# Kent Academic Repository



**Al-Hilaly, Youssra K, Marshall, Karen E, Lutter, Liisa, Biasetti, Luca, Mengham, Kurtis, Harrington, Charles R, Xue, Wei-Feng, Wischik, Claude M and Serpell, Louise C (2022) *An Additive-Free Model for Tau Self-Assembly*. Methods in molecular biology (Clifton, N.J.), 2551 . pp. 163-188. ISSN 1940-6029.**



## Downloaded from

https://kar.kent.ac.uk/115115/ The University of Kent's Academic Repository KAR

## The version of record is available from

## This document version

Author's Accepted Manuscript

## DOI for this version

## Licence for this version

UNSPECIFIED

## Additional information

## Versions of research works

### Versions of Record

If this version is the version of record, it is the same as the published version available on the publisher's web site. Cite as the published version.

### Author Accepted Manuscripts

If this document is identified as the Author Accepted Manuscript it is the version after peer review but before type setting, copy editing or publisher branding. Cite as Surname, Initial. (Year) 'Title of article'. To be published in ***Title of Journal*** , Volume and issue numbers [peer-reviewed accepted version]. Available at: DOI or URL (Accessed: date).

## Enquiries

If you have questions about this document contact ResearchSupport@kent.ac.uk. Please include the URL of the record in KAR. If you believe that your, or a third party's rights have been compromised through this document please see our Take Down policy (available from https://www.kent.ac.uk/guides/kar-the-kent-academic-repository#policies).

Methods in Molecular Biology: Protein aggregation

# An additive-free model for tau self-assembly

Youssra K Al-Hilaly[1,2, *], Karen E Marshall[2], Liisa Lutter[3], Luca Biasetti[2], Kurtis Mengham[2], Charles R Harrington[4,5], Wei-Feng Xue[3], Claude M Wischik[4,5], Louise C Serpell[2]

[1] Chemistry Department, College of Science, Mustansiriyah University, Baghdad, Iraq

[2] Sussex Neuroscience, School of Life Sciences, University of Sussex, E Sussex, UK

[3] School of Biosciences, University of Kent, Canterbury, UK

[4] Institute of Medical Sciences, University of Aberdeen, Aberdeen, AB25 2ZP, UK

[5] TauRx Therapeutics Ltd., Aberdeen, AB24 5RP, UK

***corresponding author Youssra K Al-Hilaly, email:**

**youssra.alhilaly@uomustansiriyah.edu.iq**

**An additive-free model for tau self-assembly**

**Abstract**

Tau is a natively unfolded protein that contributes to the stability of microtubules. Under pathological conditions such as Alzheimer's disease (AD), tau protein misfolds and self-assembles to form paired helical filaments (PHFs) and straight filaments (SFs). Full-length tau protein assembles poorly and its self-assembly is enhanced with polyanions such as heparin and RNA *in vitro* but a role for heparin or other polyanions *in vivo* remains unclear. Recently, a truncated form of tau (297-391) has been shown to self-assemble in the absence of additives which provides an alternative *in vitro* PHF model system. Here we describe methods to prepare *in vitro* PHFs and SFs from tau (297-391) named dGAE. We also discuss the range of biophysical/biochemical techniques used to monitor tau filament assembly and structure.

**Keywords: Self-assembly, cross-beta, tau, paired helical filament, electron microscopy, thioflavin S fluorescence, tyrosine fluorescence, circular dichroism, atomic force microscopy, X-ray fibre diffraction**

## 1. **Introduction**

Tau is associated with a group of neurodegenerative diseases known as tauopathies, which includes Alzheimer's disease (AD), frontotemporal dementia and chronic traumatic encephalopathy. Tau is a microtubule-associated protein that contributes to the stability of microtubules **[1]**. It is an intrinsically disordered or natively unfolded

protein and as such it has a highly flexible conformation **[2]**. The N-terminal domain is negatively charged followed by a positively charged microtubule-binding repeat domain (MTBD) made up of imperfect repeats and an upstream proline-rich region in the form of Ser-Pro and/or Thr-Pro motifs. The Ser and Thr residues frequently undergo phosphorylation **[3]**.

The microtubule-associated protein tau (*MAPT*) gene is located on chromosome 17 and alternative splicing leads to the production of six major tau isoforms ranging from 37-46 kilodaltons (kDa) **[4, 5]**. Of the six isoforms of tau, three possess three repeats (3R) and three contain four repeats (4R). Within a healthy brain, tau is phosphorylated in the cytoplasm and non-phosphorylated in the nucleus **[6, 7]**. However, in AD and other tauopathies, tau can become hyperphosphorylated **[8]** or fragmented **[9]** and self-assemble via multiple steps to eventually form tau filaments (PHFs and SFs). These filaments accumulate intracellularly as neurofibrillary tangles and are believed to play a central role in pathology and disease progression **[10]**.

Filaments extracted from AD tissue share the β-sheet rich structure with amyloid and display a cross β-structure by X-ray fibre diffraction **[11]**. Recent advances in cryo-electron microscopy have resolved several structures for tau filaments **[12-14]**. Full-length tau and various tau fragments have been used in structural studies to investigate self-assembly mechanisms and structure *in vitro*. However, these studies often depend on a cofactor, such as heparin, to induce filament assembly **[15-17]**. Furthermore, it has been observed that filaments assembled using these cofactors are very different from those found in AD tissue **[18, 19]**. Therefore, it is clear that there is a need for a more physiologically and structurally relevant form of tau required to better understand tau self-assembly and pathology **[20, 21]**.

dGAE is the name given to a truncated form of tau comprising residues 297-391. This region includes repeats 3 and 4 of the 4-repeat isoforms of tau. dGAE represents a stable core region of full-length tau and contains the region that has been resolved by cryoEM of tau filaments extracted from tauopathy tissues **[12, 14]**. In the absence of additives, dGAE has been observed to self-assemble into PHFs and SFs that share the cross-β characteristics of amyloid and that morphologically resemble the filaments found within neurofibrillary tangles in AD brains **[20, 21]**.

Many biophysical and biochemical techniques can be used to study tau assembly and structure **[22]**. Thioflavin S (ThS) along with Thioflavin T (ThT) is commonly used to monitor fibrillogenesis, and tyrosine fluorescence and circular dichroism (CD) can be used to track the conformational changes. Atomic force microscopy and transmission electron microscopy are frequently used to visualize tau filaments morphology and could provide low-resolution structural information. Further structural details for tau filaments can be gained by X-ray fibre diffraction (XRFD). In this chapter, we describe methods to prepare *in vitro* PHFs and SFs from tau (297-391) and we explain a range of biophysical /biochemical techniques used to monitor tau filament assembly and structure.

