Synthesis and Purification of Peptide Trimers for Tau R3 Inhibition and Disaggregation

Fall 2025, Undergraduate Laboratory Internship

Laboratory of Chemical Biology (Prof. YoungSoo Kim)

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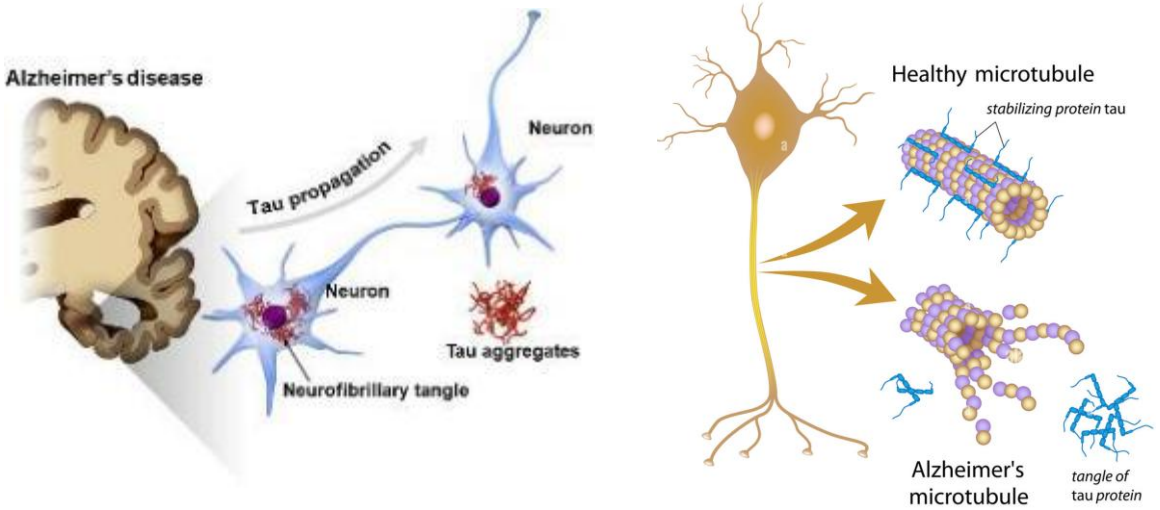
Experimental Objectives

- **Synthesis and Purification**: Synthesize five candidate peptide trimers designed to inhibit and disaggregate TauR3 protein—a primary pathological feature of Alzheimer's Disease—using Solid-Phase Peptide Synthesis (SPPS), followed by HPLC purification.
- **Efficacy Testing**: Conduct Inhibition and Disaggregation Assays on the synthesized peptide trimers to evaluate their impact on TauR3.



Takeda S. Tau propagation as a diagnostic and therapeutic target for dementia: potentials and unanswered questions. Frontiers in neuroscience. 2019 Dec 13;13:1274.

https://www.alamy.com/stock-photo/neurofibrillary-tangle.html?sortBy=relevant

Theoretical Background: TauR3 Protein

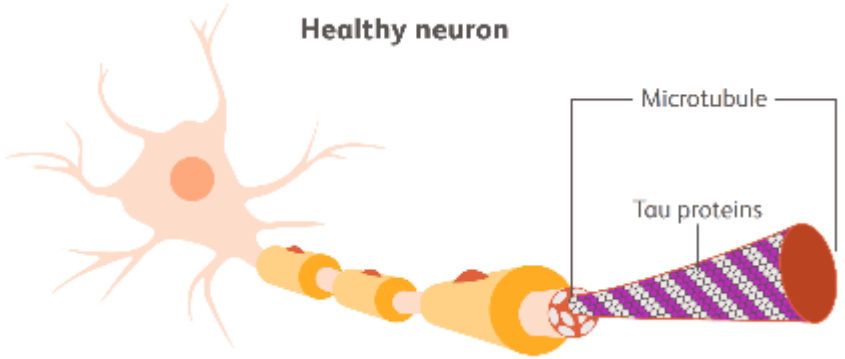
Function: Tau proteins play a critical role in stabilizing microtubules within brain cells (neurons).

