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TITLE:

In Vitro Assay for Studying the Aggregation of Tau Protein and Drug Screening

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KEYWORDS:

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SHORT ABSTRACT:

The tau aggregation assay described in this manuscript mimics the anticipated features of *in vivo* tau misfolding and aggregation.

LONG ABSTRACT:

Aggregation of tau protein and formation of paired helical filaments is a hallmark of Alzheimer's disease and other tauopathies. Compared to other proteins associated with neurodegenerative diseases, the reported *in vitro* aggregation kinetics for tau protein are less consistent presenting a relatively high variability. Here we describe the development of an *in vitro* aggregation assay that mimics the expected steps associated with tau misfolding and aggregation *in vivo*. The assay uses the longest tau isoform (huTau441) which contains both N-terminal acidic inserts as well as four microtubule binding domains (MBD). The *in vitro* aggregation is triggered by addition of heparin and followed continuously by thioflavin T fluorescence in a 96 well microplate format. The tau aggregation assay is highly reproducible between different wells, experimental runs and batches of the protein. The aggregation leads to tau PHF-like morphology which is very efficient in seeding the formation of *de novo* fibrillar structures. In addition to its application in studying the mechanism of tau misfolding and aggregation, the current assay is a robust tool for screening drugs that could interfere with the pathogenesis of tau.

INTRODUCTION:

Alzheimer's disease is a devastating neurodegenerative disorder that is histopathologically defined by the accumulation of extracellular senile plaques of aggregated Amyloid beta[1] and intracellular neurofibrillary tangles containing aggregated hyperphosphorylated tau protein[2].

Physiological tau is monomeric and presented as six unique isoforms containing 0-2 N terminal inserts and 3 or 4 microtubule binding domains[3,4] arising from alternative splicing and an average of 2-3 phosphorylations. It is believed that hyperphosphorylation, misfolding and self-aggregation into fibrillary structures constitute the key elements in tau pathogenesis, as pathologically assessed in demented individuals[5,6].

The aggregated neurofibrillary tau tangles are a hallmark not only for AD but also for other tauopathies, including frontotemporal lobar degeneration (FTLD), Pick's disease, progressive supranuclear palsy (PSP), fronto-temporal dementia (FTD) and primary age-related tauopathy (PART)[2]. From a biochemical point of view, understanding the mechanism of tau misfolding and aggregation could shed light on the pathological processes associated with AD and other tauopathies. In addition to the scientific aspect, robust *in vitro* aggregation assays are valuable tools for screening of drug candidates[7-10]. It is believed that the aggregation of tau follows a nucleation dependent polymerization process (NDP)[11-14]. The NDP kinetics is sigmoidal and starts with an energetically unfavorable nucleation step followed by a fast energetically downhill aggregation process.

Unlike other amyloidogenic proteins, including the prion protein, amyloid beta and α-synuclein, tau does not spontaneously aggregate under physiological conditions and even extreme pHs or high temperatures are non-conducive for aggregation[15]. This is most probably due to the hydrophylic interactions present in the tau aggregation interface. However, tau aggregates efficiently at physiological concentrations when inducers such as heparin[16] or other polyanions[17,18] are being used.

Previous efforts to set up *in vitro* tau aggregation assays have shed some light onto the details of tau misfolding and aggregation, but they came short of mimicking what is believed to be the *in vivo* tau aggregation kinetics. In most cases, the tau aggregation kinetics was lacking the initial lag phase associated with tau nucleation. This might have been the consequence of using very high tau protein concentrations, presence of aggregates in the starting tau protein preparations and/or use of tau fragments with much higher aggregation propensity than the more physiological full length tau protein[19-23]. Furthermore, previous studies did not address the reproducibility and robustness aspect of tau aggregation kinetics.

Here, we describe a robust *in vitro* tau aggregation assay which mimics the main characteristics of a nucleation dependent polymerization with an initial lag phase corresponding to the tau nucleation followed by an exponential growth phase. Furthermore, the generated recombinant tau aggregates are fibrillar in nature and have an extremely high seeding potency. The assay is highly reproducible also between tau batches and represents a valuable tool to screen for aggregation inhibitors.

PROTOCOL:

1. Reagent Preparation

1.1 **Reaction buffer**

1.1.1 Prepare reaction buffer: 0.5 mM TCEP in PBS, pH 6.7 by dissolving TCEP dry powder (MW= 286.65 g/mol) in PBS stock solution, pH 7.4.

Note: The presence of TCEP is due to bridging aggregation studies using wild type tau protein which contains cysteines. In the current protocol, TCEP is only used to adjust the pH of PBS from 7.4 to 6.7 and plays no role in regulating any redox reactions.

1.1.2 Mix thoroughly and filter the solution through a sterile 0.22 µm pore size PES membrane filter.

1.1.3 Aliquot and store at -80 ˚C.

1.1.4 Thaw on the bench and stabilize at RT before use.

1.2 huTau441

1.2.1 Remove huTau441 (for protein expression and purification see Apetri *et al.*[24]) from -80 ˚C freezer.

1.2.2 Thaw on the bench and equilibrate to room temperature (RT).

1.2.3 Spin tube with protein stock for 5 min at 12,000 x g at 20-25 ˚C to eliminate air bubbles.

1.2.4 Measure the concentration of huTau441by absorption at 280 nm using an extinction coefficient of 0.31 mL $mg^{-1}cm^{-1}$

1.3 Thioflavin T

1.3.1 Prepare 500 µM thioflavin T (ThT) stock solution by dissolving 10 mg of ThT dry powder (MW=318.86 g/mol) in 35 mL reaction buffer.

1.3.2 Mix thoroughly, vortex 3 times for 20 seconds at maximum speed and filter the solution through a sterile 0.22 µm pore size PES membrane filter.

1.3.3 Determine concentration by absorption measurements at 411 nm using an extinction coefficient of 22,000 M^{-1} cm^{-1} and adjust ThT concentration to 500 µM. Store at RT protected from light. Prepare fresh every 2 months.

1.4 Heparin

1.4.1 Prepare fresh 55 µM heparin solution by dissolving 1 mg of HMW heparin dry powder (MW = 17-19 kDa) in 1 mL reaction buffer at RT.

1.4.2 Shake vigorously and vortex 2 times for 5 seconds.

1.4.3 Filter solution through a sterile 0.20 µm pore size PES membrane filter (syringe).

2. Continuous Mode ThT Aggregation Assay on a Multi-Mode Microplate Reader

Note: ThT dye is added to the reaction to monitor huTau441 aggregation kinetics in a continuous mode (automatic measurements). Although the reaction can be followed by a regular fluorometer using a conventional cuvette, the manual nature of the operation limits the frequency of measurements and compromises the accuracy of the recorded kinetic curves. For this reason, an automatic multi-mode microplate reader is used.