**Materials**

All buffers and solutions should be made using ultrapure water purified to reach a resisitivity of 18 MΩ.cm at 25 °C. To minimise the loss of tau proteins (e.g. dGAE) and the possible effect of the internal surface of Eppendorf, protein low binding tips and Eppendorf tubes should be used throughout preparation. All solutions and buffers should be passed through a 0.22-μm pore size polyethersulfone (PES) or polyvinylidene fluoride (PVDF) filter prior to use to reduce bacterial growth and

remove contaminants. Do not filter solutions containing protein due to potential loss from binding to the filter membrane.

**2.1 Self-assembly of dGAE filaments**

1. Aliquot of dGAE of desired concentration.
2. Phosphate buffer (PB) pH 7.4; 10 mM.
3. 200 mM Dithiothreitol (DTT).
4. Eppendorf thermomixer.
5. Parafilm.

**2.2 Thioflavin S fluorescence**

1. 20 mM 3-(N-morpholino)propane sulfonic acid (MOPS) buffer; dissolve 4.2 g MOPS in 1 mL filtered milli-Q water, adjust the pH with 1 M NaOH to 6.8.
2. 5 μM ThS in 20 mM MOPS buffer pH 6.8.
3. 10 mm Quartz cuvette.
4. Spectrofluorimeter.
5. Distilled water, Ethanol, 2% Hellmanex and lens cleaning tissue (Whatman) for cuvette cleaning.

**2.3 Tyrosine fluorescence**

1. 10 mm quartz cuvette.
2. Spectrofluorimeter.
3. Distilled water, ethanol, 2% Hellmanex and lens cleaning tissue (Whatman) for cuvette cleaning.

### 2.4 Circular Dichroism

1. Nitrogen pre-purged circular dichroism spectrometer.
2. dGAE soluble protein or filaments in 10 mM phosphate buffer, pH 7.4.
3. Quartz cuvette of appropriate pathlength.
4. Distilled water, ethanol, 2% Hellmanex and lens cleaning tissue (Whatman) for cuvette cleaning.

### 2.5 X-ray fibre diffraction (XRFD)

#### 2.5.1 X-ray fibre diffraction sample preparation

1. dGAE filaments in salt-free buffer.
2. Filtered milli-Q water.
3. Refrigerated centrifuge.
4. Petri dish.
5. Glass capillaries (Harvard Apparatus Ltd, Fircroft way, Edenbridge, Kent, UK), (1.5 mm OD X 1.17 ID borosilicate).
6. Teflon slide.
7. Wax.
8. Glue.
9. Plasticine.
10. Hot plate.
11. Razor blade.
12. Glass cutter pen.

#### 2.5.2 X-ray fibre diffraction data collection

1. Goniometer head (Hampton).
2. X-ray source (CuKalpha) and detector.

**2.5.3 X-ray fibre diffraction data analysis and modelling**

Computer workstation running Mosflm/ fit2d/ fibrefix/ CLEARER (and latest version of java).

**2.6 Negative stain transmission electron microscopy (TEM)**

1. Formvar/carbon coated 400 mesh copper grids (Agar Scientific, Essex, UK).
2. Filter paper.
3. 0.22-µm filtered milli-Q water.
4. 2% (w/v) uranyl acetate.
5. Tweezer, anti-magnetic with thin tip.

**2.7 Block preparation**

3. Class II Type A2 biological safety cabinet.
4. Scalpel blade, #22.
5. 4% paraformaldehyde.
6. 0.1% glutaraldehyde.
7. PBS 1x.
8. Ethanol (30%, 50%, 75%, 90%, 100%).
9. Unicryl Resin.
10. BEEM capsule.

**2.8 Brain sectioning**

1. Tweezers, antimagnetic.

2. Razor blade.
3. Hexagonal 300-mesh nickel grids, 3.05 mm.
4. Ultra Diamond Knife – Wet 45° 2.5 mm.
5. Ultramicrotome.
6. Glass knives.

## 2.9 Immunogold labelling TEM

### 2.9.1 Immunogold labelling TEM of brain tissue sections

1. Fixed sections from Alzheimer's patient brain from middle frontal gyrus.
2. A modified phosphate-buffered saline (PBS+), pH 8.2, containing 1% BSA, 500 µl/L Tween-20, 10 mM Na EDTA, and 0.2 g/L $NaN_3$.
3. Filter paper.
4. Distilled water.
5. 0.5% (w/v) aqueous uranyl acetate.
6. Petri dish.
7. 24-well plate.
8. Parafilm.
9. Plastic container with lid.
10. Tissue paper.
11. Tweezer, antimagnetic with thin tip.
12. Blocking solution: Normal Goat Serum (1:10 dilution in PBS+)
13. Primary antibodies, T22 rabbit polyclonal anti-tau antibody (ABN454; Merck Millipore, Darmstadt, Germany) 1:1000 dilution in PBS+, Total Tau rabbit polyclonal anti-tau antibody (SAB4501821; Sigma-Aldrich Ltd; Gillingham,

UK) 1:1000 dilution in PBS+, and mAb 423 that recognises tau C-terminally truncated at Glu-391 **[23, 24]**, 1:20 dilution in PBS+.

14. An appropriate secondary probe (1:10 dilution in PBS+): goat anti-rabbit IgG or goat anti-mouse IgG with 10 nm gold nanoparticles (GaR10 or GaM10) (BBI solutions, Crumlin, UK).

### 2.9.2 On-grid immunogold labelling TEM

1. dGAE filaments.
2. A modified phosphate-buffered saline (PBS+), pH 8.2, containing 1% BSA, 500 µl/L Tween-20, 10 mM Na EDTA, and 0.2 g/L $NaN_3$.
3. Formvar/ carbon-coated 400 mesh copper TEM support grids (Agar Scientific, Essex, UK).
4. Filter paper.
5. Filtered milli-Q water.
6. 2% (w/v) aqueous uranyl acetate.
7. Petri dish.
8. Parafilm.
9. Tweezer, antimagnetic with thin tip.
10. Blocking solution: Normal goat serum (1:10 dilution in PBS+).
11. Primary antibody, mAb 423 that recognises tau C-terminally truncated at Glu-391 **[23, 24]**, 1:20 dilution in PBS+.
12. Goat anti-mouse IgG with 10 nm gold nanoparticle (GaM10) as a secondary probe (BBI solutions, Crumlin, UK) 1:10 dilution in PBS+.