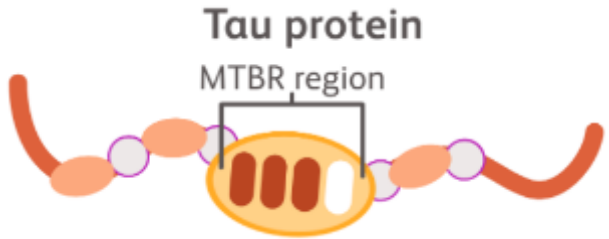
Structure (MTBR): The region where the tau protein binds to microtubules is called the Microtubule Binding Repeat (MTBR). Tau variants are classified based on these repeats:

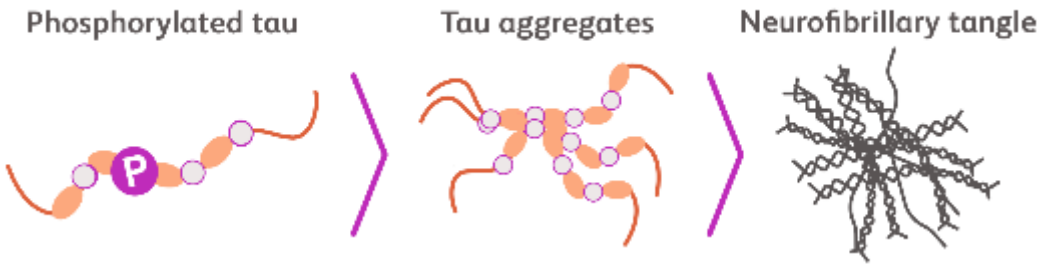
- **3R Tau** (Tau R3): Contains three binding repeats.
- **4R Tau**: Contains four binding repeats.

In Alzheimer's Disease, 3R and 4R tau proteins typically aggregate in an approximate 1:1 ratio.

Pathology: The Neurofibrillary Tangles (NFTs) found in the brains of Alzheimer's patients consist of "Paired Helical Filaments" (PHFs) formed by the co-aggregation of TauR3 and 4R tau.







Siddiqua A, Margittai M. Three-and four-repeat Tau coassemble into heterogeneous filaments: an implication for Alzheimer disease. Journal of Biological Chemistry. 2010 Nov 26;285(48):37920-6.
https://www.bms.com/media/media-library/scientific-media-resources/tau-alzheimers-disease-fact-sheet.html

Pathological Mechanism

Normal **TauR3** is essential for brain function; however, in the environment of Alzheimer's Disease, the following degenerative processes occur:

1. **Hyperphosphorylation**: Triggered by factors such as Amyloid-beta (Aβ), phosphate groups excessively attach to the tau protein.

2. **Microtubule Dissociation**: Hyperphosphorylated TauR3 detaches from the microtubules. As a result, the microtubules—which serve as the structural scaffold of the neuron—collapse, leading to the failure of the intracellular transport system.

3. **Formation of Toxic Aggregates**: The dissociated Tau R3 proteins entangle to form oligomers, which eventually develop into neurofibrillary tangles (NFTs), ultimately causing neuronal cell death.