2.1 Instrument set up

2.1.1 Turn on the computer and the multi-mode microplate reader. Let the equipment stabilize for 10 minutes.

2.1.2 Start the software and prepare a protocol.

2.1.2.1 Select the protocol type: standard protocol (Data reduction is performed independently for each plate).

2.1.2.2 Set the temperature at 37 ˚C and select preheatment before continuing the protocol.

2.1.2.3 Set the kinetic run: Run Time 50 h / Measurement interval: 15 min.

2.1.2.4 Set orbital shaking at 425 cpm (3 mm) in continuous mode.

2.1.2.5 Select the read method: Fluorescence intensity – Endpoint/Kinetic – Monochromators Wavelengths: Excitation 440 nm (20 nm bandwidth)/ Emission 485 nm (20 nm bandwidth) – Optics position: Top – Normal read speed – Read height: 4.50 mm

2.1.2.6 Start the run using the created protocol. Name the experiment, select the destination of the newly created file and allow the instrument to pre-equilibrate to desired temperature.

2.2 Spontaneous huTau441 conversion

2.2.1 Prepare the reaction sample in a 1.5 mL tube. Use 200 µL mix per reaction and at least 4 replicates (reaction volume for 4 replicates = 800 µL).

2.2.2 Prepare 800 µL reaction sample (in case of 4 replicates) containing 15 µM huTau441, 8 µM heparin and 50 µM ThT. Start by mixing the protein with the reaction buffer, add heparin

and ThT and mix well by pipetting up and down 5 times. Respect the indicated order for reagent addition.

2.2.3 Spin samples at 12000 x g and 25 ˚C for 5 min to eliminate air bubbles.

2.2.3 Dispense 200 µL of reaction sample per well in 96-well microplates (96-well black solid microplate, well-volume 360 µL, flat bottom). Avoid formation of air bubbles.

2.2.4 Seal microplate to avoid evaporation.

2.2.5 Place microplate in multi-mode microplate reader and start measurements.

2.2.6 After completion of experiment, remove plate from equipment and export data to a data processing software.

2.3 Quality check of the conversion, seed collection and storage.

2.3.1 Remove the sealer from the plate and pool the different replicates in 1.5 mL tubes. Mix well the aggregated sample in the wells by pipetting up and down 2 times before collecting it. Aggregates tend to deposit on the bottom of the well.

2.3.2 Mix thoroughly in the 1.5 mL tube by pipetting up and down 5 times and dispense 10-20 µL on a mica surface for analyzing the aggregates by AFM (for further details see Apetri *et al.*[24]).

2.3.3 Harvest the aggregates by spinning the 1.5 mL tube at 20,000 x g and 4 ˚C for 1 hour. Aggregates form a pellet.

2.3.4 Separate and analyze supernatant by S-MALS to confirm the absence of monomeric tau in the sample indicating a successful conversion into aggregates (for further details see Apetri *et al.*[24]).

2.3.5 Label the 1.5 mL tube containing the remaining aggregates (pellet)indicating initial huTau441 protein concentration and sample volume. Snap freeze the aggregates and store at -80 ˚C.

2.4 Seeded reaction

2.4.1 Remove huTau441 aggregates from -80 ˚C freezer. Add the volume of reaction buffer indicated on the label (initial sample volume) and let the tube stabilize to RT. Resuspend the aggregates by pipetting up and down 5 to 8 times.

2.4.2 Sonicate the aggregated sample. For a 200 µL sample (15 µM huTau441), sonicate on ice using a 1/8" microtip (from 100 µL up to 10 mL) for a total period of 15 s using pulses of 1 s and pauses of 2 s at 30% amplitude (sonicator 250 Watts). Re-equilibrate sample to RT.

Note: The employed sonication conditions lead to a homogeneous population of tau fibrils with lengths of 20-50 nm[24].

2.4.3 Prepare the reaction sample in 1.5 mL tubes. Use 200 µL mix per reaction and at least 4 replicates (reaction volume for 4 replicates = 800 µL).

2.4.4 Prepare 800 µL reaction sample containing 15 µM huTau441, 8 µM heparin and 50 µM ThT. Start by mixing the protein with the reaction buffer, add heparin and ThT and mix well by pipetting up and down 5 times. Respect the indicated order for reagent addition.

2.4.5 Spin samples at 12000 x g and 25 ˚C for 5 min to eliminate air bubbles.

2.4.6 Dispense 200 µL of reaction sample per well in 96-well (96-well black solid microplate, well-volume 360 µL, flat bottom). Avoid formation of air bubbles.

2.4.7 Homogenize thoroughly the preformed fibril sample by repetitive up and down pipetting (5 times) and add to each well the amount corresponding to the desired percentage of seeds. For a 200 µL well total volume, 2 µL of preformed seed addition is a 1% (v/v). Mix by pipetting up and down 3 times when adding to the well and avoid formation of air bubbles.

2.4.8 Seal microplate to avoid evaporation.

2.4.9 Place microplate in multi-mode microplate reader and start measurements.

2.4.10 Remove plate from equipment and export data to a spreadsheet.

REPRESENTATIVE RESULTS:

Recombinant huTau441 containing the C291A and C322A mutations and N-terminal His and C-terminal C-tags was expressed and purified as previously described[24]. The huTau441 batches are highly pure as visualized on SDS-PAGE and virtually 100% monomeric as assessed by S-MALS (**Figure 1**). The aggregation of 15 µM huTau441 was induced by the addition of 8 µM HMW heparin and the reaction was followed continuously by ThT fluorescence using a multimode microplate reader. The excitation wavelength was 440 nm (bandwidth 20 nm) whereas emission was measured at 485 nm (bandwidth 20 nm). The assay is highly reproducible, with results from 10 individual wells being virtually indistinguishable (**Figure 2A**). The morphologies of the ThT positive huTau441 aggregates were assessed after 50 h by AFM. Aggregated hutau441 is a homogeneous mixture of fibrillar structures of different lengths similar to reported *ex vivo* morphologies (**Figure 2B**). Furthermore, the final reaction mixture does not contain monomer, suggesting a full conversion into aggregates as shown by S-MALS measurements (**Figure 2C**). The kinetics of huTau441 aggregation in independent experimental runs are very similar as emphasized by similar sigmoidal curves and indistinguishable lag and growth phases (**Figure 3**). The high level of reproducibility is maintained when different batches of protein are used (**Figure 4**). Furthermore, preformed huTau441 aggregates are very efficient