## 2.10 Atomic force microscopy imaging

### 2.10.1 Atomic force microscopy sample preparation

1. Metal disc (magnetic stainless steel, 15 mm diameter, Agar Scientific).
2. Highly oriented pyrolytic graphite (HOPG) (10 x 10 x 2 mm plates, standard quality with mosaic angle of 3.5° ± 1.5°, Agar Scientific).
3. Double-sided tape.
4. Electrical (PVC) tape.
5. Petri dish or specimen storage box.
6. Milli-Q water.
7. Syringe filter (0.22 μm pore size).
8. Sterile syringe (10 mL).
9. Sterile Falcon tube.
10. Sterile pipette tips.
11. Nitrogen gas source (e.g. nitrogen gas cylinder).
12. Tweezers (e.g. Dumont biology tweezers).

### 2.10.2 Atomic force microscopy image data collection and processing

1. Bruker Multimode 8 scanning probe microscope with Nanoscope V controller.
2. Bruker ScanAsyst-Air probes (nominal spring constant of 0.4 N/m and nominal tip radius of 2 nm).
3. Computer workstation running Bruker Nanoscope Analysis.

### 2.11 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

1. dGAE prepared with and without DTT.
2. 10 mM phosphate buffer (PB) diluted from a stock solution of 200 mM, pH 7.4.

3. Mixer for microcentrifuge tubes with temperature control.
4. Bicinchonicic (BCA) assay kit.
5. Clear bottom 96 well plates.
6. Fluorimeter capable of reading absorbance at 562 nm.
7. 2 mg/mL bovine serum albumin (BSA).
8. Precast Tris-glycine protein mini gels (*see* **Note 1**).
9. Tris/glycine/SDS running buffer: Diluted to 1X from a 10X pre-prepared stock (*see* **Note 1**). Final concentration 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3.
10. Laemlli sample buffer: Purchased as a 4X stock (*see* **Note 1**) and diluted to 1X with sample (*see* **Note2**).
11. Electrophoresis chamber and cassette holder (*see* **Note 1**).
12. Protein standards ladder (*see* **Note 3**).
13. Coomassie stain.
14. Rocker.
15. Clean containers for gel.

### 2.11 Dot blot

1. dGAE prepared with and without DTT.
2. 10 mM phosphate buffer (PB) diluted from a stock solution of 200 mM, pH 7.4.
3. Mixer for microcentrifuge tubes with temperature control.
4. Rocker.
5. Clean containers for gel.
6. Nitrocellulose membranes 0.2 μm pore size.

7. Tris-buffered saline (TBS).
8. Tris-buffered saline with 0.1 % Tween (TBS-T).
9. Blocking solution (5% non-fat dry milk in TBS-T).
10. Primary antibody T22 diluted in blocking solution 1:1000.
11. Secondary antibody goat anti-rabbit IgG HRP conjugate diluted in blocking solution 1:1000.
12. Enhanced chemiluminescence (ECL) reagent.
13. Clean plastic wallets.
14. ECL imaging system.

## 3. Methods

### 3.1 Self-assembly of dGAE filaments

1. Incubate 100 μM dGAE with and without 10 mM DTT in phosphate buffer (10 mM, pH 7.4) at 37 °C with agitation at 700 oscillations per minute for 48-72 h (*see* **Note 4**). Under these conditions, straight filaments will be the main morphology (Figure 1, a).
2. Incubate 300 μM dGAE in phosphate buffer (10 mM, pH 7.4) containing 10 mM DTT at 37 °C with agitation at 400 oscillations per minute for 48 h using a thermomixer. Under these conditions, PHF will be the main morphology of the filaments. (Figure 1, b).

### 3.2 Thioflavin S fluorescence

1. Mix 20 μL dGAE with 280 μL ThS (5 μM in 20 mM MOPS buffer) then vortex briefly and leave for 3 minutes.

2. Place in 10 mm quartz cuvette and insert into spectrofluorimeter.
3. Set the excitation wavelength to 440 nm.
4. Set an emission scan between 450-600 nm.
5. Set excitation and emission slits to 5 and 10 nm respectively. Set the sample compartment at a temperature of 21°C, scan rate to 600 nm/min and average three spectra for each measurement. (Figure 2, a).
6. Collect the spectrum for the blank and subtract it from the sample's reading.

**3.3 Tyrosine fluorescence**

1. Place dGAE sample in 10-mm quartz cuvette and insert into spectrofluorimeter.
2. Set the excitation wavelength to 280 nm and an emission scan between wavelength 290-500 nm. Set the sample compartment to 21 °C with a scan rate of 600 nm/min. Average three spectra for each measurement (Figure 2, b).
3. Collect the spectrum for the blank and subtract it from the sample spectrum.

**3.4 Circular dichroism**

1. For CD measurements, either collect spectra for the whole sample, or centrifuge the aggregated dGAE samples at the final time point using 20,000 g, 4°C for 30 minutes. Separate the supernatant and the pellet into two Eppendorf tubes. Resuspend the pellet into an appropriate amount of phosphate buffer (10 mM, pH 7.4).
2. Place supernatant fraction or resuspended pellet in appropriate quartz cuvette.
3. Use a water bath to equilibrate samples at 21°C.
4. Take scans between 180-300 nm using a spectropolarimeter (*see* **Note 5**).
5. Average three spectra for each measurement.

6. For baseline, collect the CD spectrum for the buffer and subtract it sample spectra.
7. Convert the CD data of the supernatant into molar ellipticity ($deg.cm^2.dmol^{-1}$) when concentration is known (Figure 2, c). CD data for pellet is retained in mdeg (*see* **Notes 6 and 7**).

## 3.5 X-ray fibre diffraction

### 3.5.1 X-ray fibre diffraction sample preparation

1. For XRFD, collect pellet from dGAE (500 µL) assembly solution by centrifuging at 20,000 x g for 30-45 minutes at 4°C. You need a large amount of pellet, if the pellet is not large enough, use a larger volume of dGAE.
2. Wash the pellet three times with milli-Q water (400-600 µL) by centrifugation 20,000 g for 15 minutes at 4°C to remove excess salts.
3. Suspend the resulting pellet with 10-20 µL of milli-Q water.
4. Using a glass cutter pen, cut the glass capillaries into 20-30 mm and fill one end with preheated wax.
5. Put two wax-tipped capillary tubes ~ 10 mm away in petri dish using plasticine.
6. Place 10 µL of the suspended pellet between wax-tipped capillary tubes and allow to dry overnight at room temperature (*see* **Note 8**).
7. Move the two capillary tubes away from one another gently, the aligned filaments will stick to one end of the capillary tubes (Figure 3, a).
8. To align the filaments as a film, place 10 µL of the resuspended pellet on a Teflon slide and leave it overnight at room temperature. The following day, gently take

the film off using a razor blade. Using a tiny amount of glue, stick the film to the end of wax-tipped capillary tube (Figure 3, a).