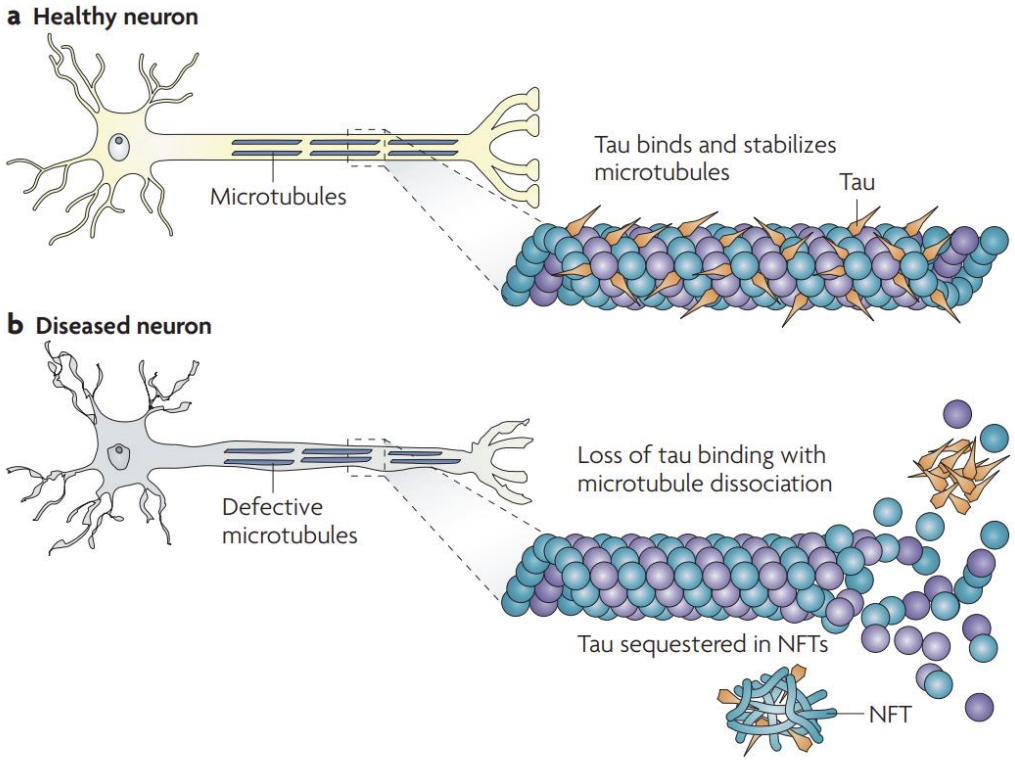


Figure 2 | **Tau in healthy neurons and in tauopathies. a** | Tau (also known as MAPT) facilitates microtubule stabilization within cells and is particularly abundant in neurons. Microtubules serve as 'tracks' that are essential for normal trafficking of cellular cargo along the lengthy axonal projections of neurons. **b** | It is thought that tau function is compromised in Alzheimer's disease and other tauopathies. This probably results from both tau hyperphosphorylation, which reduces the binding of tau to microtubules, and the sequestration of hyperphosphorylated tau into neurofibrillary tangles (NFTs), which reduces the amount of tau that is available to bind microtubules. The loss of tau function leads to microtubule instability and reduced axonal transport, which could contribute to neuropathology.

Brunden KR, Trojanowski JQ, Lee VM. Advances in tau-focused drug discovery for Alzheimer's disease and related tauopathies. Nature reviews Drug discovery. 2009 Oct;8(10):783-93.

Cowan CM, Bossing T, Page A, Shepherd D, Mudher A. Soluble hyper-phosphorylated tau causes microtubule breakdown and functionally compromises normal tau in vivo. Acta neuropathologica. 2010 Nov;120(5):593-604.

Research Rationale

1. **D-Amino Acid Peptides**: Short D-amino acid peptides can bind to tau fibrils, facilitating their disaggregation and inhibition.

2. **Proteolytic Stability**: Unlike standard L-amino acid peptides, D-amino acid peptides are not easily degraded by endogenous enzymes. This high metabolic stability allows them to remain in the bloodstream or brain for longer durations, effectively targeting specific sites on the tau protein.

3. **Therapeutic Advantages**: Compared to antibody-based therapeutics, D-peptides are smaller in molecular size, which reduces the risk of an immune response and potentially increases the efficiency of crossing the Blood-Brain Barrier (BBB).

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How short peptides disassemble tau fibrils in Alzheimer's disease

Ke Hou, Peng Ge, Michael R. Sawaya, Liisa Lutter, Joshua L. Dolinsky, Yuan Yang, Yi Xiao Jiang, David R. Boyer, Xinyi Cheng, Justin Pi, Jeffrey Zhang, Jiahui Lu, Romany Abskharon, Shixin Yang, Zhiheng Yu, Juli Feigon & David S. Eisenberg

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A novel peptide-based tau aggregation inhibitor as a potential therapeutic for Alzheimer's disease and other tauopathies

Anthony Aggidis, George Devitt, Yongrui Zhang, Shreyasi Chatterjee, David Townsend, Nigel J. Fullwood, Eva Ruiz Ortega, Airi Tarutani, Masato Hasegawa, Amber Cooper ... See all authors

First published: 03 October 2024 | https://doi.org/10.1002/alz.14246 | VIEW METRICS

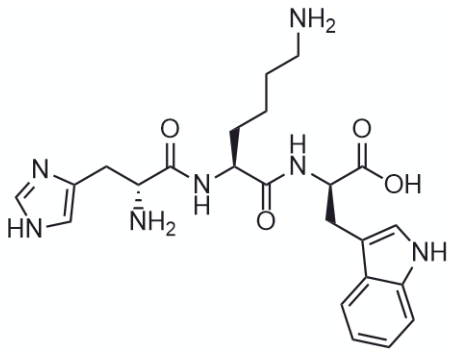
Co-authors David Allsop and Nigel J. Fullwood are now deceased.