in recruiting tau monomer and inducing formation of *de novo* tau aggregates. Amounts as low as 0.0025% (v/v) of preformed tau aggregates are capable to bypass nucleation and trigger generation of *de novo* fibrils (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1: Recombinant huTau441 is monomeric and highly pure. A) Purity assessment under denaturing conditions on a 4-12% SDS-PAGE gel including molecular weight standard protein ladder (in kDA) (Lane 1); flow through from the final C-tag affinity purification step (Lane 2); column wash fraction (Lane 3), eluted huTau441 protein peak, before (Lane 4) and after buffer exchange (Lane 5), respectively). B) S-MALS analysis of the huTau441. Protein shows to be > 99.9% monomeric with a molar mass of 51 kDa and does not contain aggregates or fragments.

Figure 2: Aggregation of recombinant huTau441 is highly reproducible and leads quantitatively to fibrillar structures. A) Kinetics of heparin induced huTau441 aggregation monitored continuously in 96 well microplate format by ThT fluorescence. Concentrations of huTau441 and heparin are 15 μM and 8 μM, respectively. The 10 individual curves correspond to the conversion of the same protein batch in ten wells of the same microplate and are characterized by an average (with SD) lag phase of 14.2 ± 0.38 h and t_{50} of 18.8 ± 0.40 h. Kinetic data was fitted with a 5-parameter logistic curve with a linear decline in the upper asymptote. Lag phase and t_{50} were calculated by interpolation between predicted values by linear regression. Lag phase is calculated based on the 3% increase of ThT fluorescence signal. Curve fitting and summary statistics are obtained using IBM SPSS statistics version 20.0.0.2. B). Tau aggregates show PHF-like morphologies as indicated by AFM imaging at 50 h; C). S-MALS analysis of monomeric huTau441 (t = 0 h) and the supernatant of the reaction mixture at the completion of the reaction (t = 50 h). At the 50 h time point the reaction mixture was centrifuged for 1h at 20,000 X g and 4 ˚C and the resulted supernatant injected on S-MALS. The disappearance of the monomer peak confirms the complete aggregation of tau.

Figure 3: The tau aggregation assay shows high reproducibility between independent experimental runs. The two panels display four individual kinetics traces for spontaneous huTau441 aggregation collected in two independent experiments using the same batch of huTau441 protein. Concentrations of huTau441 and heparin are 15 μM and 8 μM, respectively. Kinetics are characterized by an average (with SD) lag phase of 12.2 ± 0.18 h and t_{50} of 17.8 ± 0.8 h (Run 1) and 11.6 ± 0,52 h and t_{50} of 17.8 ± 0.23 h (Run 2), respectively. Kinetic data was fitted with a 5-parameter logistic curve with a linear decline in the upper asymptote. T_{50} were calculated by interpolation between predicted values by linear regression. Lag phase is calculated based on the 3% increase of ThT fluorescence signal.

Figure 4: The tau aggregation assay shows high reproducibility between different batches of huTau441 protein. Each panel shows four replicates corresponding to a specific huTau441 batch. The aggregation of each batch was followed in independent experiments. Concentrations of huTau441 and heparin are 15 μM and 8 μM, respectively. Aggregation kinetics corresponding to the four individual tau batches are characterized by an average (with

SD) lag phase of 15.3 ± 0.38 h and t_{50} of 21.1 ± 0.46 h (Batch 1); lag phase of 12.5 ± 0.07 h and t_{50} of 19.8 ± 0.34 h (Batch 2); lag phase of 15.1 ± 0.34 h and t_{50} of 21.9 ± 0.86 h (Batch 3) and lag phase of 11.5 ± 0.29 h and t_{50} of 17.8 ± 0.29 h (Batch 4), respectively. Kinetic data was fitted with a 5-parameter logistic curve with a linear decline in the upper asymptote. Lag phase and t_{50} were calculated by interpolation between predicted values by linear regression. Lag phase is calculated based on the 3% increase of ThT fluorescence signal.

Figure 5: Preformed hutau441 aggregates have high seeding activity. To initiate seeding, sonicated tau aggregates were added to monomer hutau441. From red to blue, each color of the different kinetic curves represents the different amount of added seeds: 1.25%, 0.63%, 0.31%, 0.16%, 0.08%, 0.04%, 0.02%, 0.01%, 0.005% and 0.0025% (v/v), respectively. Spontaneous conversion of hutau441 is represented in black. All conditions were tested in quadruplicate. The four kinetic replicates associated with a certain concentration of seeds are highly reproducible and in most cases indistinguishable. Concentrations of hutau441 and heparin are 15 μM and 8 μM, respectively. The addition of small amounts of pre-formed sonicated fibrillar structures eliminates the initial lag phase observed in the spontaneous conversion and the effect is proportional with the amount of seeds. From red to blue, the difference in t_{50} observed (t_{50} spont.conv-t_{50} seeding) is 18.9, 18.40, 17.8, 17.3, 16.6, 16.1, 15.4, 14.7, 14.2 and 13.7 hours, respectively. Spontaneous conversion of hutau441 is characterized by an average t_{50} (with SD) of 19.85 ± 0.54 h. Kinetic data was fitted with a 5-parameter logistic curve with a linear decline in the upper asymptote. t_{50} were calculated by interpolation between predicted values by linear regression.

DISCUSSION:

Despite numerous efforts, the tau aggregation kinetics reported in the literature to date lack the desired level of reproducibility and/or some of the features of a nucleation dependent polymerization[19-23,25]. This is often emphasized by the lack of a lag phase, inefficient seeding and non-fibrillar nature of tau aggregates. The reason for these shortcomings can vary and includes sub-optimal tau protein quality (fragmentation, presence of aggregates, low purity, *etc*.), choice of protein and inducing reagents and or experimental conditions. Another complicating factor are the two cysteine residues located around the tau aggregation interface that can form intra- or inter- molecular disulfide bridges depending on the redox environment and affect the efficiency of tau aggregation. In most approaches reducing reagents such as DTT or TCEP have been used to maintain the cysteine residues in reduced forms and thus increase levels of reproducibility[25]. Furthermore, the extinction coefficient of tau is very low which leads to difficulties in accurate measurements of protein concentration.