**3.5.2 X-ray fibre diffraction data collection**

1. Mount the aligned samples on a goniometer head.
2. Collect the diffraction pattern using a Rigaku rotating anode fitted with Saturn CCD detector, using an oscillation of 0.5°. For example; exposure times of 30 or 60 seconds with a specimen to detector distance of 50 mm or 100 mm, respectively (Figure 3, b).

**3.5.3 X-ray fibre diffraction data analysis and modelling**

1. X-ray pattern can be examined using a range of different programmes including ipmosflm or imosflm [25]; Fit2D, Adxv or Clearer **[26]** as well as many others.
2. The X-ray diffraction settings must be introduced to ensure accurate measurements of diffraction signals can be made on the meridian and equator of the X-ray fibre diffraction pattern.
3. The diffraction pattern obtained from amyloid fibrils generally share the "cross-β" pattern composed of a 4.76 Å meridional arising from hydrogen bonding of β-strands and approximately 10-12 Å equatorial diffraction signals arising from the sheet spacing; as well as additional reflections on the equator arising from protofilament packing and peptide chain length (Figure 4).
4. Further analysis can be conducted to analyse the diffraction pattern using Clearer **[27]** or other suitable X-ray fibre diffraction analysis programmes to gain more information regarding the organisation of the peptide or protein within the

amyloid fibril structure by determining the size of the smallest repeating unit or helical pitch. Further details are given in **[27, 28]**.

5. Modelling of the peptide can be conducted using a modelling programme to generate a plausible model structure to fit the size of the smallest repeating unit, taking into account the hydrogen bonding distance of 4.76 Å and the determined β-sheet separation.
6. If a modelled structure can be generated within a smallest repeating unit, a simulated fibre diffraction pattern can be calculated using Clearer **[26]** or other suitable software, to evaluate the consistency of the model to fit the experimental diffraction data **[26]**. Further details are given in **[27, 28]**.

## 3.6 Negative stain transmission electron microscopy

1. Place 4 μL of the assembly solution on Formvar/carbon film coated grid and incubate for 1 minute. Blot away excess using filter paper on the edge of the grid.
2. Wash the grid with 4 μL filtered milli-Q water and blot dry using filter paper.
3. Place 4 μL of 2% (w/v) aqueous uranyl acetate onto the grid for 30 seconds and then blot any excess dye and allow to air dry.
4. Examine and image the grids on a transmission electron microscope with compatible camera.

## 3.7 Block preparation

1. The following protocol describes the preparation of a sample block from frozen human brain tissue.

2. Transport the frozen tissue on dry ice to preserve structure and integrity to a Class II Type A2 biological safety cabinet.
3. Excise a sample of around 1 $mm^3$ using a scalpel blade (#22), place it immediately into a 1.5 ml Eppendorf tube containing a fixative solution (4% paraformaldehyde, 0.1% glutaraldehyde in PBS 1×) and leave at 4 °C overnight.
4. The following day wash out the fixative solution using PBS five times over a period of 4 h. Keep the sample rotating at 4 °C during the washes. This washing was necessary to remove residual aldehyde which can interfere with labelling in subsequent steps.
5. Following the buffer rinse, dehydrate the sample using solutions of increasing Ethanol concentrations (30%, 50%, 75%, 90%, 3× 100%, each for 20 min).
6. Following the 100% EtOH step, prepare the sample for embedding in resin (*see* **Note 9**) by primary incubation in a 100% EtOH : resin (2 : 1) solution for 2 h followed by a secondary incubation using a 1 : 2 ratio (30 min for both incubation steps) before moving into complete resin overnight at 4 °C.
7. The following day replace the resin with fresh resin and move the sample into a BEEM capsule. Resin polymerisation is performed under UV light for 3–4 days at 4 °C until the resin is fully polymerised (*see* **Note 10**). Remove the finished block from the BEEM capsule and prepare for ultrathin sectioning.

### 3.8 Brain sectioning

1. Mount tissue block on the sample holder of the ultramicrotome
2. Locate the region of tissue. Depending on the shape of the mould, the sample might require more or less trimming preparation.

3. If a pyramidal capsule was used, trim the excess resin using a glass knife until the layer containing the tissue is reached. At this point use a razor blade to delineate a trapezoidal shape (Figure 5, a) containing the region of interest (*see* **Note11**).
4. If the tissue is already located on a planar surface, then proceed to delineate the region of interest using a razor blade. Make sure to remove the excess resin around the region. Mount the trimmed block on the ultramicrotome and begin trimming away the layers of resin using a glass knife.
5. Utilize a glass knife until the layer of tissue is reached.
6. Switch to a diamond knife and set the section thickness to 200 nM.
7. Align the edge of the knife with the face of the block so that the block's reflection is parallel as well as constant throughout while the knife moves perpendicular to the sample (Figure 5, b).
8. Move the knife stage back a little and fill the well of the knife with distilled water until it reaches the edge of the blade, then remove water using a small pipette (or a syringe) until the water reflection on the knife's edge appears silver (*see* **Note 12**).
9. Once the block is aligned, proceed to cut at 200 nM until a full section is achieved (*see* **Note 13**). When there is also experimental interest in the depth at which the section is collected, it is imperative to provide extra care to the alignment step in order not to waste any of the tissue.
10. Once a full section of tissue has been collected, set the ultramicrotome to a thickness of 60 nM and begin collecting individual sections (*see* **Note 14**). Use an eyelash brush to separate the sections, pick up a nickel grid with a self-

closing tweezer and position it underneath the section. Gently raise the grid towards the surface and collect the section (*see* **Note 15**).

11. Leave the grid to dry for 5 -10 minutes.

## 3.9 Immunogold labelling

### 3.9.1 TEM immunogold labelling of brain tissues

1. Make 20 µL drops of the blocking solution (normal goat serum1:10 in PBS+) on parafilm that is fixed in a petri dish.
2. Block the brain sections in normal goat serum for 30 minutes at room temperature by inserting the grid carefully inside the drop (*see* **Note 17**).
3. Make drops of primary antibodies by pipetting 20 µL of the primary antibody onto parafilm that is fixed inside a petri dish.
4. Transfer the grid from the blocking solution into the primary antibody.
5. Put the petri dish inside a plastic container that has a wet tissue to provide a humid environment, and incubate overnight at 4°C (*see* **Note 16**).
6. Use 24-well plate to rinse the grid three times for 2 minutes using PBS+. Add 1 mL PBS+ in each hole and put the grid carefully inside the hole (*see* **Note 17**).
7. Make 20 µL drop of an appropriate secondary antibody on parafilm that is fixed inside a petri dish.
8. Transfer the grid into the drop (*see* **Note 17**) and incubate for 1 h at room temperature.
9. Wash grids three times for 10 minutes with PBS+ and then with distilled water four times for 5 minutes. For washing, fill the holes of 24-well plates to the top with either PBS+ or distilled water, place the grids carefully on the top of

the solution, do not let it sink, and make sure the brain section facing down (Figure 6, a).

