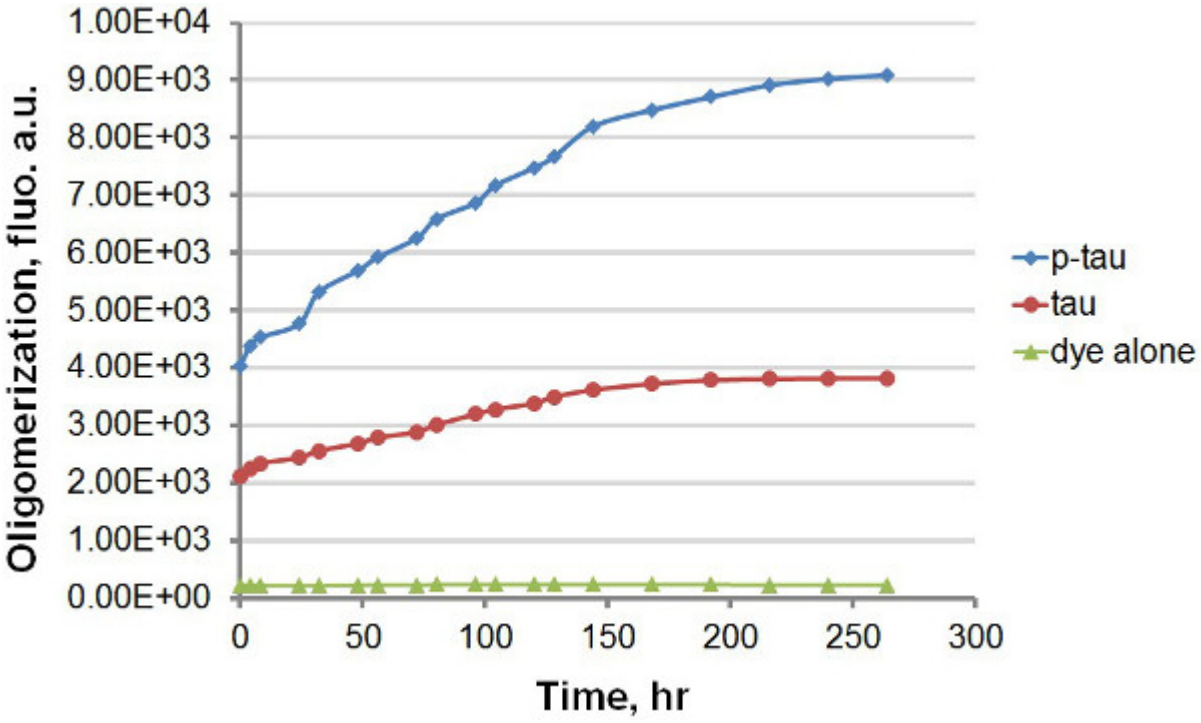

Figure 4. Aggregation curves for ThT alone, tau and p-tau in the continual measurement mode in the presence of ThT. Each reaction consisted of 0 or 50 µM tau or p-tau, 30 µM heparin, and 30 µM ThT in aggregation buffer. Reactions were incubated at 37 °C in a 96-well plate. At different time points, the plate was removed from the incubator and loaded to the plate reader for fluorescence reading (excitation 450 nm, emission 510 nm). Between readings, the plate was kept in the incubator without agitation under a cover. The presence of ThT significantly

slowed down aggregation, but, importantly, the hyperphosphorylated tau still exhibited a faster rate of aggregation than did its unmodified counterpart. Note that the scale of time is in hours.