We focused especially on a few parameters and quality attributes that we considered crucial for a robust, reproducible and representative aggregation profile for tau protein: eliminating the possibility of intra- and inter- molecular disulfide formation, generating a highly pure tau monomer and improving the accuracy of concentration determination. All these reagent related questions are potential attention points that we considered critical for optimal assay development. To address these issues, full length huTau441 was expressed with two mutations, C291A and C322A, and with N- and C- terminal tags. Mutation of the cysteine residues has

minimal impact on the tau protein while eliminating the otherwise very difficult to control disulfide bridging. Expressing the protein with relatively short N- and C- terminal tags allowed us to pursue a two-step affinity purification protocol which led to very high purity, integrity and monomer content. Furthermore, we introduced a F8W mutation that increased the extinction coefficient of the protein and allowed much more accurate concentration measurements[24].

In addition to using high-quality protein reagents, other assay parameters were also optimized. The optimal tau : heparin ratio was identified to be around 0.5 (M/M) which is in line with previously published studies[26]. Furthermore, mechanical and optical instrumental settings are crucial to ensure reproducibility and the optimal parameters might differ to some extent depending on the manufacturer.

The aggregation of tau described in this assay shows characteristics that are associated with tau pathogenesis in AD and related tauopathies. The process starts with an initial lag phase corresponding to the formation of high energy nuclei and is followed by a rapid growth phase that corresponds to fibril growth. The lag time is sufficiently long to open a broad window to study in detail the seeding process covering a broad range of seed concentrations (**Figure 5**) while still not too long so protein degradation and/or non-specific aggregation are avoided (**Figure 2**). These secondary events could especially happen when an intrinsically disordered protein such as huTau441 is exposed for long periods of time to physiological conditions. The obtained tau aggregates display the morphology of PHFs isolated from brains of AD patients and are very efficient in recruiting monomeric tau and converting it to *de novo* generated aggregates, a process referred to as seeding. The assay is highly reproducible, the kinetic curves being virtually indistinguishable between wells, experimental runs and protein batches. Although the current assay focuses only on the longest tau isoform, huTau441, the application can be adapted to study the conversion of other forms of tau (Ameijde *et al.*, Acta Neuropathologica Communications, in press) Furthermore, it enables mechanistic studies focused on the interplay of tau isoforms and possibly shed light on the differences between tau pathogenesis in AD where both 3R and 4R isoforms are present in PHFs and PICK's disease or Frontotemporal dementia where tau pathology contains mainly 3R and 4R tau isoforms, respectively[27].

The very high reproducibility of the assay should allow readers to implement it with relative ease in their specific lab settings. The assay mimics what is believed to be the *in vivo* misfolding and aggregation of tau, enabling mechanistic studies that will shed light on tau pathogenesis and it constitutes a valuable tool for screening drug candidates and evaluate their interference with the different steps of the pathogenesis process.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1_ai

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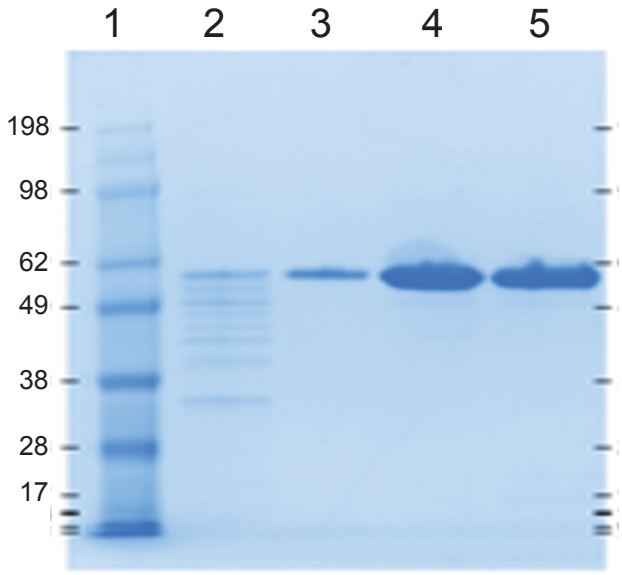
A



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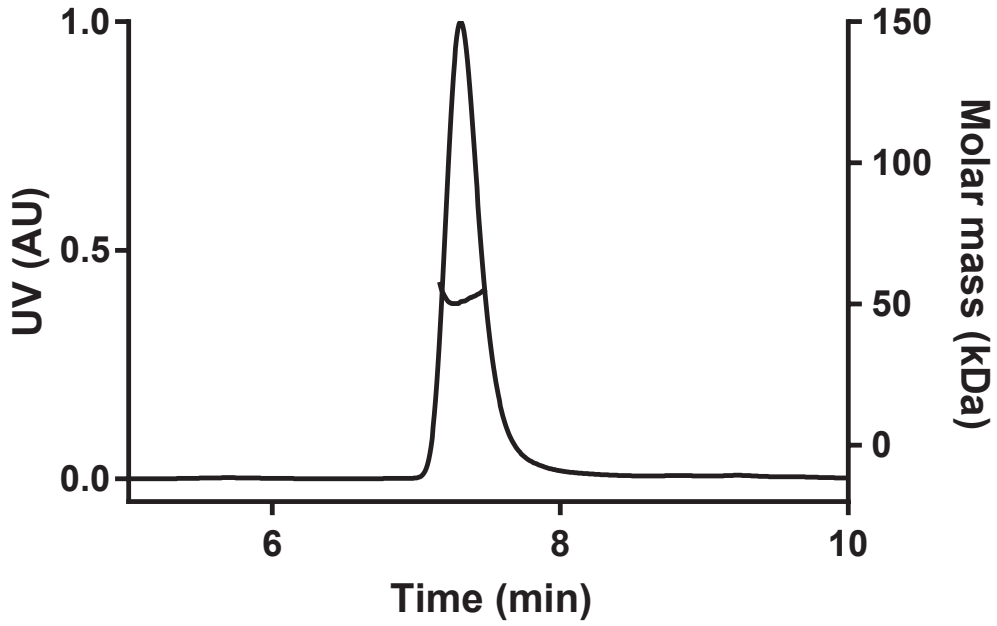