10. Stain the grid with 0.22-µm filtered 0.5% aqueous uranyl acetate for 1 hour.
11. Rinse the grids with distilled water 5 times for 2 minutes using 24-well plate as explain in step 9.
12. Examine and image the grid on a transmission electron microscope with compatible camera (Figure 6, b).

### 3.9.2 Negative stain immunogold labelling TEM

1. Make 20 µL drop of the blocking solution (normal goat serum diluted 1:10 in PBS+) on parafilm that is fixed in petri dish.
2. Pipette 4 µL aliquots of the dGAE fibrils onto Formvar/ carbon coated grid, and leave it for 1 minute.
3. Remove the excess by filter paper on the edge of the grid and leave 2-3 minutes to dry, but do not leave it to over dry, as this could result in less binding of the primary antibody with the epitope.
4. Block the grid in normal goat serum for 15 minutes at room temperature by placing the grid carefully on the top of the drop. Make sure the grid side that has the sample facing down and do not let the grid sink inside the drop.
5. Prepare a 20 µL drop of the primary antibody on parafilm that is fixed in a petri dish. Transfer the grid from the blocking solution to the primary antibody by placing the grid gently on the top of the drop.
6. Incubate the grid with primary antibody for 2 h at room temperature.
7. Rinse the grid three times for 2 minutes using PBS+. For rinsing, make 20 µL drops of PBS+ on parafilm that is fixed inside a petri dish.

8. Make 20 µL drops of the secondary antibody on a parafilm that is fixed inside a petri dish.
9. Immunolabel the grid with a 10 nm gold particle-conjugated secondary probe for 1 h at room temperature.
10. Rinse the grid five times, 2 minutes each with PBS+ and then five times, 2 minutes each with milli-Q water. For rinsing, make 20 µL drops of PBS+ or milli-Q water on parafilm that is fixed inside a petri dish.
11. Finally, stain the grid with 0.22-µm filtered 2% aqueous uranyl acetate for 30 sec and dry.
12. Image the grid by TEM (Figure 6, c).

### 3.10 Atomic force microscopy imaging

#### 3.10.1 Atomic force microscopy sample preparation

1. Prepare the metal disc for sample support by cutting out a small square of double-sided tape that is smaller than the highly oriented pyrolytic graphite ( HOPG ) plate (e.g. 5 x 5 mm piece) and apply one side of the tape firmly to the metal disc (*see* **Note 18**). Then place the HOPG plate on the second adhesive side of the double-sided tape and gently press down on it to firmly attach it to the metal specimen disc.
2. Prepare the HOPG plate for sample deposition by cleaving off the top layer of the surface immediately prior to sample deposition. This cleaving step can be done by applying a strip of PVC tape that covers the entire HOPG surface and then removing it by swiftly pulling from one end of the strip in an upward motion while holding the metal disc down using appropriate tweezers. Make

sure that a complete layer of HOPG has been cleaved off by checking the bottom of the removed PVC tape. If some areas were not cleaved repeat this step until a whole layer is cleaved in a single motion (*see* **Note 19**).

3. Store the disc with freshly cleaved HOPG in a covered Petri dish to prevent contamination of the surface. A sample storage box with lid can also be used.
4. Deposit 20 μL of the sample containing 300 μM monomer equivalent concentration of the assembled fibrils to the HOPG surface using a sterilised pipette tip. Leave the sample in the covered Petri dish for 30 minutes.
5. Sterile filter milli-Q water by aspirating it into a syringe, attaching a syringe filter to the syringe, and pushing down on the plunger. The first syringe volume of water is discarded. Subsequently, place the syringe filter above a sterile Falcon tube to deposit the sterile filtered milli-Q water into the tube. 200 μL of the sterile filtered water is recommended per sample.
6. Place tissue paper on the workstation and prepare a gentle steady stream of nitrogen gas. Hold the metal disc with the HOPG plate and deposited sample above the tissue paper by grabbing and holding it with tweezers. Then slightly tilt the disc and wash the HOPG surface by pipetting 200 μL of sterile-filtered milli-Q water onto it with a sterile pipette tip. Quickly dry the surface with the stream of nitrogen gas for approximately 30 s.

### 3.10.2 Atomic force microscopy image data collection

1. Prepare the AFM for imaging by placing the disc onto the atomic force microscope sample stage (Figure 7, a). Load the AFM probe into the probe holder and insert the loaded holder into the AFM head.

2. Image the sample using the ScanAsyst in air peak-force tapping imaging mode (Bruker) by following the standard procedure from the instrument manufacturer. Choose a scan size and number pixels per line according to the aim of the study. To maximise structural detail using a smaller scan size and more pixels per line (e.g. 3 μm × 3 μm scan size with 2048 pixels per line) reduces the pixel size and results in higher resolution data whereas a larger scan size and less pixels per line (e.g. 5 μm × 5 μm scan size with 1024 pixels per line) enables collection of information on more individual particles within the same timeframe (*see* **Note 20**).

### 3.10.3 Atomic force microscopy data processing and analysis

1. Flatten images using Bruker Nanoscope Analysis software to remove the tilt and bow imaging effects by following instructions of the instrument manufacturer.
2. Further image processing and analysis of the AFM image data can be carried out using desired algorithms written in any programming language (e.g. MATLAB, Python, etc.) by exporting the image data in either binary or text format in Bruker Nanoscope Analysis.

## 3.11 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

1. If separating supernatant and pellet (*see* **Note 21**), following agitation spin the sample in a microcentrifuge for 30 minutes at 16100 x g. Remove the supernatant and transfer to another low-bind tube, leaving a small volume of buffer covering the fibrils to keep them hydrated. Store the fibril pellet at 4 °C while determining the protein concentration in the supernatant.