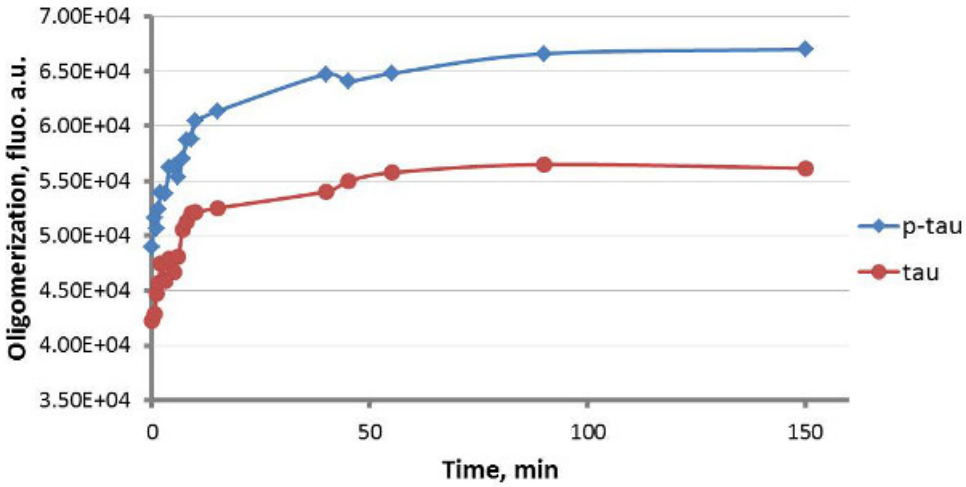

Figure 5. Small-scale tau aggregation assays in the presence of ThS. *In vitro* heparin-induced aggregation of 6 µM tau and p-tau was assessed in the continual measurement mode with thioflavin S as the indicator dye. In addition to the protein, each reaction contained 30 µM heparin and 20 µM ThS in the HEPES aggregation buffer (10 mM HEPES pH 7.5, 5 mM DTT, 0.1 mM EDTA). All ingredients except heparin were mixed and equilibrated at RT. After adding heparin, the reaction was transferred to the cuvette and placed in the sample holder. Fluorescence was recorded immediately as T_0, and continued for about 2 hr or until the fluorescence increase slowed down to near zero. Because of the relatively short reaction period, the entire reaction was carried out at RT in the same cuvette.

	tau	p-tau
60 - 100 µM tau	50 µl	0 µl
60 - 100 µM p-tau	0 µl	50 µl
300 µM heparin	10 µl	10 µl
aggregation buffer	39 µl	39 µl
100 mM DTT	1 µl	1 µl

Table 1. Aggregation mixture components for no-dye, terminal assay.

	tau	p-tau	Dye alone
60 - 100 µM tau	50 µl	0 µl	0 µl
60 - 100 µM p-tau	0 µl	50 µl	0 µl
300 µM heparin	20 µl	20 µl	20 µl
3 mM thioflavin T	2 µl	2 µl	2 µl
Aggregation buffer	126 µl	126 µl	176 µl
100 mM DTT	2 µl	2 µl	2 µl

Table 2. Aggregation mixture components for with-dye, continual assay on a plate reader.

	tau	p-tau	Dye alone
60 - 100 µM tau	5 µl	0 µl	0 µl
60 - 100 µM p-tau	0 µl	5 µl	0 µl
300 µM heparin	20 µl	20 µl	20 µl
3 mM thioflavin S	1.5 µl	1.5 µl	1.5 µl
Aggregation buffer	171.5 µl	171.5 µl	176.5 µl
100 mM DTT	2 µl	2 µl	2 µl

Table 3. Aggregation mixture components for with-dye, continual assay on a compact spectrofluorometer.

Discussion

This protocol demonstrates different assay conditions and instruments that detect the phosphorylation-dependent fast tau aggregation kinetics. In the terminal assay, the fluorescence dye ThT is added to a portion of the reaction removed from the master mix at each time point. Amyloid binding-induced fluorescence is then measured [26]. In the second, with-dye mode, tau aggregation is carried out in the presence of ThT or ThS, rendering this type of reaction suitable for real-time automatic assessment of the growth of tau aggregates. Each of these methods has its pros and cons.