Figure 2_ai

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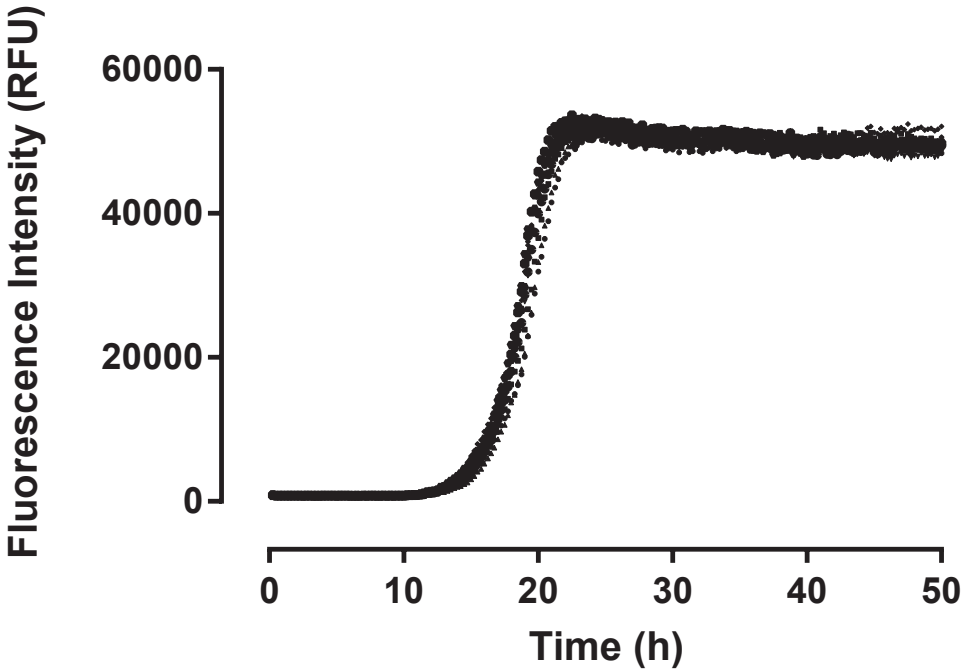
A



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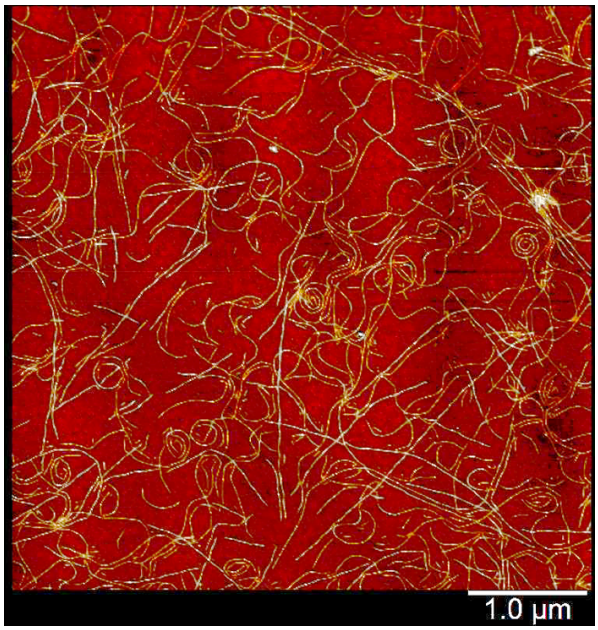

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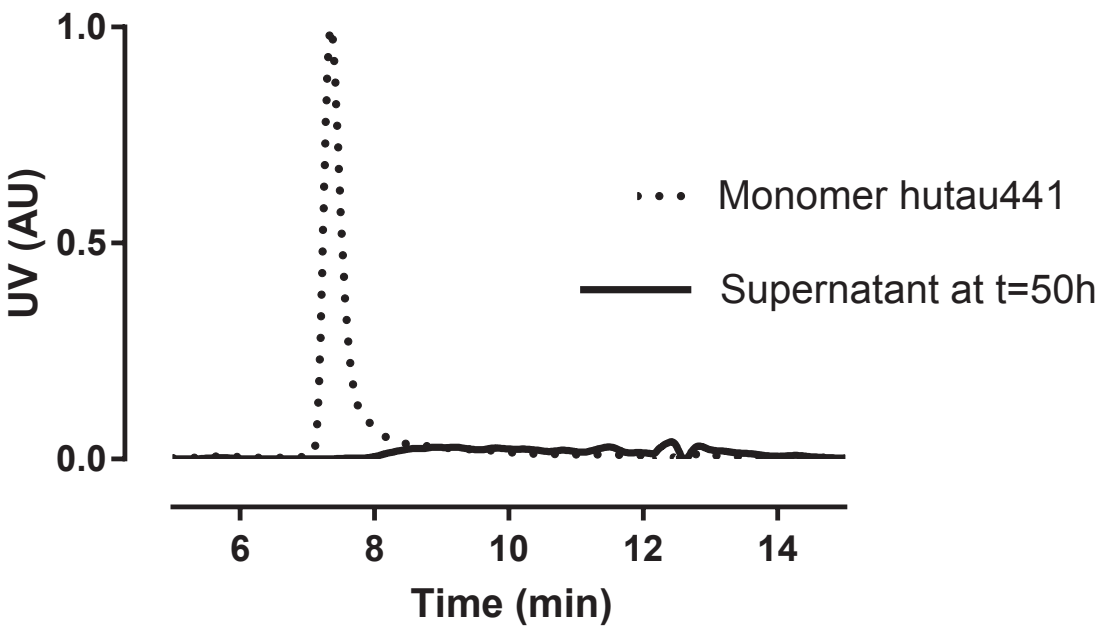