Hou K, Ge P, Sawaya MR, Lutter L, Dolinsky JL, Yang Y, Jiang YX, Boyer DR, Cheng X, Pi J, Zhang J. How short peptides disassemble tau fibrils in Alzheimer's disease. Nature. 2025 Aug 28;644(8078):1020-7.

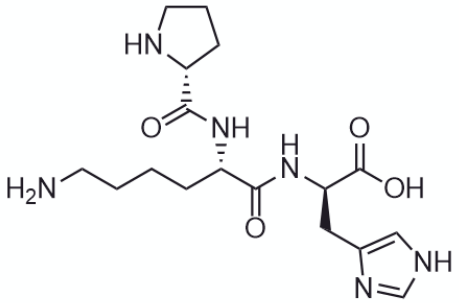
Aggidis A, Devitt G, Zhang Y, Chatterjee S, Townsend D, Fullwood NJ, Ortega ER, Tarutani A, Hasegawa M, Cooper A, Williamson P. A novel peptide-based tau aggregation inhibitor as a potential therapeutic for Alzheimer's disease and other tauopathies. Alzheimer's & Dementia. 2024 Nov;20(11):7788-804.

Experimental Procedure

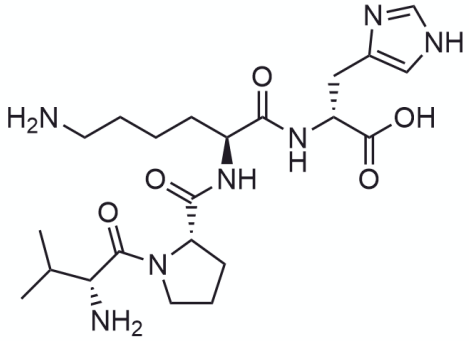
Five different short peptide trimers will be synthesized using the SPPS (Solid-Phase Peptide Synthesis) method, followed by Tau R3 Inhibition and Disaggregation Assays to evaluate their efficacy.



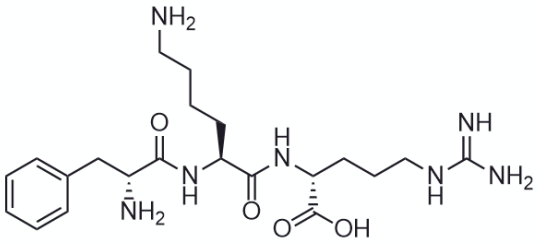
D-His-Lys-D-Trp



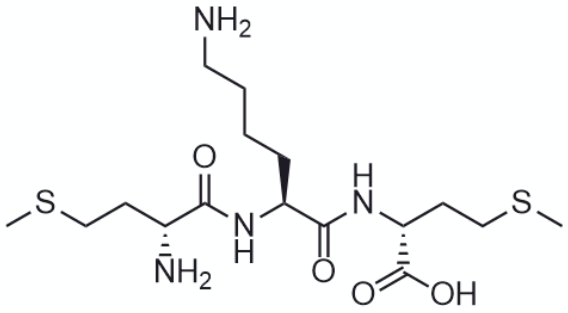
D-Pro-Lys-D-His



D-Val-Pro-Lys-D-His



D-Phe-Lys-D-Arg



D-Met-Lys-D-Met

$X_DK(X_D)$ Peptide Trimer Synthesis

- Scale : 0.5 mmol
- Resin : Wang Resin (0.85 mmol/g scale)
- L form amino acid

1. Resin swelling (4 hr)
- Wang resin (0.85 mmol/g), Fmoc-Lys(Alloc)-OH
- 0.5 mmol

2. Fmoc-Lys(Alloc)-OH (symmetric anhydride) (2 hr)
- Amino acid 10 eq. + DIC 5 eq. + DMAP (catalyst)

3. Fmoc deprotection (5 min + 10 min)
- 20% piperidine (0.1 M oxyma), double deprotection

4. Amino acid (aa1) coupling (40 min)
- Amino acid 5 eq. (Dissolve in 10 mL DMF) + DIC 5 eq. + Oxyma (DIEA) 5 eq. (10 mL)