The terminal-mode reaction is conducted with only those ingredients needed for tau aggregation. Diluting and mixing the reaction with thioflavin T drastically slows down the rate of fluorescence increase, essentially stops the reaction for fluorescence quantification. This method thus is also compatible with manual operation. However, because the reaction is practically terminated upon ThT addition, a large amount of tau may be required for plotting an aggregation curve. Another potential caveat for this method is that frequent access to the reaction mixture may introduce microbial or proteolytic contamination, or protein oxidation. In contrast, the with-dye mode allows the generation of amyloid in the presence of ThT or ThS. The advancement of aggregation can be monitored continuously without ever disturbing the reaction. This feature is particularly attractive when establishing an automated assay platform. However, different dyes may elicit specific responses. Indeed, ThT retards tau and p-tau aggregation significantly, but ThS has little effects (compare **Figures 3 and 5**). There are several other fluorescence dyes, including Congo red and thiazines, which have been used in histological and cell biology studies for PHF formation. At least one report stated that some of these dyes may induce tau aggregation in tissue culture cells [45]. Therefore, when choosing these compounds for amyloidogenesis kinetics studies, caution shall be practiced and that different dyes may have to be compared.

With respect to the choice of instrument, the single-sample fluorometer used in the first approach is highly reliable, but the operation may be laborious when more than a few reactions are to be compared. Using multiple cuvettes can help avoid cross-contamination between reactions, although the cost of these fragile quartz cuvettes may be prohibitive to some. Conversely, the multi-well microplate reader can examine multiple reactions at the same time. The use of disposable 96-well plates is advantageous as well. With a heating element, a microplate reader can be a dedicated device for monitoring multiple reactions at 37 °C for an extended period of time. However, evaporation might be a concern. DiNitto *et al.* overlaid a similar reaction with mineral oil to prevent evaporation[46].

Some precautions are to be taken for the above protocols to ensure consistent and quantitative results. Firstly, tau and p-tau spontaneously form amyloid aggregates over time, particularly when in a high concentration. It is thus imperative to freeze all the aliquots of protein preps, and thaw only the needed volume before the experiments. Even so, some aggregates, including the granular intermediates detectable by ThT [47] might have formed during the preparation of recombinant proteins. A substantial initial fluorescence reading of a typical aggregation reaction is thus common. Nonetheless, adding a pre-spinning step and transferring the supernatant to a separate tube, even without a visible protein pellet, can reduce, and maintain consistent, initial fluorescence of the same batch of tau and p-tau prep. Secondly, the working stock solution of ThT (*i.e.*, 60 µM) is stable at RT for less than a week before the fluorescence diminishes. It is thus advised to re-make the 60 µM ThT every several days.

One prominent reason underlying studies of p-tau aggregation is the development of novel AD diagnostics and therapeutics. Compounds that inhibit or revert recombinant tau aggregation have been identified from high-throughput screens and targeted tests [18,40,41,48]. Efficacy of these compounds for p-tau aggregation remains to be elucidated. These screens were carried out in the terminal mode by dispensing a common aggregation mix without the dye to individual multiplate wells with different compounds. Typically after O/N incubation, ThT or ThS is added for fluorescence measurement, revealing the inhibitory power of many compounds. The with-dye approach mentioned above and by Rankin *et al.*[49] has yet to be incorporated to high-throughput screens. Now with hyperphosphorylated tau available for kinetic and pharmaceutical studies of paired helical filament formation, Alzheimer's disease drug discovery is likely to advance farther.

Finally, it is worth noting that the study of p-tau aggregation is of critical importance to not only tauopathies, but can also impact an even wider population. For example, there are reports that the neurofibrillary tangles are detectable in some patients of chronic traumatic encephalopathy, such as professional American football players and boxers [50-52]. Similar correlation has also been reported for single or repetitive traumatic brain injury patients including soldiers [53]. Protocols described in this work may thus help the discovery and development of new therapeutics targeting p-tau aggregates in neuronal cells.

Disclosures

We have nothing to disclose.

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