Figure 3_ai

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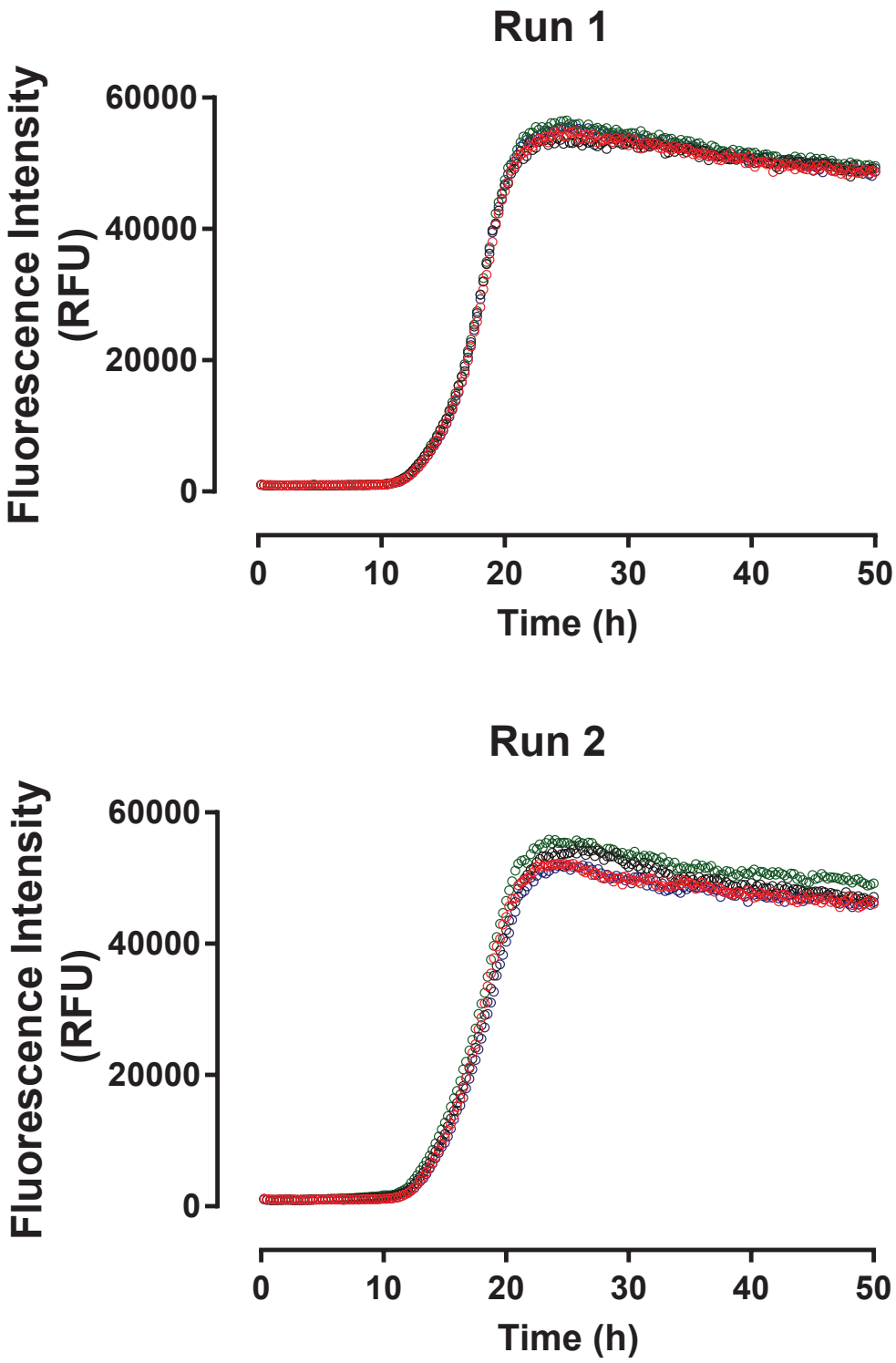

Figure 4_ai

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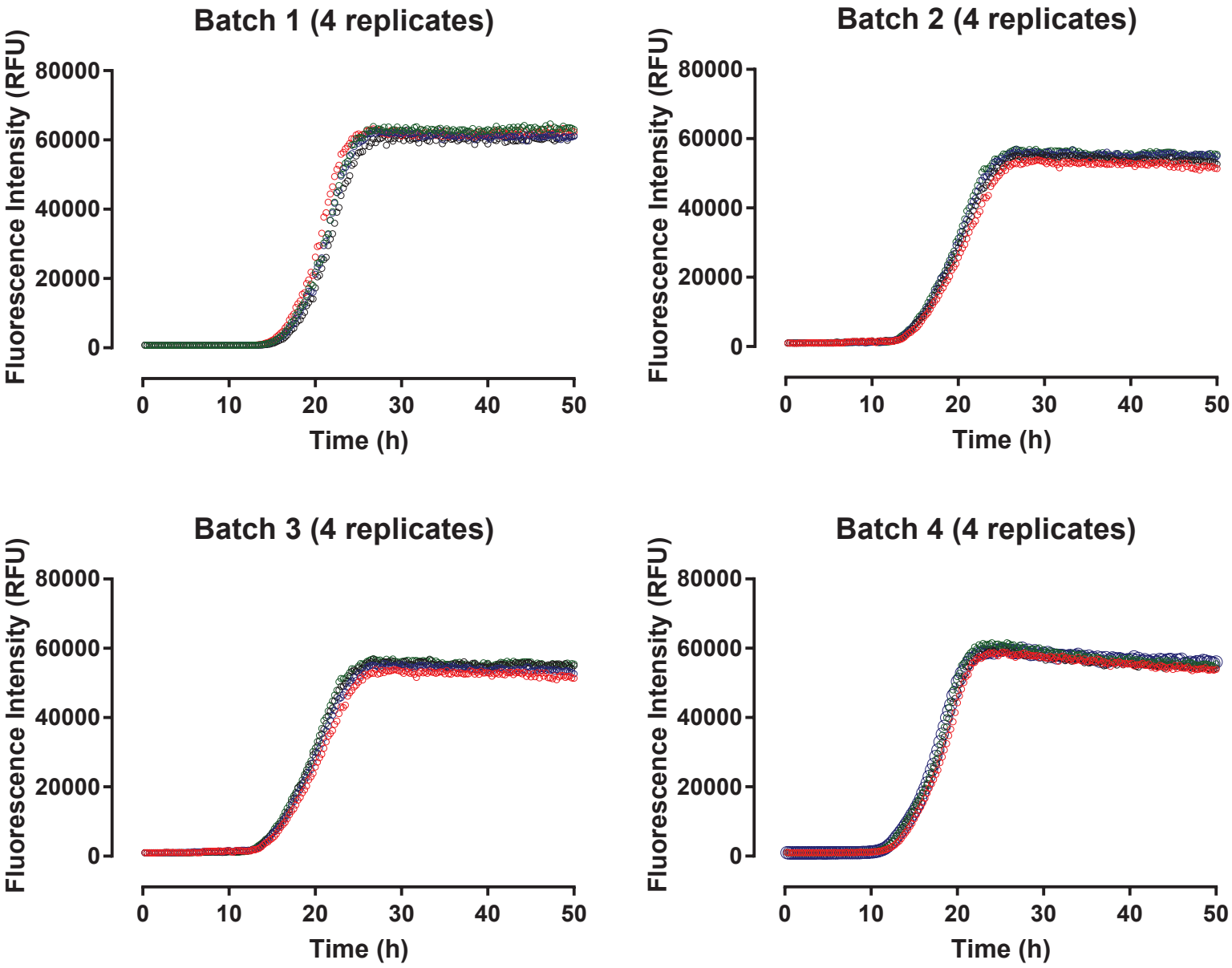