2. Determine the protein concentration in the supernatant fraction using a BCA assay. This information can be used to determine the proportion of dGAE that has fibrillised and the volume of 10 mM PB to add to bring the final concentration of fibrils to 100 µM (*see* **Note 22**).
3. Non-reducing SDS-PAGE.

   i. Mix dGAE sample with 4X Laemlli sample buffer at a ratio of 3:1 (*see* **Note 23**) and incubate at room temperature for 5 minutes (*see* **Note 24**).

   ii. Prepare the running buffer by diluting 1:10 to achieve a 1X stock.

   iii. Prepare the gel apparatus. Remove the tape from the bottom of the gel and rinse out the wells (*see* **Note 25**) with water. Clip the gel into the frame and insert into the electrophoresis chamber. If only one gel is being used, use a buffer dam on the other side (*see* **Note 26**). Pour running buffer in between the gels or gel and buffer dam to the top so that the wells are submerged. Pour remaining running buffer into the outside of the chamber up to approximately halfway.

   iv. Load around 5 µL of ladder standard into the first well. Load the samples into the wells of the gel using gel loading tips, taking care to avoid spilling any sample into adjacent lanes.

   v. When all the samples are loaded, attach the lid ensuring the electrodes are plugged in the correct way round (red to red and black to black). Run the gel at 200 V for 30-40 minutes and switch off the power supply after the bromophenol blue has reached near to the bottom of the gel (*see* **Note 27**).

   vi. Remove the gel holder from the chamber and unclip, taking out the gel in the cassette. Pour enough MQ water to cover the gel into a clean container that has sufficient space for the gel to move freely. Carefully prise apart the

plastic cassettes and either gently pick the gel up by the bottom edge and move into the water in the container, or rinse it off the plastic using MQ water from a wash bottle. Place on a rocker for 5 minutes, then remove the water by carefully pouring (taking care not to pour the gel out of the box), or by using a 10 or 25 mL pipette. Replace with fresh water and incubate for another 5 minutes. After pouring off the second wash, add enough Coomassie stain to cover the gel and place on a rocker for 1 hour, covered. Pour off the stain and de-stain using water overnight, changing the water 2-3 times. Image the gel using a bio-imaging scanner (Figure 8).

### 3.12 Dot blot with dGAE

1. Remove samples from freezer (if frozen) and thaw at 4 °C (*see* **Note 28**). Briefly centrifuge at low speed (approximately 5000 rpm for 30 seconds) to pull down all liquid then vortex carefully so that the liquid remains at the bottom of the tube.
2. Cut nitrocellulose membrane to a size that will fit all samples and into a container. Dot the mixture onto the membrane, a total volume of 3 μL should be applied, 1-1.5 μL at a time. Wait for the first dot to almost dry completely before adding the next (*see* **Note 29**). Allow to dry completely then transfer into a container (*see* **Note 30**).
3. Wash the membrane twice in TBS-T for 2 minutes each, then once in TBS for 2 minutes (*see* **Note 31**). Place the membrane in the container on a slow rocker to ensure complete and even coverage of the membrane.
4. Pour off the TBS then add blocking solution for 1 hour.

5. Pour off the blocking solution and add primary antibody. Incubate for one hour.
6. Wash three times in TBS-T for 10 minutes each.
7. Add secondary antibody and incubate for one hour.
8. Wash twice in TBS-T for 10 minutes each, then once in TBS for ten minutes. Prepare the ECL reagent.
9. After the final wash, prepare a paper towel, one piece of clean plastic and a clean plastic document holder. Remove the membrane using blunt forceps and drip dry using the paper towel to absorb excess buffer from the edge of the membrane (*see* **Note 32**). Lay down onto the clean plastic and using a pipette drop the ECL reagent over the membrane so that it is completely covered. Leave for 3 minutes then drip off onto paper towel as before and when the excess reagent has drained, place onto a new clean and dry plastic document holder and cover. Image using an ECL imaging system (Figure 9).

**Notes**

1. We use the Bio-Rad Mini-PROTEAN TGX system with a Tetra Cell chamber and any Kd gels, but alternative equivalents could be used. Running and sample buffers are also purchased from Bio-Rad.
2. For example, use 9 μL sample + 3 μL 4X sample buffer for a 15-well gel that holds a maximum volume of 15 μL per well.
3. The molecular weight marker ladder should go down to at least 5 kDa, preferably 2 kDa.
4. Always use protein low bind tips and tubes when preparing samples containing dGAE. Wrap Parafilm around the lid to prevent evaporation. Do

not completely fill the tube; this prevents the solution mixing properly and can result in less efficient assembly. Similarly, do not underfill. A final volume of 500-1000 µL is recommended for a 1.5 mL microcentrifuge tube.

5. If the HT signal reaches above 600 mV, the reading is no longer reliable.
6. Measure the protein concentration of the supernatant using a BCA assay.
7. The protein concentration for the pellet (filaments) cannot be determined using any known protein concentration protocols, due to insolubility of the filaments, thus, the CD data of the pellet cannot be converted into molar ellipticity. However, you could follow the instruction in note 23 to determine the approximate protein concentration for pellet.
8. Sometimes slow evaporation of the sample gives better alignment, keep the sample overnight in the fridge.
9. The resin used in this protocol was Unicryl. Any hydrophilic acrylic resin or resin mixture of preference that is suitable for immunolabelling on section can be used.
10. Utilize a BEEM capsule holder to maintain the samples vertically to not allow the tissue sample to move through the resin.
11. It is advised to use a rectangle trapezium shape as it allows for easier separation of individual sections from the ribbon. Alternative shapes include square, suited for images at ultra-low magnification, and rectangle, used for sequential sectioning as well as when using tissue of variable thickness.
12. If too much water is added in the well, it might overflow and wet the outside layer of the diamond knife's edge. If this occurs remove the knife from the stage, drain the water and clean the edge dry before trying again.

13. If the water level appears to be appropriate but the block becomes wet once the cutting begins it is advised to repeat the same procedure as 13. Alternatively, it is possible to begin removing some of the water through the use of a syringe while gently drying the block with a microfilter paper tissue. As the block encounter the knife and undergoes a full rotation, continue to dry it very carefully when it slows down. This should stop the block from getting wet as well as calibrating the water level to the right amount.
14. The interference colour of the section varies depending on its thickness. A section of 60 – 90 nm is expected to reflect a silver hue. A thickness lower than 60 nm reflects a grey colour, while a section with a thickness up to 150 nm has a gold hue. The colour of the section can be used as a good indicator for its thickness.
15. The pull up method had the advantage of placing the section wherever you want on the grid, without wrinkles. On the other hand, it does require a certain amount of practice and skill. An easier way to pick up a section is by using the press method. This can be achieved by approaching the section with the grid not from underneath but from the top and pressing on it. This method is rather straightforward, but it may result in wrinkles in the specimen, as well as over layering of some of its areas.
16. Keeping the samples inside a plastic container that has a wet tissue will prevent the sample from dryness.
17. Keep your brain section facing up, otherwise, you might lose it.
18. Positioning the tape slightly off-centre is convenient for moving the disc around as it results in one side of the disc having more space, thus making it easier to grab onto with tweezers.