5. Fmoc deprotection (5 min + 10 min)
- 20% piperidine (0.1 M oxyma), double deprotection

$X_DK(X_D)$ Peptide Trimer Synthesis

- Scale : 0.5 mmol
- Resin : Wang Resin (0.85 mmol/g scale)
- L form amino acid

6. Boc_2O protection (18 hr)

- Boc_2O 1 mL + DIEA 0.01 mL + DMF 2 mL

7. Alloc deprotection (2 hr)

- 0.25 eq. $Pd(PPh_3)_4$ + 24 eq. DMBA (135.21) in 20 mL DCM

8. 20% Piperidine washing (2 min)

9. AA2 Coupling (40 min)

10. Fmoc deprotection (5 min + 5 min)

11. Cleavage & Ether precipitation (3.5 hr) total 4 mL

- TFA : TIS : DODT : DW = 92.5 : 2.5 : 2.5 : 2.5

HPLC Peptide Purification

1. Solvent, Sample Preparation

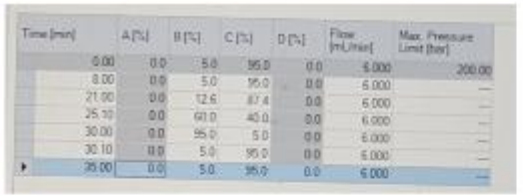
- Solvent A: HPLC-grade H2O + 0.1% Trifluoroacetic acid (TFA)
- Solvent B: HPLC-grade Acetonitrile (ACN) + 0.1% TFA
- Sample: Dissolve crude peptide in Solvent A (use <10% ACN or DMSO if needed for solubility).
- Filtration: Filter sample through a 0.22 µm PVDF membrane filter.

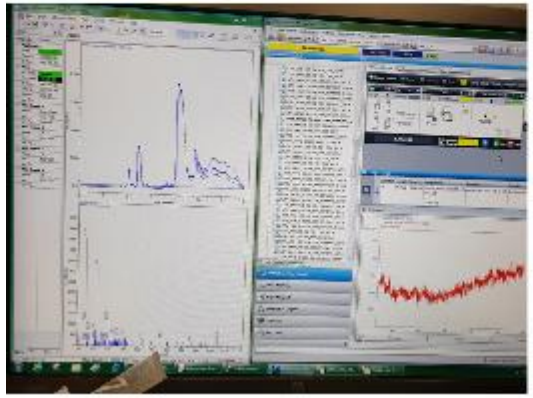
2. Column Equilibration (Pre-Injection)

- Set pump to isocratic mode at method starting condition (95% A / 5% B)
- Flush 10-20 column volumes of starting mobile phase

3. Analytical Scouting (Identify peak retention time)

- Column: C18, 4.6 x 250 mm, 5 µm
- Flow Rate: 1.0 mL/min
- Detection: 220 nm (Backbone), 280 nm (Trp/Tyr/Phe)
- Gradient:
  0.0 min → 5% B
  30.0 min → 95% B
- Objective: Note the retention time (tR) of the target peak.





HPLC Peptide Purification

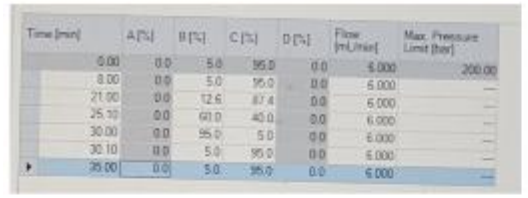
4. Preparative Run (Purification)

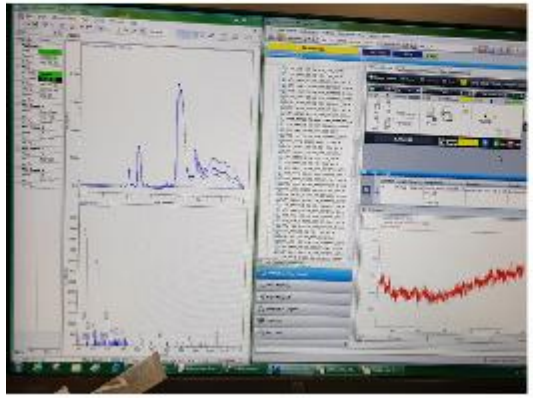
- Column: C18, 4.6 x 250 mm, 5 µm
- Focused Gradient (i.e. peak flattening):
  - Start: (%B at tR) - 10%
  - End: (%B at tR) + 10%
  - Duration: 20-30 minutes
- Fraction Collection (Agilent 1260):
  - Mode: Peak-based (Slope/Threshold)
  - Threshold: 50-100 mAU (adjust based on sample concentration)