Figure 5_ai

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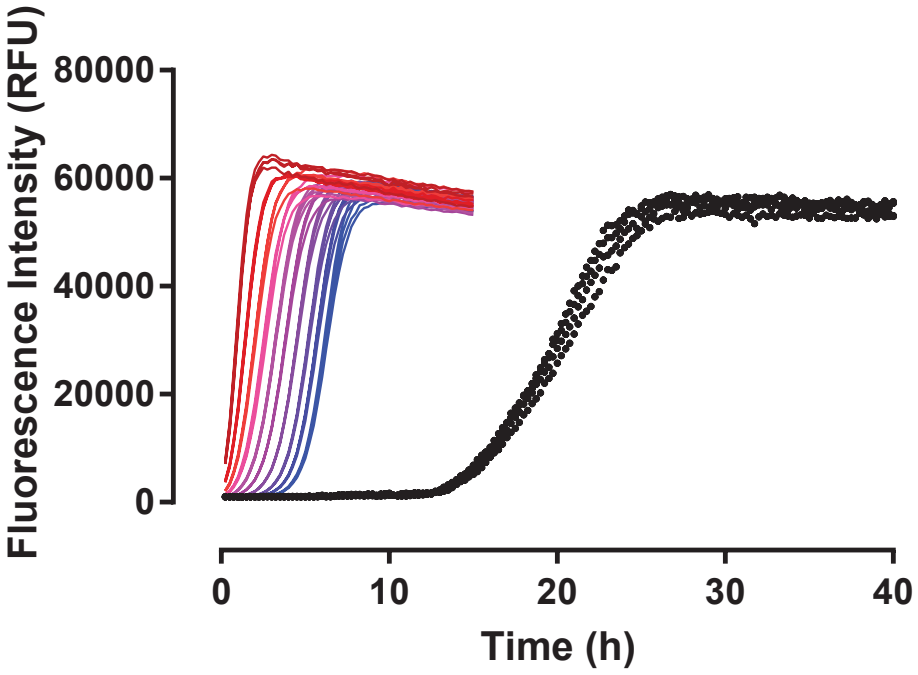

Table of Materials

Click here to download Table of Materials JoVE_Materials.xlsx

Name of Material/ Equipment	Company	Catalog Number
Thioflavin T	Sigma-Aldrich	T3516-5G
Heparin	Sigma-Aldrich	H3393-50KU
TCEP	Sigma-Aldrich	75259-1G
PBS	Gibco-Life Technologies	10010-015
0.22 µm sterile filter	Corning	431160
0.20 µm sterile serynge filter	Corning	431229
96-well microplates	Thermo Scientific	9502867
Microplate sealers	R&D Systems	DY992
Synergy Neo2 Multi-Mode Microplate Reader	Biotek	Synergy Neo2
Eppendorf Tubes	Eppendorf	0030 120.086
Ultrasonics-Branson SFX250	Branson	101-063-966R

Comments/Description
dry powder (Mw = 318.86 g/mol)
dry powder (Mw = 17-19 kDa)
dry powder (MW= 286.65 g/mol)
Sterile, pH 7.4 (1X)
PES membrane
PES membrane
Black, flat botton
Adhesive strips
Hybrid Technology, Gen5 Software
1,5 ml tubes
1/2" Solid Horn and 1/8" microtip

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Author(s):	Rosa Crespo, Wouter Koudstaal and Adrian Apetri

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Department:	
Institution:	Maarten Santman Senior Legal Counsel & Corporate Secretary
Title:	
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Janssen Vaccines & Prevention B.V.
Archimedesweg 6
2333CN Leiden
The Netherlands

July 2, 2018

Dear Dr. Nguyen,

Please find our revised manuscript entitled **"***In vitro* assay for studying the aggregation of tau protein and drug screening" [JoVE58570] that we hope is now acceptable for publication in Jove. We have taken into account the reviewers' comments and have added the details requested for the protocol and provided clarifications in the text where requested.

A point-by-point response to the specific comments of the reviewers follows.

Sincerely Yours,

Adrian Apetri, Ph.D.
Janssen Prevention Center, Janssen Pharmaceutical Companies of Johnson & Johnson

Response to reviewers' comments

Editorial comments:

Changes to be made by the Author(s):

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.*

We have proofread the manuscript.

2. Figure 1: Please label the ladder (kDa, Da?).

We have now specified the unit of the ladder (kDA) in the legend of Figure 1.

3. Please tone down the title: Delete "Highly Reproducible"

Thank you for your suggestion. Although the assay has a very high reproducibility in the variety of formats we have tested it is indeed conceivable that some non-voluntary alteration in different lab settings would still induce variability. We have eliminated the "highly reproducible" part from the title of the manuscript.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Synergy Neo, Eppendorf, etc.

We have removed all commercial language and used generic terms instead. The commercial products are listed in the Table of materials and reagents.

5. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

We have improved the protocol by including more experimental details.

6. 1.2.3: What happens after spinning? Resuspension?

We have adjusted the text to indicate that the specific spin is to remove air bubbles.

7. 1.3.3: Adjust to what concentration?

We have added the exact ThT concentration (i.e. 500 μM)

8. 2: Continues Mode? Please revise for clarity.

We have added "automatic measurements" to clarify what we mean by "continuous mode"

9. 2.4.7: How many times for the up and down pipetting? How much is added and what is the desired seed percentage?

We added the missing details to the revised version of the manuscript

10. Please do not abbreviate journal titles.

We removed journal abbreviations from the manuscript. The references were inserted using the Jove template which comes with abbreviations. Please clarify if the comment refers to abbreviation of journal names in the reference list or if it is limited to the manuscript.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Aggregation of tau is intrinsic to Alzheimer's disease and other neurodegenerative diseases. Study of the aggregation of tau using recombinant proteins has been hampered by variable results, both in reaction kinetics and the fibrillarity of the end products. The authors describe a highly reproducible way to generate recombinant fibrillar tau aggregates. This is a very useful method that can be employed for study of tau aggregation per se, as well as for the study of tau seeding in cells or animals.

Major Concerns:

- The authors do not directly compare their protocol to other protocols, which makes it difficult to assess for other scientists how they could improve their own protocols. A clearer description/discussion of what makes this protocol so robust would be helpful; is it the tau protein, the mutations, the heparin concentration for example.