19. Freshly cleave the HOPG surface every time immediately prior to a new sample being deposited.
20. Standard tapping mode or a force-distance curve-based image mode on instruments from other manufacturers can also be employed to image the samples. When collecting high resolution images in tapping mode ensure that only light contact is made with the sample to avoid the deformation of both the sample and probe surfaces which can cause imaging artefacts. If using a force-distance curve-based image mode such as the ScanAsyst mode on Bruker instruments, this can be accomplished by lowering the peak-force set point. Additionally, Z-limit can be lowered to within a suitable range to increase vertical digital resolution.
21. Depending on the application or type of analysis required, samples may be used whole, or separated into fibrils and low molecular weight species that remain in the supernatant following centrifugation. It must be acknowledged that "whole" means that there will be a range of differently sized species present, ranging from monomer through to protofibrils, oligomers and insoluble large assemblies (including fibrils). Furthermore, although agitation and heating has stopped, there is still likely to be some association and dissociation of species to and from the ends of fibrils. This protocol is not designed to separate out intermediate sized species, the details of these procedures can be found elsewhere.
22. As an example, 500 μL of a 100 μM dGAE solution was prepared then agitated for several days to assemble into fibrils. After centrifugation, the concentration of protein in the supernatant was 10 μM. Therefore 90% of the dGAE had assembled into fibrils that formed a pellet, and the fibril

concentration in the original 500 µL would be 90 µM. To achieve a final concentration of 100 µM fibrils, resuspend the pellet in 450 µL.

23. To avoid spillover do not use the maximum capacity of the well. We recommend using at least 10% less that the specified well volume, e.g. if the maximum volume is 15 µL, use a maximum volume of 13.5 µL.
24. Samples are generally not boiled and no reducing agent is added to the sample buffer. This is to keep the species in the sample as close to native as possible, while still being able to separate using standard SDS-PAGE instead of native PAGE (Figure 8).
25. Attach a gel loading tip to a regular wash bottle using Parafilm to easily insert the tip into the wells.
26. Make sure the gels are tightly fitted into the frame and ensure there is no gap at the bottom to avoid leakage.
27. The time taken to run the gel will vary depending on the system used. Check every ten minutes or so and ensure that the bromophenol blue in the Laemlli sample buffer does not run off the bottom of the gel; stop the current if it is near the bottom regardless of the time elapsed. The 2-kDa marker in the ladder can also be used to ensure the gel does not run for too long.
28. Although it is preferable not to freeze samples as there is a risk that the freeze-thawing process might affect the structural integrity of self-assembled species, under some circumstances e.g. time course assays it is very difficult or impossible to assay all time points in one go without freezing. Preliminary testing from our lab has shown there is no signal loss in either SDS-PAGE or dot blotting with frozen samples of dGAE.

29. It can be almost impossible to see the dot when dry, therefore try not to allow to dry completely before adding the next. A grid of approximately 1 cm squares can be marked out using a pencil to aid application. Adding all 3 μL in one go leads to the protein spreading out over the membrane.
30. If observation of total protein is required, before moving onto the next step washing with TBS-T, add ponceau S stain and incubate on a rocker for 5 minutes, rinse with MQ water for 5 minutes each, image, repeat rinse with MQ water for another 5 minutes followed by two washes with TBS-T for 5 minutes each to remove the stain, then continue with blocking and the rest of the procedure.
31. We have found that washing the membrane before blocking increases signal to noise (Figure 9).
32. Do not lay the membrane down completely onto the paper towel.

**References**


1. Weingarten, M.D., et al., *A protein factor essential for microtubule assembly.* Proc Natl Acad Sci U S A, 1975. **72**(5): p. 1858-62.
2. Mukrasch, M.D., et al., *Structural polymorphism of 441-residue tau at single residue resolution.* PLoS Biol, 2009. **7**(2): p. e34.
3. Wang, Y. and E. Mandelkow, *Tau in physiology and pathology.* Nat Rev Neurosci, 2016. **17**(1): p. 5-21.
4. Goedert, M., et al., *Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease.* Neuron, 1989. **3**(4): p. 519-26.
5. Fichou, Y., et al., *The elusive tau molecular structures: can we translate the recent breakthroughs into new targets for intervention?* Acta Neuropathol Commun, 2019. **7**(1): p. 31.
6. Bukar Maina, M., Y.K. Al-Hilaly, and L.C. Serpell, *Nuclear Tau and Its Potential Role in Alzheimer's Disease.* Biomolecules, 2016. **6**(1): p. 9.
7. Kimura, T., et al., *Phospho-Tau Bar Code: Analysis of Phosphoisotypes of Tau and Its Application to Tauopathy.* Front Neurosci, 2018. **12**: p. 44.
8. Iqbal, K., et al., *Tau in Alzheimer disease and related tauopathies.* Curr Alzheimer Res, 2010. **7**(8): p. 656-64.

9. Wischik, C.M., et al., *Isolation of a fragment of tau derived from the core of the paired helical filament of Alzheimer disease.* Proc Natl Acad Sci U S A, 1988. **85**(12): p. 4506-10.
10. Goedert, M., *Tau protein and the neurofibrillary pathology of Alzheimer's disease.* Trends Neurosci, 1993. **16**(11): p. 460-5.
11. Berriman, J., et al., *Tau filaments from human brain and from in vitro assembly of recombinant protein show cross-beta structure.* Proc Natl Acad Sci U S A, 2003. **100**(15): p. 9034-8.
12. Fitzpatrick, A.W.P., et al., *Cryo-EM structures of tau filaments from Alzheimer's disease.* Nature, 2017. **547**(7662): p. 185-190.
13. Falcon, B., et al., *Novel tau filament fold in chronic traumatic encephalopathy encloses hydrophobic molecules.* Nature, 2019. **568**(7752): p. 420-423.
14. Falcon, B., et al., *Structures of filaments from Pick's disease reveal a novel tau protein fold.* Nature, 2018. **561**(7721): p. 137-140.
15. Perez, M., et al., *Polymerization of tau into filaments in the presence of heparin: the minimal sequence required for tau-tau interaction.* J Neurochem, 1996. **67**(3): p. 1183-90.
16. Kampers, T., et al., *RNA stimulates aggregation of microtubule-associated protein tau into Alzheimer-like paired helical filaments.* FEBS Lett, 1996. **399**(3): p. 344-9.
17. Dregni, A.J., et al., *In vitro 0N4R tau fibrils contain a monomorphic β-sheet core enclosed by dynamically heterogeneous fuzzy coat segments.* Proceedings of the National Academy of Sciences, 2019. **116**(33): p. 16357.
18. Zhang, W., et al., *Heparin-induced tau filaments are polymorphic and differ from those in Alzheimer's and Pick's diseases.* Elife, 2019. **8**.
19. Fichou, Y., et al., *Heparin-induced tau filaments are structurally heterogeneous and differ from Alzheimer's disease filaments.* Chem Commun (Camb), 2018. **54**(36): p. 4573-4576.
20. Al-Hilaly, Y.K., et al., *Tau (297-391) forms filaments that structurally mimic the core of paired helical filaments in Alzheimer's disease brain.* FEBS Lett, 2020. **594**(5): p. 944-950.
21. Al-Hilaly, Y.K., et al., *Alzheimer's Disease-like Paired Helical Filament Assembly from Truncated Tau Protein Is Independent of Disulfide Crosslinking.* J Mol Biol, 2017. **429**(23): p. 3650-3665.
22. Vadukul, D.M., Y.K. Al-Hilaly, and L.C. Serpell, *Methods for Structural Analysis of Amyloid Fibrils in Misfolding Diseases.* Methods Mol Biol, 2019. **1873**: p. 109-122.
23. Novak, M., J. Kabat, and C.M. Wischik, *Molecular characterization of the minimal protease resistant tau unit of the Alzheimer's disease paired helical filament.* Embo j, 1993. **12**(1): p. 365-70.
24. Novak, M., et al., *Difference between the tau protein of Alzheimer paired helical filament core and normal tau revealed by epitope analysis of monoclonal antibodies 423 and 7.51.* Proceedings of the National Academy of Sciences of the United States of America, 1991. **88**(13): p. 5837-5841.
25. Leslie, A.G.W., *Recent changes to the MOSFLM package for processing film and image plate data.* oint CCP4 + ESF-EAMCB Newsletter on Protein Crystallography, 1992. **26**.