5. Fraction Analysis & Recovery

- Purity Check: Re-inject 10 µL of collected fractions into the Analytical Method.
- Pooling: Combine fractions with >95% peak area integration.
- Evaporation: Rotovap at <30°C to remove Acetonitrile.
- Lyophilization: Freeze-dry the aqueous remainder to obtain peptide + TFA salt powder






Tau R3 Inhibition Assay (Protocol)

ThT Assay Protocol: Tau R3 Fibrillization Inhibition
Target: Tau R3 (50 uM)
Inhibitor: Peptide Trimer (3-mer)

I. PEPTIDE & STOCK PREPARATION

1. Peptide Trimer Stock (1 mM):
   - Dissolve lyophilized peptide trimer in 100% DMSO to a concentration of 1 mM.
   - Store aliquots at -80 °C.

2. Working Solution Preparation:
   - Thaw 1 mM peptide stock.
   - Dilute the stock with DW until the final DMSO content is exactly 5.5% before mixing with Tau R3.

II. INCUBATION PROCEDURE

Perform at 10:00 AM daily (e.g. Sunday to Thursday) to create the 5, 4, 3, 2, 1-day and 18 hr timepoints.

1. Setup Two Reaction Groups:
   - Control Group: 50 uM TauR3 + Vehicle (5.5% DMSO in DW).
   - Test Group: 50 uM TauR3 + Peptide Trimer (in 5.5% DMSO/DW).

2. Conditions:
   - Seal tubes (Protein LoBind) to prevent evaporation.
   - Incubate at 37 °C (ensure consistent shaking)

3. Labeling:
   - Ensure tubes are marked by Day (5 through 1) and Group (Control vs. Peptide).

III. REAGENTS

Prepare the following reagents before step IV (seeding & plate loading)

1. ThT Working Solution (5 uM):
   - Buffer: 50 mM Glycine buffer, pH 8.5.
   - Preparation (5 mM Diluted Stock):
      a. Weigh 1.16 mg ThT; dissolve in 728 uL Glycine buffer.
         - Calculation: 5mM = [(1.16mg) / (318.86g/mol)] / x L
      b. Dilute 5 mM stock 1:1000 in Glycine buffer to reach 5 uM.
         - Volume: Prepare 2000 uL (2 mL).
         - Procedure: 2 mL glycine buffer - 2 uL + 2 uL of 5 mM ThT stock.
   - Storage: PROTECT FROM LIGHT (wrap in foil) until use.

2. 0-Hour & Blank Prep:
   - Prepare Blank: 5.5% DMSO in DW.
      - Procedure: 100 uL DW - 5.5 µL DW + 5.5 uL DMSO.
   - 0-Hour Normalization Solution (10% DMSO)
   - Purpose: Added to 0-hour samples to match solvent conditions.
   - Procedure: In 120 uL DW, remove 12 uL DW and add 12 uL DMSO.

Tau R3 Inhibition Assay (Protocol)

IV. SEEDING & PLATE LOADING

1. Plate Type: 96-well half-area black flat-bottom plate (Corning #3694).
   Note: Half-area plates require lower volumes and reduce background noise.

2. Loading Order (In Triplicate):
   General Seeding (Blank and Incubated Samples)
   - Order: Blank -> Day 5 -> Day 4 -> Day 3 -> Day 2 -> Day 1 -> 18hr.
   - For BOTH Control and Drug Test tubes at each timepoint:
     a. Add 25 uL of sample per well (run in triplicates).
     b. Add 75 uL of 5 uM ThT solution to each well.

3. 0-Hour Specific Seeding:
   - Order of addition for 0-hour wells:
     a. Add 75 uL of ThT solution.
     b. Add 50 uL of 10% DMSO solution.
        - Procedure: 120 uL DW - 12 uL DW + 12 uL DMSO.
     c. Add 25 uL of the fresh 0-hour TauR3 sample.

4. Incubation: Let the plate sit for 5 minutes at Room Temperature (RT).

V. MEASUREMENT PARAMETERS (Tecan Infinite 200 PRO)

- Mode: Fluorescence Bottom Reading
- Excitation Wavelength: 450 nm
- Emission Wavelength: 485 nm
- Replicates: Triplicate (n=3)