We thank to the reviewer for the comment and for encouraging us to further stress the most important factors in the assay optimization process. We have elaborated in the revised version and we hope that this strengthens the manuscript. We focus in the first three paragraphs of the discussion section on the importance of protein quality, its monomer content and the ability to keep it in a state where disulfide bridging is not possible. We also discuss the importance of accurate protein concentration assessment, the adjustment we made to the tau protein and the optimization of heparin concentration and instrument settings. Furthermore, figure 1 shows the high purity and monomer content of tau, one of the most important quality attributes in our assay.

- The authors use tau with mutated cysteines to prevent disulfide bridge formation, but they still use a reducing agent in their reactions. This should be discussed as a particular advantage of their system appears to be that this can be omitted?

The use of TCEP is due to its required presence when aggregation employing wild type Tau containing cysteines is performed. In our optimization efforts we compared side by side different forms of tau and for a fair comparison we decided to use the same buffer. It is conceivable that for this particular tau variant, the presence of TCEP will only lower the pH of PBS from 7.4 to pH 6.7 but will not affect the conversion. We added a sentence to the revised version of the manuscript to explain.

- Sonication is very different between apparatuses, would it be possible for the benefit of the scientific community to make a generalized addition to this part of the methodology so that it becomes easier to implement? Perhaps of how the optimal settings were determined for this machine?

We added a sentence to the revised manuscript related to the parameters of our sonicator and also a statement as how we optimized sonication to achieve a homogeneous population of short fibers of 20-50 nm in length.

- A claim is made in the discussion that the optimal tau : heparin ratio was identified. In the manuscript only 2 concentrations of heparin were used, apparently without affecting the tau aggregation. This should be clarified and if there are data on which this is based it would be good to show or at least discuss. In addition, what determined the reaction being optimal? lag phase? fibrillarity? etc.

We thank the reviewer for the observation and we clarified in the revised version of the manuscript how optimization was performed including a reference to previous studies.
We have expanded in our revised manuscript the discussion on the importance of having high quality reagents, an optimal lag phase and that we aimed to set up an assay that mimics what is believed to be the *in vivo* tau aggregation process with the corresponding kinetic profile showing quantitative conversion, seeding activity and fibrillar morphology of aggregates.

Minor Concerns:
- for tau and ThioT the extinction coefficients are in different units, please unify.

We have listed in the revised version the extinction coefficient for tau protein in both mass and molar units.

- it would be good to note whether ThT should be made fresh for every experiment or that simple concentration adjustment suffices.
We clarified in the revised version of the manuscript the details regarding preparation, shelf life and storage of ThT.

Reviewer #2:
Manuscript Summary:
The manuscript "Highly reproducible in vitro assay for studying the aggregation of tau protein and

drug screening" by Rosa Crespo et al. explains the method of producing highly reproducible tau aggregation triggered by heparin in vitro. With this method, the author claims to achieve reproducible tau aggregation between different wells, runs, and batches of protein. Thus, this method has huge implications in the field of tau pathogenesis. Although, the author has made attempt to explain details of methodology; however, there are some concerns that should be considered.

Major Concerns:
(1) The author should attempt to show the sensitivity of huTau441 aggregation using the lower amount of huTau441 aggregates up to 0.0001% (v/v) or less. The amount of huTau441 seed in 0.01% (v/v) is considerably high. At a high concentration, any unrelated protein aggregate could cross-seed huTau441 aggregation.

We are know showing 10 different concentrations of seeds in the 0.0025- 1.25 % range (adjusted Figure 5).-The concentration of seeds used in our assay is significantly lower than shown in other publications. Also, seeding displayed in our experiments is concentration dependent and reproducible (kinetic traces for all four replicates corresponding to each concentration of seeds are virtually indistinguishable) and leads to tau aggregates with clear fibrillar morphology. Furthermore, we are not aware of systematic studies showing seeding efficiency of "any unrelated protein aggregate" and we do not think that the concerns are justified.

(2) The author should also test the specificity of huTau441 aggregation in this condition against other protein aggregates such as, alpha-Synuclein and amyloid beta.

We thank the reviewer for the constructive suggestion. However, while we acknowledge the importance of these scientific questions, the suggested experiments are not germane to this article. Our intention is to report on a robust tau aggregation assay that mimics what is believed to be the mechanism of *in vivo* tau pathogenesis. The specificity of tau aggregates as seeds in the conversion is confirmed by multiple things:

- Concentration dependent seeding potential
- Seeding efficiency at extremely low seed concentrations
- Homogeneous populations of *de novo* generated tau fibrils

(3) The lag phase of unseeded huTau441 aggregation is very short. It is possible that huTau441 preparation contains pre-aggregated seed or heparin concentration is too high. It is well known that these two factors highly modulate the aggregation of Tau. Author should attempt to centrifuge purified huTau441 at a high-speed 100,000Xg to remove the pre-aggregates rather than 12000Xg. On the other hand, if possible, the author should re-optimize the conditions with different concentrations of heparin, concentration of huTau441 protein, temperature and shaking speed.

Previous reports focused on tau aggregation showed very short lag phases if any. The lag phase for the spontaneous conversion of huTau441 has been optimized in our assay set up efforts to 12-15 hours. We consider this lag phase as optimal since it allows a broad time window to study seeding while still avoiding protein degradation, non-specific aggregation and/or artifacts that could appear when intrinsically unfolded proteins such as tau are incubated for extended periods of time under physiological conditions.

We appreciate the concern of the reviewer regarding the possible presence of tau aggregates in the starting material. Purity, integrity and absence of aggregates are some of the crucial parameters we have focused on. Thus, we have dedicated a full figure (Figure 1) to show the quality attributes of our monomer tau preparations. Thus, SDS-PAGE shows that huTau441 preparations contain no degradation products and are virtually 100 % intact. Furthermore, SEC-MALS analysis shows that the huTau441 preparation is 100 % monomeric as emphasized by a single peak in the chromatographic profile and a MW of 51 kDa (theoretical MW is 49 kDa). These observations eliminate the potential presence of any detectable traces of aggregates in the starting material. Also, the fact that the final "aggregated" material does not contain fragments validates our optimized lag time as optimal for this assay.
Moreover, the high reproducibility of the assay in different experiments and while using different batches should fully eliminate concerns regarding the quality of the protein reagents used in this study.

In order to better explain our rational in optimizing the lag time, we included a short paragraph in the revised version of the manuscript.

Minor Concerns:

(1) It is unclear about the storage buffer of purified huTau441. It should be explained.

For the protein purification we have referenced one of our previous publications. We also added the formulation buffer, PBS + 0.5 mM TCEP, pH 6.7 to the revised version of the manuscript.