26. Zibaee, S., et al., *A simple algorithm locates beta-strands in the amyloid fibril core of alpha-synuclein, Abeta, and tau using the amino acid sequence alone.* Protein Sci, 2007. **16**(5): p. 906-18.
27. Makin, O.S. and L.C. Serpell, *X-ray diffraction studies of amyloid structure.* Methods Mol Biol, 2005. **299**: p. 67-80.
28. Morris, K.L. and L.C. Serpell, *X-ray fibre diffraction studies of amyloid fibrils.* Methods Mol Biol, 2012. **849**: p. 121-35.

**Figure captions**

**Figure 1:** Straight dGAE filaments produced *in vitro* using 100 μM dGAE in 10 mM PB, pH 7.4, 10 mM DTT (a). Paired helical filaments produce *in vitro* using 300 μM dGAE under reducing conditions in 10 mM PB, pH 7.4.

**Figure 2:** Typical graphs for a) ThS fluorescence and b) tyrosine fluorescence. CD spectra showing the two different secondary structures for dGAE fractions: c) supernatant shows a random coil structure whereas d) pellet display a strong β-sheet secondary structure.

**Figure 3:** Schematic to illustrate different alignment methods resulting in a fibre bundle and a film. The fibre bundle can give rise to a partially aligned sample consisting of laterally associated fibrils. The film results in a flat structure consisting of randomly aligned fibrils, but when the film is rotated to display the edge, this gives a highly aligned texture. X-ray fibre diffraction from the fibre bundle of tau filaments **[21]** results in diffraction with partial alignment. A film collected edge-on, results in well-oriented pattern providing more information in the resulting diffraction pattern.

**Figure 4:** Schematic diagram of a partially aligned bundle of fibrils resulting in a diffraction pattern collected from amyloid fibrils with a cross-β structure (illustrated by the stripy fibre). The meridional diffraction signal is found on the vertical axis of the diffraction pattern, parallel to the mounted fibrils. The diffraction signal arises from the repeating distance between the “stripes” or β-strands. The equatorial

diffraction signals are found on the horizontal axis of the pattern and derive from spacing across the fibre and will comprise of β-sheet spacing as well as other signals from peptide chain length and the filament packing.

**Figure 5:** (A) Schematic showing trimming of the sample block. Locate the region of interest in the tissue, make a series of incisions in a trapezium shape then use a razor blade to trim the top of the block in a pyramidal shape. (B) Steps required to align the face of the block with the edge of the knife. As you move the knife closer, a thin shiny line will start to appear on the surface of the block. This is the reflection of the edge of the diamond knife. At this point, rotate the knife's stage in the required direction in order for the reflection to be parallel with the knife's edge. Once this has been achieved, continue the sample's descent very carefully and keep monitoring the distance between the reflection and the edge. If it appears as it is shrinking, the top of the sample is closer to the knife than its lower part; adjust the inclination of the block from the block's stage so that the reflection appears once again at the same distance as it did at the beginning. If the distance is increasing, then do the opposite. Note that you might need to move the knife's stage back a little when the block's inclination is being adjusted, in order not to hit the block. Once the knife's reflection and its distance from the knife's edge are parallel and constant, respectively, throughout the cutting motion, then it is time to fill the knife with water and move the stage closer to begin the cutting process.

**Figure 6:** Schematic diagram explaining the grid washing using 24-well plate (a). Immunogold labelling for b) NFTs of tau from AD brain section immunogold labelled with total tau anti-tau antibody c) PHFs formed *in vitro* from dGAE and labelled with mAb 423 anti-tau antibody.

**Figure 7:** Atomic force microscopy uses a small, sharp probe tip attached to an oscillating cantilever to record sample surface topology. In this protocol, the sample was deposited onto the surface of a freshly cleaved HOPG plate attached to a metal disc, and subsequently placed on the AFM sample stage for nano-scale imaging as showing in schematic (a). (b) AFM images of dGAE filaments. A 5 × 5 μm AFM image of dGAE filaments with inset representing a cropped 4× magnified view. (Reproduced from **[20]** with permission from Wiley.

**Figure 8:** SDS-PAGE of dGAE fibrils prepared with (lanes 1-4) and without (lanes 5-8) DTT under different denaturation conditions. Lane 1/5: heating to 95 °C for 8 minutes with reducing agent (β-mercaptoethanol (βME) diluted 1:10 into Laemlli sample buffer). Lane 2/6: heating (95 °C, 8 minutes) without reducing agent. Lane 3/7: incubating at room temperature in Laemlli sample buffer containing βME 1:10 for 5 minutes. Lane 4/8: incubating at room temperature in Laemlli sample buffer without reducing agent. Heating to 95 °C for 8 minutes denatures fibrils, that are seen stuck in the well of the gel when samples are incubated at room temperature, but does not denature the 24 kDa dimeric species, suggesting that it is very stable. The presence of SDS alone is able to partially denature fibrils into monomer and dimer (lanes 4 and 8). 9 μg of protein was loaded.

**Figure 9:** Example of a dot blot using a whole solution of 100 μM dGAE with 10 mM DTT over time using the polyclonal anti-tau oligomer antibody T22. Aliquots were removed at each time point and stored at -20 °C then dotted onto a membrane. A comparison of membranes that were either washed or not washed before blocking are shown. The signal can be seen to decrease over time as dGAE fibrillises.